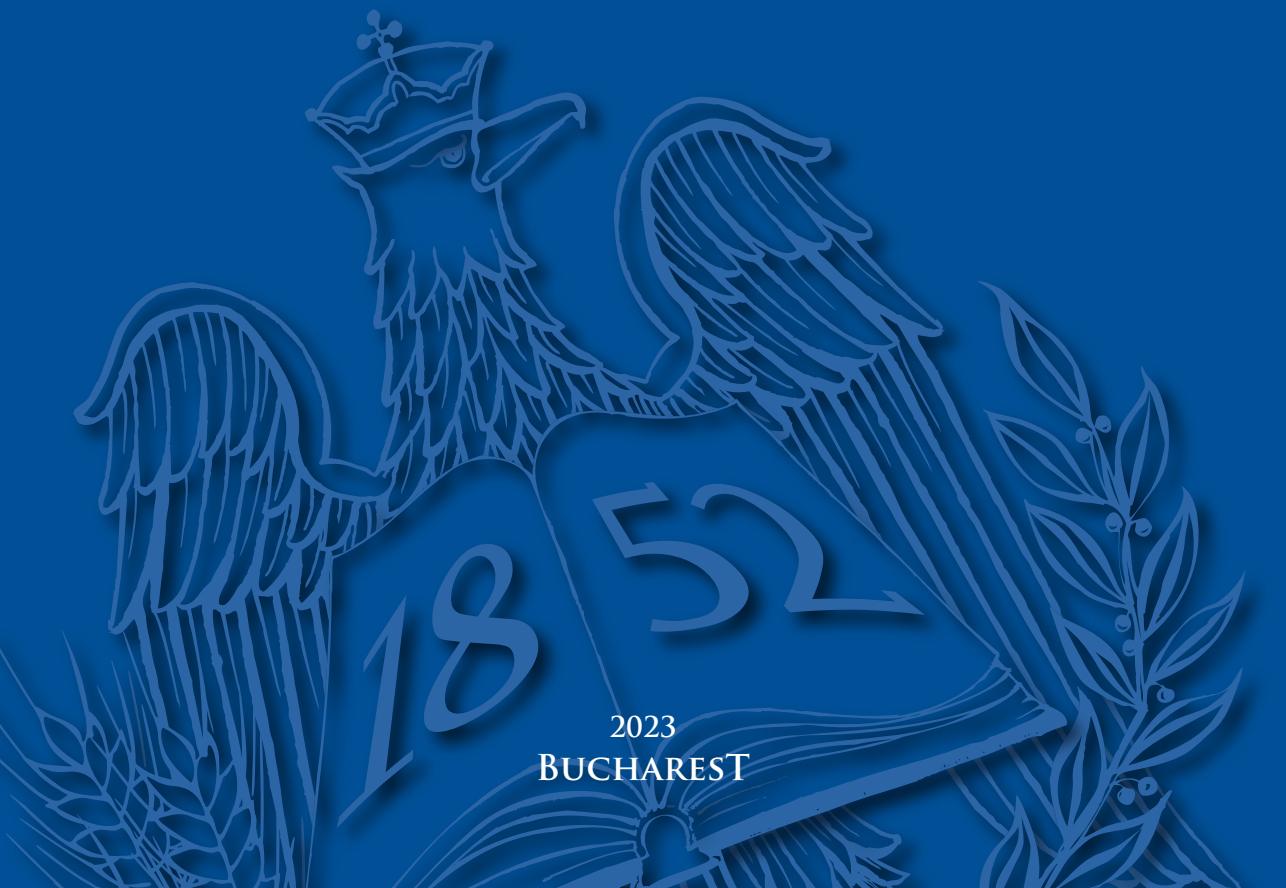
University of Agronomic Sciences
and Veterinary Medicine of Bucharest
Faculty of Veterinary Medicine

SCIENTIFIC WORKS

SERIES C. VETERINARY MEDICINE

VOL. LXIX (2)



2023
BUCHAREST

SCIENTIFIC WORKS
SERIES C. VETERINARY MEDICINE
VOLUME LXIX (2), 2023

University of Agronomic Sciences
and Veterinary Medicine of Bucharest
Faculty of Veterinary Medicine

SCIENTIFIC WORKS
SERIES C.
VETERINARY MEDICINE

Volume LXIX (2)

2023
BUCHAREST

PUBLISHERS:

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania - Faculty of Veterinary Medicine
Address: 105 Splaiul Independentei, District 5, Zip code 050097, Bucharest, Romania
Phone: + 40 213 182 564, Fax: +40 213 182 888
E-mail: veterinarymedicinejournal@usamv.ro, Webpage: http://veterinarymedicinejournal.usamv.ro

CERES Publishing House
Address: 29 Oastei Street, District l, Bucharest, Romania
Phone: + 40 21 317 90 23, E-mail: edituraceres@yahoo.com, Webpage: www.editura-ceres.ro

To be cited: Scientific Works. Series C. Veterinary Medicine, Vol. LXIX (2), 2023

ISSN 2065-1295, ISSN 2343-9394 (CD-ROM), ISSN 2067-3663 (Online), ISSN-L 2065-1295

International Database Indexing:
Index Copernicus; CABI; Google Scholar; Scipio; OCLC; PNB (Polish Scholarly Bibliography); Cite Factor; Research Bible; Universal Impact Factor

SCIENTIFIC COMMITTEE

1. Larry ADAMS - Purdue University College of Veterinary Medicine, Indiana, USA
2. Sarah BAILLIE - Bristol Veterinary School, University of Bristol, UK
3. Cinzia BENAZZI - Veterinary Medical Sciences, University of Bologna, Italy
4. Laurentiu BENGA -Veterinary Laboratory of the Central Unit for Animal Research and Welfare Affairs, University Hospital, Heinrich Heine University Dusseldorf, Germany
5. Cornel CĂTOI - Faculty of Veterinary Medicine, USAMV Cluj-Napoca, Romania
6. Emilia CIOBOTARU-PÎRVU - Faculty of Veterinary Medicine, USAMV of Bucharest, Romania
7. Mario CODREANU - Faculty of Veterinary Medicine, USAMV of Bucharest, Romania
8. Cristin COMAN - National Institute for Medical-Military Research and Development "Cantacuzino", Romania
9. Gheorghe DĂRĂBUŞ - Faculty of Veterinary Medicine, USV from Timisoara, Romania
10. Doina DANEŞ - Faculty of Veterinary Medicine, USAMV of Bucharest, Romania
11. Claudio GENCHI - Dep. of Veterinary Sciences and Public Health, University of Milan, Italy
12. Viorel HERMAN - Faculty of Veterinary Medicine, USV from Timisoara, Romania
13. Cornel IGNA - Faculty of Veterinary Medicine, USV from Timisoara, Romania
14. Mariana IONIŢĂ - Faculty of Veterinary Medicine, USAMV of Bucharest, Romania
15. Anja KIPAR - Institute of Veterinary Pathology, Vetsuisse Faculty Zurich, University of Zurich, Switzerland
16. Horst Erich KÖNIG - Institute of Anatomy, Histology and Embryology, University of Veterinary Medicine Vienna, Austria
17. Ioan Liviu MITREA - Faculty of Veterinary Medicine, USAMV of Bucharest, Romania
18. Liviu Dan MIRON - Faculty of Veterinary Medicine, USV of Iasi, Romania
19. Dumitru MILITARU - Academy of Agricultural and Forestry Sciences, Bucharest, Romania
20. Manuella MILITARU - Faculty of Veterinary Medicine, USAMV of Bucharest, Romania
21. Sorin MORARIU - Faculty of Veterinary Medicine, USV from Timisoara, Romania
22. Laurent OGNEAN - Faculty of Veterinary Medicine, USAMV of Cluj-Napoca, Romania
23. Kurt PFISTER - Ludwig-Maximilians University, Munich, Germany
24. Gheorghe SAVUŢA - Faculty of Veterinary Medicine, USV of Iasi, Romania
25. Georghe SOLCAN - Faculty of Veterinary Medicine, USV of Iasi, Romania
26. Marina SPÎNU - Faculty of Veterinary Medicine, USAMV of Cluj-Napoca, Romania
27. Andreea Iren ŞERBAN - Faculty of Veterinary Medicine, USAMV of Bucharest, Romania
28. Dana TĂPĂLOAGĂ - Faculty of Veterinary Medicine, USAMV of Bucharest, Romania
29. Constantin VLĂGIOIU - Faculty of Veterinary Medicine, USAMV of Bucharest, Romania

SUMMARY

FUNDAMENTAL SCIENCES

CLINICAL SCIENCES

ANIMAL PRODUCTION, PUBLIC HEALTH AND FOOD QUALITY CONTROL

EXPERIMENTAL MEDICINE

FUNDAMENTAL SCIENCES

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

COMPARATIVE LEVELS OF LEAD AND CADMIUM IN SHEEP WOOL AND COW HAIR

Emanuela BADEA[1], Gheorghe GORAN[1], Cristina ȚOCA[2], Cristian MUȘETESCU[1]

[1]Faculty of Veterinary Medicine, USAMV of Bucharest, 105 Splaiul Independenței, 050097, District 5, Bucharest, Romania
[2]IDAH of Bucharest, 63 Doctor Staicovici, 050557, District 5, Bucharest, Romania

Corresponding author email: emanuela.badea@gmail.com

Abstract

This study aimed to examine the use of wool from sheep and hair from cows raised in the commune of Bran, Romania as a possible indicator of environmental exposure to lead and cadmium. In addition, the samples collected from sheep and cows were analysed both unwashed and washed in order to determine a possible difference between lead and cadmium concentrations. The samples were analysed for heavy metal concentrations using Inductively Coupled Plasma Mass Spectrometry. Statistical analysis showed that there are no significant differences between the concentrations of lead and cadmium in washed and unwashed sheep wool and cow hair, respectively.

Key words*: lead, cadmium, sheep, cows, ICP-MS.*

INTRODUCTION

Lead and cadmium are heavy metals that can cause severe health hazards in both humans and animals when consumed in high concentrations. Lead and cadmium toxicity have been extensively studied and have been found to cause a range of health problems, including developmental delays, anemia, and neurological disorders in individuals exposed to lead, or kidney damage, respiratory illness, and cancer in individuals exposed to cadmium. Exposure can occur through contaminated food, water, and air. Prevention efforts, such as reducing industrial emissions and monitoring food safety, are essential to minimize exposure (De Francisco et al., 2003; Flora et al., 2012; Godt et al., 2006; Klaassen et al., 2009; Satarug et al., 2003; Satarug et al., 2011).

The contamination of the environment with these metals is a global concern, and the contamination of the food chain is a significant source of exposure for humans and animals. (Chary et al., 2008; Kumar et al., 2019; Sonone et al., 2020). Several studies have researched the possibility of using animal hair as a bioindicator of environmental pollution (Kośla et al., 2003; Kozak et al., 2002; Skibniewska et al., 2011; Skibniewski et al., 2013).

Comparative levels of lead and cadmium in sheep wool and cow hair have been a subject of research for many years. Several studies have been conducted to determine the concentration of these heavy metals in animal hair and their potential risks to human and animal health. The results of these studies have shown that both sheep wool and cow hair can accumulate significant amounts of lead and cadmium from the environment (Hristev et al., 2008; Patra et al., 2007; Patra et al., 2006; Tuncer, 2019).

The variation in the concentration of lead and cadmium in sheep wool and cow hair may be attributed to several factors, including the geographical location, the type of soil, the type of vegetation in the grazing areas, and the feed that the animals receive. Additionally, the age of the animals, the breed, and the nutritional status of the animals can also influence the accumulation of heavy metals in hair or wool.

In this context, this study aimed to assess the level of contamination of cows and sheep raised in the rural region of Bran, Romania by analysing cow hair and sheep wool samples. The samples were analysed both unwashed and washed to see if the environmental exterior contamination of the samples could influence the concentrations of Pb and Cd found in hair and wool.

MATERIALS AND METHODS

Wool and hair samples were collected from sheep (n = 6) and cows (n = 6) that were raised in the commune of Bran, Romania. Two samples were collected from each individual, so that the first sample was analysed unwashed, while the second sample was washed prior to analysis. Wool and hair samples were washed with warm water, then left to soak in water for 48 hours, drained, and soaked in water for an additional 48 hours. Samples were then drained, and left to soak in 98° alcohol for 24 hours. After this process, all samples were rinsed with distilled water and left to dry at room temperature. All wool and hair samples were weighed to 0.5 g and placed in polypropylene tubes. Samples were then disintegrated by cold wet mineralisation; 5 ml of HNO_3 and 1 ml of HCl were added to each sample. After mineralization was complete, in two weeks at room temperature, ultrapure water was added in each sample to 10 ml.

All hair samples were analysed using a Perkin-Elmer Elan DRC II ICP-MS (RF Power 1500 W; Nebulizer PFA-100; Sample Uptake Rate ≈175 µL/min - self aspiration; Spray Chamber Cyclonic; Nebulizer Flow Set for ≤1.5% oxides CeO+/Ce+). Calibration curves were developed using standard solutions of 0.005 ppm, 0.01 ppm, 0.1 ppm, 1 ppm, 5 ppm, 10 ppm, obtained by dilution from a multi-element ICP MERCK standard solution containing 100 mg/L of Pb and Cd. Statistical analysis was performed using SPSS software. For a higher accuracy, given the small number of samples in each group, the median was used to compare Pb and Cd concentrations between groups. The Mann-Whitney test was applied to the obtained values of Pb and Cd concentrations in wool and hair samples.

RESULTS AND DISCUSSIONS

Lead and cadmium median concentrations in cow hair and sheep wool samples depending on the method of preparation of samples, along with the results of the statistical analysis, are presented in Table 1.

Figures 1 and 2 show the mean concentrations of lead and cadmium depending on sample method of preparation in cow hair and sheep wool samples, respectively.

The median concentrations of Pb in cow hair samples was different for washed and unwashed samples. It was visible that unwashed samples had higher concentrations of Pb (365.30 ppb) than washed samples (172.41 ppb), however these differences were not statistically significant.

The median concentrations of Pb in sheep wool were also higher in unwashed samples (298.88 ppb) compared to washed samples (234.45 ppb), but still with no statistical significance.

Table 1. Lead and cadmium median concentrations (ppb) in cow hair and sheep wool samples depending on the method of preparation of samples

Sample type		N	Pb	Cd
Unwashed	Cattle	6	365.30[a]	37.07[a]
	Sheep	6	298.88[a]	36.73[a]
Washed	Cattle	6	172.41[a]	31.03[a]
	Sheep	6	234.45[a]	36.84[a]

Values with different superscripts between rows in a column vary significantly at $p < 0.05$.

Hristev et al. (2008) studied the lead content in washed and unwashed wool of sheep. The obtained concentrations were much higher compared to the ones found in the present study, the authors also finding a significant statistical difference between the concentrations of Pb in unwashed wool (15.3 ppm) versus washed wool (8.15 ppm).

Regarding Cd concentrations in cow hair, the difference between unwashed samples (37.07 ppb) and washed samples (31.03 ppb) was not statistically significant.

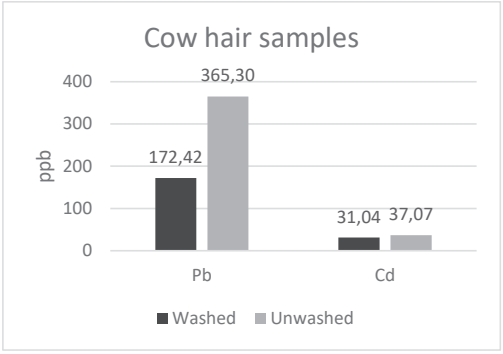


Figure 1. Mean Pb and Cd concentrations in cow hair samples depending on sample method of preparation

Median Cd concentrations in sheep wool samples were 36.84 ppb in washed samples, and 36.73 ppb in unwashed samples, however these differences were not statistically significant.
Hristev et al. (2008) also studied the cadmium content in washed and unwashed wool of sheep. The obtained concentrations were a bit higher compared to the ones found in the present study, and the authors also did not find a significant difference in the concentration of Cd between unwashed wool (0.69 ppm) and washed wool (0.53 ppm).

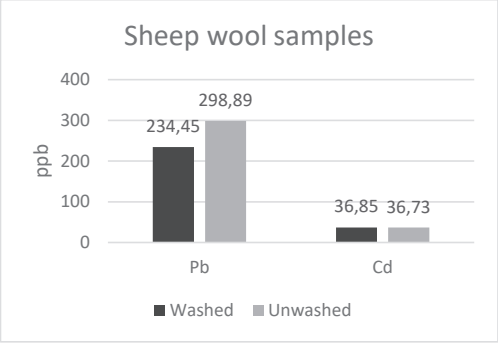


Figure 2. Mean Pb and Cd concentrations in sheep wool samples depending on sample method of preparation

The present study found that statistical analysis showed no significant differences between Pb concentrations in cows compared to sheep, nor in Cd concentrations in cows compared to sheep, as is visible in Table 1.
Patra et al. (2007) evaluated the concentrations of Pb and Cd in tail hair samples in cows raised in different polluted and non-polluted environments. Cows raised in unpolluted areas registered 2.99 ppm Pb and 0.52 ppm Cd, while the highest concentration of Pb was found in cows raised near a lead-zinc smelter (15.09 ppm) and the highest concentration of Cd was found in cows raised near a closed lead cum operational zinc smelter (5.72 ppm), with even the lowest concentrations of both heavy metals still being much higher than those found in the present study.
Rogowska et al. (2009) studied the concentrations of different heavy metals in the hair of cattle living in the area contaminated by a copper smelter, in unwashed and washed samples, over the course of 3 years. In the unwashed samples, Pb concentrations were higher compared to the concentrations found in the present study, while Cd concentrations were similar to the ones found in the present study. However, Rogowska et al. found statistically significant differences between the unwashed and washed samples, for example Cd concentrations in unwashed samples were 0.03 ppm, significantly higher ($p < 0.01$) compared to washed samples (0.014 ppm).
Tuncer (2019) evaluated the concentrations of lead and cadmium in wool samples taken from sheep raised in Centrum (industrial area) and Özalp (rural area) districts of Van province in Turkey, and concluded that sheep raised in the Central district of Van (Pb - 49.05; Cd - 0.37 ppb) had statistically significant higher levels ($p < 0.01$) of Pb and Cd than those of the sheep Özalp district (Pb - 47.02; Cd - 0.15 ppb), with the concentrations of both Pb and Cd for both districts being much lower compared to the findings of the present research.

CONCLUSIONS

Heavy metal level analysis of cow hair and sheep wool is a non-invasive method of analysis, which can be of use for environmental pollution assessment. Neither Pb nor Cd concentrations were significantly different between the two studied species, which suggest a similar level of contamination of both species.
The present study found no statistically significant differences between the concentrations of neither Pb nor Cd based on the preparation technique of the samples. Although washed samples usually had lower concentrations of both Pb and Cd, the differences were not significant. Taking into account the fact that the cows and sheep were raised in a rural, non-industrial area of Romania, it can be considered that the heavy metal pollution in the area was reduced, so further research should be performed, by analysing samples taken from animals raised in polluted areas as well, in order to be able to determine whether external contaminants present on the hair samples can significantly increase the concentrations of the analysed elements.

REFERENCES

Chary, N. S., Kamala, C. T., & Raj, D. S. S. (2008). Assessing risk of heavy metals from consuming food grown on sewage irrigated soils and food chain transfer. *Ecotoxicol Environ Saf, 69*(3), 513-524.

De Francisco, N., Ruiz Troya, J. D., & Agüera, E. I. (2003). Lead and lead toxicity in domestic and free living birds. *Avian Pathol, 32*(1), 3-13.

Flora, G., Gupta, D., & Tiwari, A. (2012). Toxicity of lead: A review with recent updates. *Interdiscip Toxicol, 5*(2), 47–58.

Godt, J., Scheidig, F., Grosse-Siestrup, C., Esche, V., Brandenburg, P., Reich, A., & Groneberg, D. A. (2006). The toxicity of cadmium and resulting hazards for human health. *J Occup Med Toxicol, 1*, 22.

Hristev, H., Penkov, D., Kmetov, V., Baykov, B., & Bliznakov, A. (2008). Lead and cadmium content in washed and unwashed wool of sheep reared in regions with increased technogenic Clarc. *Journal of Central European Agriculture, 9*(2), 305-310.

Klaassen, C. D., Liu, J., & Diwan, B. A. (2009). Metallothionein Protection of Cadmium Toxicity. *Toxicol Appl Pharmacol, 238*(3), 215–220.

Kośla, T., Skibniewska, E., Kolbuszewski, T., & Urbańska-Słomka, G. (2003). Nickel in the tissues of horses depending on their age, sex and living environment. *Pr Kom Nauk Rol Biol BTN, 51*, 115-121.

Kozak, M., Kralova, E., Sviatko, P., Bilek, J., & Bugarsky, A. (2002). Study of the content of heavy metals related to environmental load in urban areas in Slovakia. *Bratisl Lek Listy, 103*(7-8), 231-237.

Kumar, S., Prasad, S., Yadav, K. K., Shrivastava, M., Gupta, N., Nagar, S., Bach, Q.-V., Kamyab, H., Khan, S. A., Yadav, S., & Malav, L. C. (2019). Hazardous heavy metals contamination of vegetables and food chain: Role of sustainable remediation approaches - A review. *Environ Res, 179 (Pt A)*, 108792.

Patra, R. C., Swarup, D., Naresh, R., Kumar, P., Nandi, D., Shekhar, P., Roy, S., & Ali, S. L. (2007). Tail hair as an indicator of environmental exposure of cows to lead and cadmium in different industrial areas. *Ecotoxicol Environ Saf, 66*(1), 127-131.

Patra, R. C., Swarup, D., Sharma, M. C., & Naresh, R. (2006). Trace mineral profile in blood and hair from cattle environmentally exposed to lead and cadmium around different industrial units. *J Vet Med A Physiol Pathol Clin Med, 53*(10), 511-517.

Rogowska, K. A., Monkiewicz, J., & Grosicki, A. (2009). Lead, cadmium, arsenic, copper, and zinc contents in the hair of cattle living in the area contaminated by a copper smelter in 2006 - 2008. *Bull Vet Inst Pulawy, 53*, 703-706.

Satarug, S., Baker, J. R., Urbenjapol, S., Haswell-Elkins, M., Reilly, P. E., Williams, D. J., & Moore, M. R. (2003). A global perspective on cadmium pollution and toxicity in non-occupationally exposed population. *Toxicol Lett, 137*(1-2), 65-83.

Satarug, S., Garrett, S. H., Sens, M. A., & Sens, D. A. (2011). Cadmium, environmental exposure, and health outcomes. *Cien Saude Colet, 16*, 2587–2602.

Skibniewska, E. M., Skibniewski, M., Kosla, T., & Urbanska-Slomka, G. (2011). Hair zinc levels in pet and feral cats (*Felis catus*). *Journal of Elementology, 16*(3), 481–488.

Skibniewski, M., Kośla, T., & Skibniewska, E. M. (2013). Domestic cat (Felis catus) as a bioindicator of environmental lead contamination. *Environmental Protection And Natural Resources, 24*(4(58)), 47–50.

Sonone, S. S., Jadhav, S., Sankhla, M. S., & Kumar, R. (2020). Water Contamination by Heavy Metals and their Toxic Effect on Aquaculture and Human Health through Food Chain. *Letters in Applied NanoBioScience, 10*(2), 2148-2166.

Tuncer, S. S. (2019). An investigation of the lead and cadmium levels of blood serum and wool of White Karaman sheep in the Van region (Turkey). *Applied Ecology and Environmental Research, 17*(1), 1381-1387.

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

COMPARATIVE HISTOLOGICAL STUDY BETWEEN FILLET MUSCLE TRADITIONAL PREPARATION AND INDUSTRIAL PREPARATION

Andrei CĂLINOIU, Mihai-Romeo DINICĂ, Laura-Cătălina DUMITRA, Ștefania Mariana RAITA

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: andreicalinoiu95@gmail.com

Abstract

The aim of this paper is to highlight the invaluable contribution of histological techniques used to assess the quality of meat products deemed for human consumption. Additionally, histological analyses assist with the study of what effect natural vegetarian solution extracts have on the products' quality, effect reflected by the impact on preservation properties of meat products. One hundred industrially and traditionally processed and preserved fragments of tenderloin samples were prepared for histological analysis. After prelevation tissue samples were fixed with formalin solution. Samples were individually rinsed with distilled water, stained with hematoxylin-eosin and with Mallory technique. Results: muscle fibres rarely displayed their biological integrity and numerous vacuole were identified as a result of the use of brine in the industrially processed samples. Traditionally processed tenderloin displayed an unaltered muscular fibre structure. The quality of the traditionally processed product was assessed as superior when compared to the industrially processed product. Conclusion: traditional methods of preservation were assessed as giving a superior quality to the end meat product.

Key words*: histological analyses, industrial, muscle fillet, traditional.*

INTRODUCTION

Health protection is the primary objective of all EU rules and standards for agriculture, animal husbandry and the food industry (European Commission, Department - General for Agriculture and Rural Development, Department -General for Trade, *Ensuring food is safe: the veterinary and phytosanitary system of the European Union explained*, Publications Office, 2018, from https://europa.eu/european-union/topics/food-safety_ro).

The concept of food means food with nutritional and sensory properties which has a twofold role: 1) to maintain health and psycho-physical well-being, but also 2) to ensure disease prevention.

Food hides many traps for human health. Food production processes carry many critical points along the "farm to fork" journey. Primary material either gets exposed to contamination risks or, thereafter escapes various safety rules and procedures at any point in the processing chain: from the start of processing at production platforms up to the point they end up at the market stalls (Gallo et al., 2020; Dobrinas et al., 2013; Isaconi (Bulai) et al., 2019).

Our human way of life has changed profoundly over time and consequently our eating behaviors.

Modern man prefers to eat cooked food or pre-cooked food, wich is calories-rich as well as complemented by food additives. Salt is frequently used for preservation, but it has turned from a nutrient into a risk factor, being a real threat to health due to its excessive use.

The National Academy of Sciences states that "...salt improves the perception of the product's density, accentuates the sweet taste, hides metallic flavors or the taste of chemicals, having the overall effect of intensifying flavors and improving them" (Durack et al., 2008).

The World Health Organization (WHO) proposes a reduction in salt consumption to 5 g per day to reduce stroke by 23% and cardiovascular disease by 17%, which can prevent 4 million deaths globally. Recommendation goes even further for children's health with: no salt added up to the age of 14 years and 2 g per day after that until adult life. In order to reduce premature

mortality (deaths occurring before the age defined by life-expectancy at birth), WHO relaunched its international appeal to further reduce salt consumption from its current levels by 30% by 2025 (The WHO European Food and Nutrition Action Plan 2015–2020 provides a framework for action to progress towards healthy diets for all in the WHO European Region from https://www.euro.who.int/__data/ assets/pdf_file /0006/ 457611/Accelerating-salt-reduction-in-Europe.pdf).

When foodstuff composition is assessed and evaluated along with nutritional composition as well as customs and eating habits of the modern man, such evaluations can identify or confirm risks associated with nutritional imbalances. Such evaluations have the aim to draw evidence informed nutritional policies and recommend appropriate prophylactic measures.

The aim of this research is to place an emphasis on the utility of microscopic morphology for the analysis of industrially prepared fillet muscle and traditionally prepared fillet muscle; and to compare the additive content used with each of these methods of preservation.

A good quality histological analysis of any meat product can validate and confirm nutritional qualities of that product. It provides, with an in-depth insight, information on the effect and impact which some plant extracts have on the preservability as well as on the nutritional and market quality of the final product.

Interdisciplinary research remains the way forward to increasing the use of complex yet efficient and sensitive methods for the monitoring of food's quality and safety with impact on human health. This research may open new avenues in quality and safety procedures for the meat processing industry.

MATERIALS AND METHODS

The harvesting of the materials consisted of the sampling of fragments of pork and beef tenderloin prepared traditionally as well as industrially - we obtained fragments with flat faces and parallel to the side of approximately 1 cm. The tissues were processed by usual techniques, for the purpose of preservation and histological fixation, using 10% formalin solution, for a period of 48-72 hours, depending on the size of the harvested tissue. The fixative was removed by washing with distilled water. Later I included the fragments in paraffin, going through the following steps: dehydration (by passing the pieces through 6 baths of ethyl alcohol 96° for a duration of 4-6 hours for each bath), clarification (through 3 baths with benzene for a total duration of 8-9 hours), liquid paraffin impregnation (through 3 paraffin baths for 6-8 hours, in an oven at 56°C), block casting (embedding in a block of solidified paraffin). We sectioned the fragments obtained with the help of the paraffin microtome, at a size of 6 μm under a ribbon aspect. To remove the existing creases, I put the obtained sections in a heated water bath. I used the paraffin microtome in Laboratory of the discipline of Histology and Embryology of the Faculty of Veterinary Medicine of Bucharest.

We glued the sections to the slides by applying a thin layer of Mayer's ovalbumin, then they were placed in the thermostat for a very short time to achieve proper adhesion. In order to create a contrast between the tissue and cellular elements, we stained the sections using the HE (Hematoxylin Eosin) and Mallory staining methods. The staining process was preceded by dewaxing (by passing the slides in 3 successive baths of benzene for 2-5 minutes) and hydration (in 3 baths of ethyl alcohol in decreasing concentration from 90°, 80° and 70° for 5-6 minutes).

We obtained over 100 permanent histological preparations that we observed under the Motic Panthera microscope with video camera. The examination of the microscopic preparations was carried out in the laboratory of the discipline of Histology and Embryology within the Faculty of Veterinary Medicine of Bucharest. We developed and used Kiernan method (Kiernan, 2015).

RESULTS AND DISCUSSIONS

Following the microscopic examination (with the objectives of 10X, 40X and 100X) of the sections made from traditionally prepared smoked pork and beef muscle, compared to the industrially prepared one, we observed major preservation differences.

In the traditional preparation, both in pork and beef muscle, the integrity of the skeletal

striated muscle fibers has been preserved, the sarcolemma being intact, the arrangement of the nuclei is preserved. Skeletal striated muscle fibers are cylindrical in shape with nuclei located at the periphery on either side of the sarcolemma.

In the traditionally prepared pork tenderloin, the presented characteristics are supported by the sections observed under the microscope. In hematoxylin eosin staining, the cytoplasm apears pink-red, the nuclei blue-violet, the perimysium and endomysium are discolored (Figure 1).

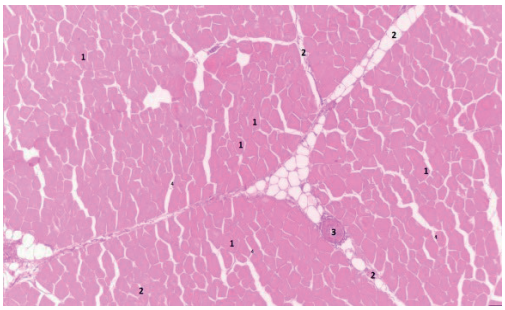


Figure 1. Traditional pork tenderloin, staining HE, ob.10 (original): 1 - transversely sectioned skeletal striated muscle fibers, 2 - perimysium, 3 - artery at the level of perimysium, 4 - endomysium

At the level of the sarcoplasm, the arrangement of the striations was preserved, which means that no noticeable changes took place at the cellular level. There are no major changes in the connective tissue. At the level of the perimysium, we observed numerous blood vessels - arteries, veins, but also nerves, arranged morphologically as in living tissues (Figure 2).

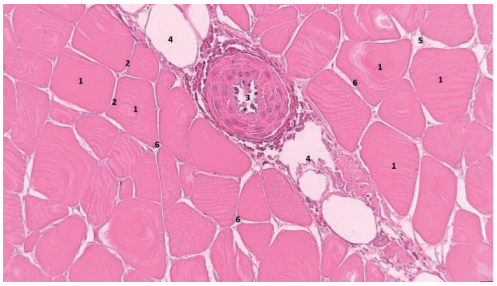


Figure 2. Traditional pork tenderloin, staining HE, ob.40 (original): 1 - transversely sectioned skeletal striated muscle fibers, 2 - sarcolemma, 3 - artery at the level of the perimysium, 4 - perimysium - numerous white fat cells, 5 - endomysium, 6 - nuclei of muscle fibers located at their periphery

Adipocytes have a typical shape with the nucleus located eccentrically, and the cytoplasm is discolored due to lipids that have been washed away by organic solvents (Figure 3).

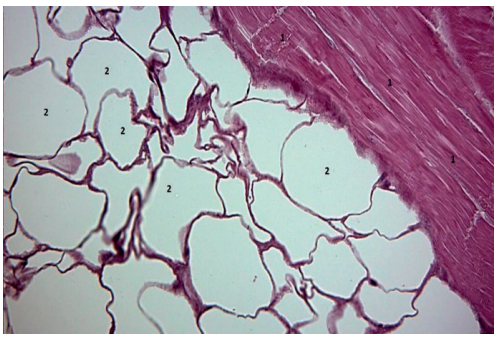


Figure 3. Traditional pork tenderloin, staining HE, ob.40 (original): 1 - longitudinally sectioned skeletal striated muscle fibers - longitudinal and transverse striations are observed, 2 - numerous white fat cells

The endomysium can be seen very well, enveloping each individual fiber, where capillaries and connective fibers are present (Figure 4).

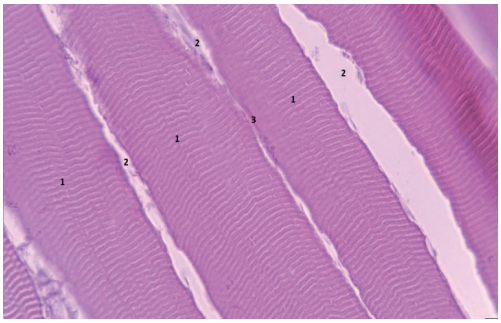


Figure 4. Traditional pork tenderloin, staining HE, ob.100 (original): 1 - longitudinally sectioned skeletal striated muscle fibers - the longitudinal and transverse striations are clearly visible, 2 - endomysium

Compared to pork muscle in cross-sectioned traditionally prepared beef muscle, the perimysium is more colorful, suggesting a richer vascularity preserved by the preparation technique, as well as muscle fibers more clearly differentiated from each other by the endomysium (Figure 5).

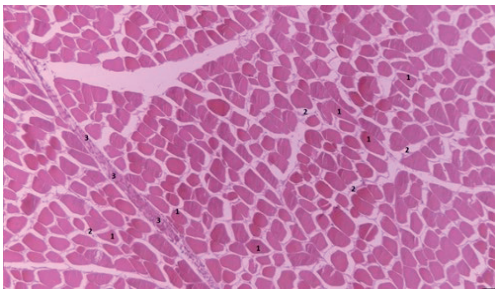


Figure 5. Traditional beef tenderloin, staining HE, ob.40 (original): 1 - transversely sectioned skeletal striated muscle fibers, 2 - endomysium, 3 - perimysium

Adipocytes show typical shape but with discolored cytoplasm because lipids have been removed by organic solvents (Figure 6).

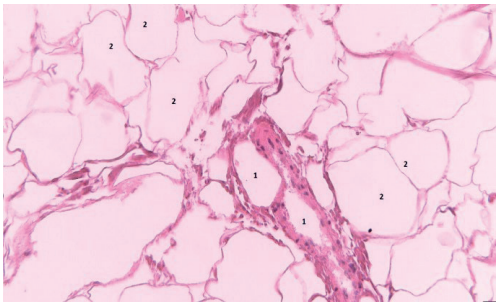


Figure 6. Traditional beef tenderloin, staining HE, ob.40 (original): 1 - blood vessels, 2 - fat cells

In Mallory staining nucleus, cytoplasm and elastic fibers are in red, red blood cells and myelin sheaths in yellow, collagen, mucus and connective tissue in blue (Figure 7).

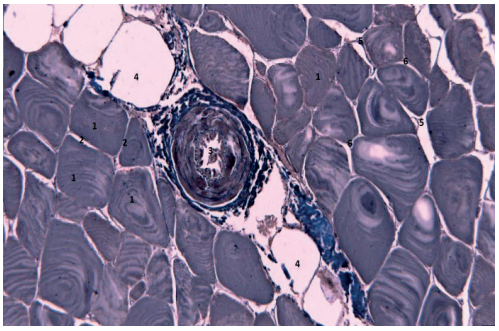


Figure 7. Traditional pork tenderloin, Mallory staining, ob.40 (original): 1 - transversely sectioned skeletal striated muscle fibers, 2 - sarcolemma, 3 - artery at the level of the perimysium, 4 - perimysium - numerous white adipose cells, 5 - endomysium, 6 - nuclei of muscle fibers located at their periphery

In the industrial preparation, the pork and beef muscle fibers show numerous vacuoles, which indicates that these muscle fillets have been injected with brine, and some fat cells are fragmented.

In the HE staining of the sections, the spacing of the spaces between the muscle fibers can be observed, the endomysium and sarcoplasm being flooded with brine (figure 8).

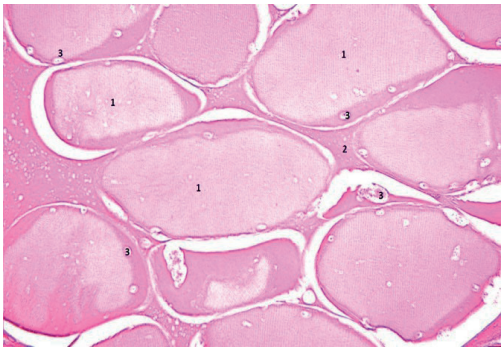


Figure 8. Industrial pork tenderloin, staining HE, ob.100 (original): 1 - transversely sectioned skeletal striated muscle fibers, 2 - endomysium, 3 - brine - both at the level of the endomysium and at the level of the sarcoplasm

In the case of industrial prepared beef muscle, in the transversal sections stained with hematoxylin-eosin, the striations of the muscle fibers are better preserved compared to industrial pork muscle, the edomysium and perimysium with preserved vasculature, as well as brine vacuoles, but in a much smaller amount compared to pork tenderloin (Figure 9).

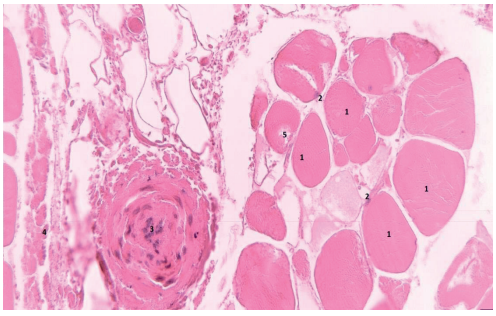


Figure 9. Industrial beef tenderloin, staining HE, ob.40 (original): 1 - transversely sectioned skeletal striated muscle fibers, 2 - endomysium, 3 - artery at the level of the perimysium, 4 - connective collagen fibers, 5 - brine vacuoles at the level of the sarcoplasm of muscle fibers

When comparing pork tenderloin with beef, we noticed that pork muscle fibers have many more vacuoles in the sarcoplasm. A brief conclusion was in the case of the beef tenderloin samples, these were injected with a smaller amount of brine (Figure 10).

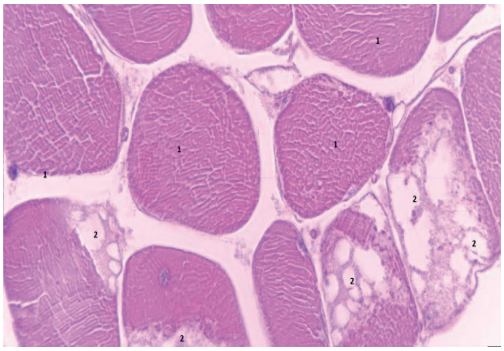


Figure 10. Industrial beef tenderloin, staining HE, ob.100 (original): 1 - transversely sectioned skeletal striated muscle fibers, 2 - brine vacuoles at the level of the sarcoplasm of muscle fibers

In Mallory's staining, collagen and connective tissue are found to be colored blue, white, discolored adipocytes, brine vacuoles at the level of the sarcoplasm are highlighted, as well as the lack of muscle fibers striations (Figure 11).

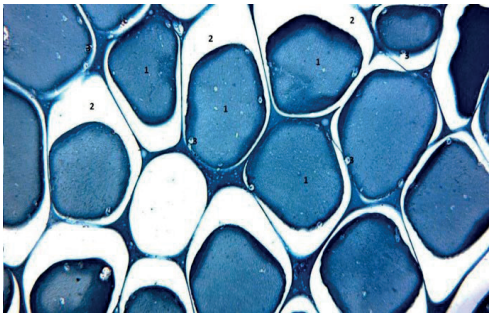


Figure 11. Industrial pork tenderloin, Mallory staining, ob.40 (original): 1 - transversely sectioned skeletal striated muscle fibers, 2 - intercellular fluid, 3 - brine vacuoles at the level of the sarcoplasm of muscle fibers

When the microscope's objective was enlarged to 100X we noticed the abundance of vacuoles of brine in industrially prepared muscle fibers, both at the level of the sarcoplasm and at the level of the endomysium (Figure 12).
In all similar samples the brine preservation method destroyed the normal histological appearance.

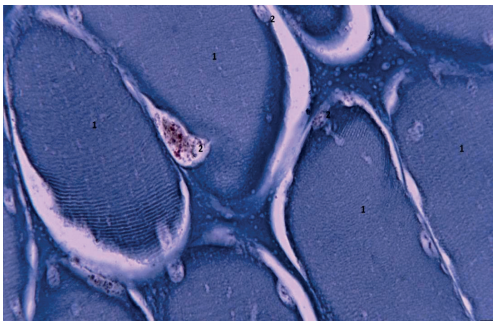


Figure 12. Industrial pork tenderloin, Mallory staining, ob.100 (original): 1 - transversely sectioned skeletal striated muscle fibers, 2 - brine vacuoles at the level of the sarcoplasm of muscle fibers

We expected to find other categories of tissues, in the observed sections, but we did not find parasites or other tissues - glandular, cartilaginous.

RESULTS AND DISCUSSIONS

With the help of optical microscopy, we were able to identify all the structures and morphological characteristics of striated muscle tissue prepared for food consumption similar to those described by Bell and Morris (2010). The special colors used allowed us to highlight the "selected" structures, with colors different from those of the other parts of the examined product, according to methods of Afanasiev (1993) and Cornilă (2011). Upon microscopic examination of pork and beef muscle sections with 10X, 40X and 100X objectives, in line with Șincai recommandations (2003), we observed that:
In the traditional preparation, the integrity of skeletal striated muscle fibers has been preserved, the sarcolemma being intact, the perimysium appears clearly around the muscle fibers, the arrangement of the nuclei is preserved, at the periphery of the fibers, the blood vessels are present (Bacha and Wood, 2011; Pavelka, 2005). Adipocytes show the typical shape with the nucleus located eccentrically, with discolored cytoplasm because the lipids have been washed away by organic solvents. These results correlate closely with the findings of Ross and Pawlina (2011) and Young and Heath (2001).
In the industrial preparation few muscle fibers kept their normal histological appearance, predominating the vacuoles which indicated

that these muscle fillets were injected with brine, the preparation method destroyed the normal histological appearance. Similar characteristics of the tissue is described by Dănacu (2015), Georgescu and Raita (2014) and Cui (2011). Adipose tissue contains fragmented fat cells (Mirancea, 2010; Yonkova et al., 2012). Comparing beef and pork tenderloins, we found a higher number of vacuoles in pork tenderloin, so it can be concluded that beef tenderloin muscle is injected with a smaller amount of brine.

CONCLUSIONS

The histological assessment and evaluation of the traditional fillet muscle structure showed 1) integrity and therefore 2) a superior quality of the end meat product in the traditionally preserved samples compared with the industrially preserved samples. Industrially preserved samples use brine. This adds to an already high salt consumption which is detrimental to human health.

ACKNOWLEDGEMENTS

This research work was carried out with the support of the staff of the Faculty of Veterinary Medicine, who helped and supported the entire work procedures for the preparation of the laboratory samples.

REFERENCES

Afanasiev, I.V., Iurina, N.A., Alio, B.V. (1993). *Research methods in histology*. Chisinau, Republic of Moldova: Ed. Universitas.

Bacha, J.JR., Wood, L.M. (2011). *Color atlas of veterinary histology, 3th edition.* New York, USA: Ed. Willy Blackwell.

Bell, S., Morris, K. (2010). *An Introduction to Microscopy.* London, UK: Ed. CRC Press.

Cazimir, I., Cornilă, N. (2011). *Practical notions of microscopic morphology, vol. II.* Bucharest, RO: Ed. Ceres.

Cui, D., Naftel, J.P. (2011). *Atlas of histology with the functional and clinical correlations.* Philadelphia, USA: Ed. Lippincott Williams & Wilkins.

Dănacu, V. (2015). *Cellular organization,* Bucharest, RO: Ed. Ars Docendi.

Dobrinas, S., Soceanu, A., Popescu, V., Stanciu, G., Suliman, S. (2013). *Quality control of some traditional meat products, Scientific Study & Research, Chemistry & Chemical Engineering, Biotechnology*, *Food Industry*, 14 (1), 29 – 40.

Durack, E., Alonso-Gomez, M. & Wilkinson, M. (2008). Salt: A Review of its Role in Food Science and Public Health. *Current Nutrition & Food Science*, 4(4), 290–297.

European Commission, Department - General for Agriculture and Rural Development, Department - General for Trade, *Ensuring food is safe: the veterinary and phytosanitary system of the European Union explained*, Publications Office, 2018, https://europa.eu/european-union/topics/food safety_ro.

Gallo, M., Ferrara, L., Calogero, A., Montesano, D., Naviglio, D. (2020). *Relationships between food and diseases: what to know to ensure food safety, Food Research International,* 137, no art. 109414.

Georgescu, B., Raita, Ș.M. (2014). *Microscopic morphology of meat and organs.* Bucharest, RO: Ed. Ceres.

Isaconi (Bulai), I.V., Gagniuc, E., Raita, Ș.M., Militaru, M. (2019). The benefits of applying the microscopic examination in the analysis of meat products, *Scientific Papers - "Ion Ionescu de la Brad" University of Agricultural Sciences and Veterinary Medicine Iasi*, vol. 62. 3, 286 – 293.

Kiernan, J.A. (2015). *Histological and Histochemical Methods. Theory and practice, 15th edition*. Banbury, UK: Ed. Scion Publishing Ltd.

Mirancea, N., Mirancea, D. (2010). *Ultrastructure of cells and tissues.* Bucharest, RO: Ed. Ars Docendi.

Pavelka, M., Roth, I. (2005). *An Atlas of tissue Biology and Pathology.* Wien, New York, USA: Ed. Springer Verlag.

Ross, M., Pawlina, W. (2011). *Histology: a Text and Atlas with Correlated Cell and Molecular Biology (international edition), 6th edition.* Philadelphia, USA: Ed. Lippincott Williams & Wilkins.

Șincai, M. (2003). *Cytohistology and specialized techniques.* Timisoara, RO: Ed. Mirton.

The WHO European Food and Nutrition Action Plan 2015–2020 provides a framework for action to progress towards healthy diets for all in the WHO European Region. https://www.euro.who.int/__data/assets/pdf_file/0006/457611/Accelerating-salt-reduction-in-Europe.pdf.

Yonkova, P., Atanasova, P., Vachkova, E., Dimitrov, R., Yovchev, D., Serbest, A., Arican, I. (2012). *Morphological changes in adipocytes from rabbit fat depots.* Bulg.J.Vet.Med. 15 Suppl. 1, 114.

Young, B., Heath, W.J. (2001). *Wheater's Functional Histology. A text and colour atlas, 4th edition.* Edinburgh, London: Ed. Churchill Livingstone.

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

MICROSCOPIC DIFFERENCES BETWEEN MUSCLE TISSUE IN BIRDS RAISED IN HOUSEHOLD VS. INTENSIVE SYSTEM

Mihai DINICĂ, Andrei CĂLINOIU, Andreea NEACŞU-NIŢU, Cristian MARIN, Ştefania Mariana RAITA

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania, 59 Marasti Blvd, Bucharest, Romania

Corresponding author email: dinica_m@yahoo.com

Abstract

Following numerous pieces of research that highlighted the value of histological techniques applied in assessing the quality and safety of meat food products, a histological examination has become mandatory in many countries. This paper describes in detail the quality characteristics of poultry meat from two different rearing systems. Fragments were taken from the breast of chickens raised in a household system and an intensive industrial system. The fragments taken were processed using the usual histological techniques and stained by the H & E (Hematoxylin & eosin) and Mallory staining methods. After analyzing the permanent histological preparations, numerous differences were observed between the two growth systems. In the household system, the muscle fibers are more developed, which shows that they do a lot of movement, at the same time, the white adipose tissue is less represented. In the industrial system, the muscle fibers show a smaller number of striations, which indicates that they do no movement, a large amount of white adipose tissue being observed. Through the histological evaluation of the chicken muscle tissue from the two different growth systems, a series of differences resulted.

Key words*: intensive system, household system, chicken meat.*

INTRODUCTION

Chicken meat is a healthy source of animal protein. It has lower rearing costs and higher efficiency than other meat species (Dalle Zotte et al., 2020). Chicken comes mainly from fast-growing (FG) broilers (e.g., Ross 308 broilers) and slow-growing (SG) broilers (e.g., Rouge label).

Slow-growing broilers complete their life cycle at a more mature age (usually at least 81 days) than conventional fast-growing lines (between 35 and 42 days) (Chabault et al., 2012). Muscle fibers are directly related to poultry growth rate and meat quality traits (Joo et al., 2013).

The number of muscle fibers formed before birth and the size of these fibers after birth determine final muscle mass, while muscle fiber characteristics affect both appearance and feed quality traits in poultry (Ismail and Joo, 2017). Fiber characteristics include fiber type composition and fiber morphological features.

For birds, skeletal muscles comprise mainly glycolytic fibers (type II, fast twitch) and oxidative fibers (type I, slow twitch). However, compared to animals, the skeletal muscles of poultry contain a relatively small number of oxidative fibers. Several studies have reported that the chicken breast in broilers consists entirely of 100% glycolytic fibers, regardless of breed (Roy et al., 2006; Verdiglione and Cassandro, 2013).

MATERIALS AND METHODS

Fragments of chicken breast raised in a household system but also raised in an intensive system were taken.

From a histological point of view, the collection of samples was carried out to preserve and fix them with formalin solution, at 10% concentration.

The duration of fixation was 24 hours, varying depending on the size of the extracted tissue. Afterward, the fixed piece was washed with distilled water to remove the fixing solution.

We integrated the sections into the coverslips by applying a thin layer of Mayer's ovalbumin, then they were placed in the thermostat for a very short time to achieve adequate adhesion.

To create a contrast between tissue and cellular elements, we stained the sections using the H&E

(hematoxylin-eosin) and Mallory staining methods.

The staining process was preceded by deparaffinization (by taking the slides in 3 successive baths of benzene for 2-5 minutes) and hydration (in 3 baths of ethyl alcohol in decreasing concentration from 90°, 80° and 70° for 5 -6 minutes).

Over 30 permanent histological slides were obtained which were studied with the help of the Motic Panthera microscope which has a video camera.

The examination of the microscopic slides was carried out in the laboratory of the discipline of Histology and Embryology within the Faculty of Veterinary Medicine of Bucharest.

RESULTS AND DISCUSSIONS

The present article does show the differences that can appear at the level of muscle fibers originating from the growth passage in two different growth systems.

In the case of chickens raised in the extensive system, the muscle fibres have a compact structure compared to the muscle fibres from chickens raised in the intensive system.

For chickens raised in the extensive system, reduced content of adipose tissue could be remarked, and skeletal striated muscle fibers show numerous striations at the level of the sarcolemma, the endomysium, which normally contains an abundant connective tissue that forms the sheath that envelops each muscle fibers is inadequately developed, which does not indicate that the birds raised in the extensive system have enough space for movement, which can lead to developed musculature and reduced adipose tissue.

Chicken breast - extensive system

Skeletal striated muscle fiber has a cylindrical shape, unbranched, with slightly rounded ends. It has a particularly long length that can reach up to 35-40 centimeters (Figure 1).

The endomysium is represented by the connective tissue that forms a thin sheath that envelops each muscle fiber.

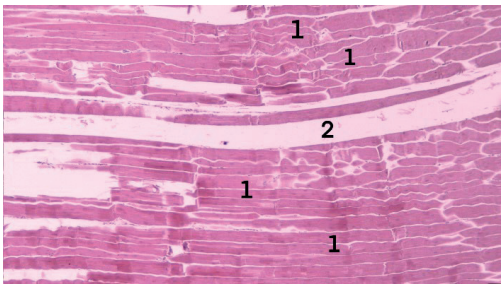


Figure 1. Chicken breast grew in an extensive system, H & E stain, obj. 10X: 1. Skeletal striated muscle fibers in the longitudinal section; 2. Endomysium - loose connective tissue

It includes a network of reticulin fibers, collagen fibers, and fibroblasts. The endomysium is inadequately developed in chicken breasts raised in the extensive system (Figure 2).

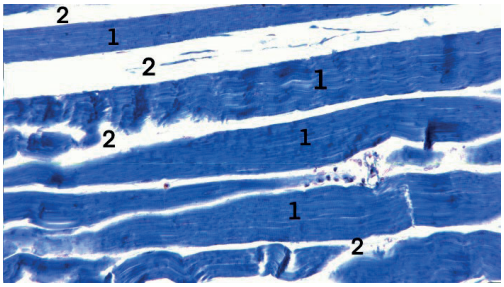


Figure 2. Chicken breast grew in the extensive system, Mallory stain, Obj. 10X: 1. Skeletal striated muscle fibers in the longitudinal section; 2. Endomysium

The endomysium is inadequately developed (Figure 3), and the sheaths formed by it are hardly visible.

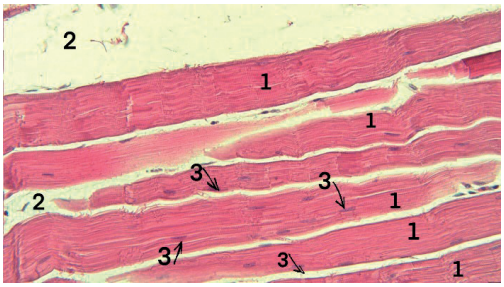


Figure 3. Chicken breast grew in an extensive system, H & E stain, obj. 40X: 1. Skeletal striated muscle fibers in the longitudinal section; 2. Endomysium; 3. Fiber nuclei muscles located on the periphery

The muscle fibers are well developed (Figure 4), the striations are accentuated and can be seen along the entire length of the fiber.

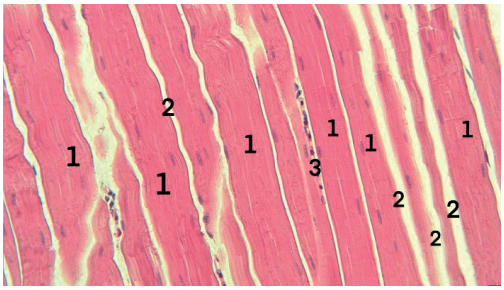


Figure 4. Chicken breast grew in an extensive system, H & E stain, obj. 40X: 1. Skeletal striated muscle fibers in the longitudinal section; 2. Nuclei of muscle fibers located at the periphery; 3. Capillary from the endomysium

The thickness of the fiber and the striations are well developed, and the nuclei are evident (Figure 5).

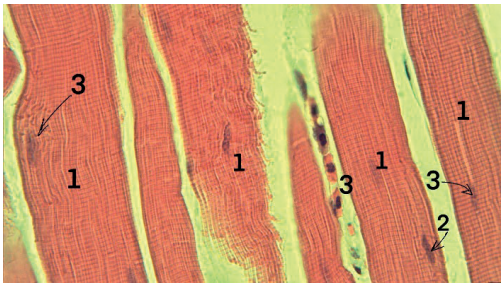


Figure 5. Chicken breast grew in an extensive system, H & E stain, obj. 100X: 1. Skeletal striated muscle fibers in the longitudinal section - the transverse and longitudinal striations at the level of the sarcoplasm; 2. The nuclei of the muscle fibers are located at the periphery; 3. Capillary from the endomysium

The thickness of skeletal striated muscle fibers is uniform at birth, but it increases with age, differentiated according to the degree and type of stress on different muscle groups.
Skeletal striated muscle fibers are well developed in chickens raised in the extensive system, being very thick and the striations very obvious (Figure 6).

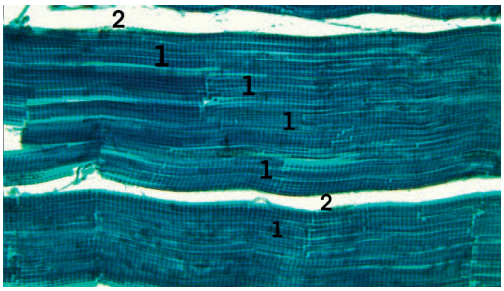


Figure 6. Chicken breast grew in an extensive system, Mallory stain, Obj. 100X: 1. Skeletal striated muscle fibers in the longitudinal section - the transverse and longitudinal striations can be observed at the level of the sarcoplasm; 2. Endomysium

For the chicken raised in the intensive system, a significantly reduced number of striations were observed at the level of the sarcoplasm, nuclei are not very evident, the endomysium forms a thick sheath that covers the muscle fiber, adipose tissue is found in large quantities and is predominant along the entire length of the fiber. This shows us the fact that birds do not have much space to move, they are raised in battery cage system. Another result that could be observed microscopically was the epimysium, which is the tissue that envelops the muscle, is highly developed and predominant (Figure 4).

Chicken breast - intensive system

The epimysium or external perimysium is a connective tissue that wraps the muscle, as an anatomical organ, anchoring it to the fascia.
In chickens raised in the intensive system, the epimysium is highly developed, it infiltrates between the muscle fibers and predominates along the entire length of the muscle fiber (Figure 7).

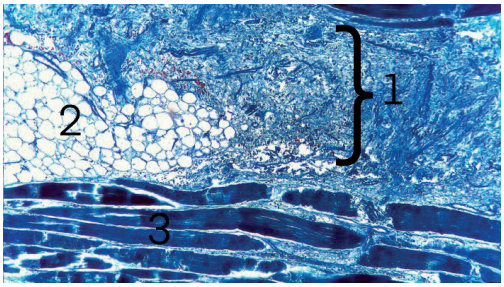


Figure 7. Chicken breast grew in an intensive system, Mallory stain, Obj. 10X: 1. Epimysium; 2. White adipose connective tissue; 3. Skeletal sciatic muscle fibers in the oblique section

The adipose cell has a spherical or ovoid shape, rarely appearing polyhedral (Figure 8).
The semilunar nucleus is eccentrically.
The cytoplasm is quantitatively reduced, being mostly replaced by a single drop of fat.

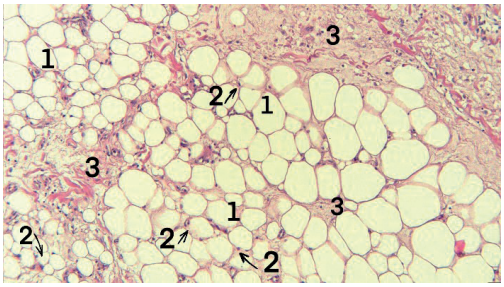


Figure 8. Chicken breast grew in an intensive system, H & E stain, obj. 10X: 1. White adipose connective tissue - adipocytes; 2. Adipocyte nuclei located at the periphery; 3. Connective matrix

Tunica media at the level of the muscular artery consists mainly of leukocytes, arranged between 2 and 20 concentric circular layers.
Elastic and collagen fibers are found among the muscle cells (Figure 9).
The two elastic limits (internal and external) appear obvious, strongly wavy, rich in glycosaminoglycans (Figure 9).

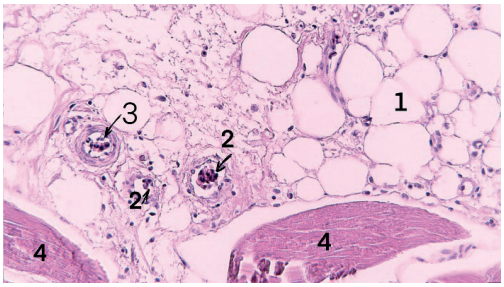


Figure 9. Chicken breast raised in an intensive system, H & E stain, obj. 40X: 1. White adipose connective tissue – adipocytes; 2. Blood vessel - vein; 3. Blood vessel - artery; 4. Striated muscle fibers - in the oblique section

Adipocytes have an eccentrically located nucleus, the cytoplasm is discoloured due to the lipids that have been washed due to organic solvents. The adipose tissue is very well developed, and the muscle tissue is barely visible (Figure 10)

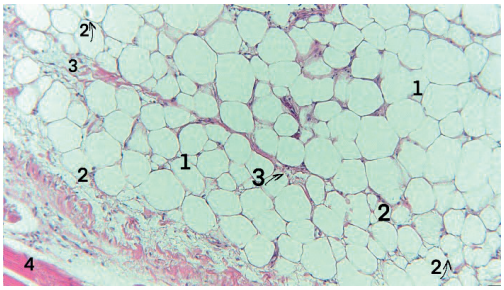


Figure 10. Chicken breast raised in an intensive system, H & E stain, obj. 40X: 1. White adipose connective tissue - adipocytes; 2. Adipocyte nuclei located at the periphery; 3. Connective matrix; 4. Striated muscle fibers - in the longitudinal section

The endomysium is well developed, envelops each muscle fiber, and presents a large thickness of the sheath that surrounds the muscle fiber (Figure 11).

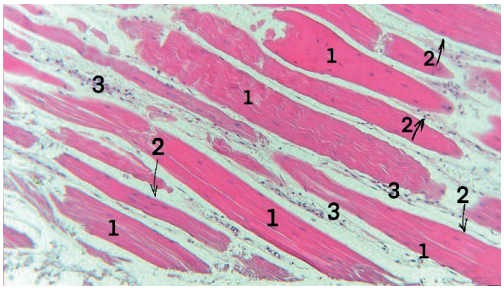


Figure 11. Chicken breast grew in an intensive system, H&E stain, obj. 40X: 1. Skeletal striated muscle fibers in the longitudinal section; 2. Nuclei of muscle fibers located at the periphery; 3. Capillary from the endomysium

The endomysium is visible, surrounding each fiber if capillaries and connective fibers are present (Figure 12).

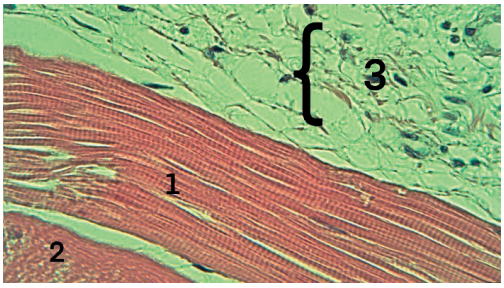


Figure 12. Chicken breast grew in an intensive system, H & E stain, obj. 100X: 1. Skeletal striated muscle fibers in the longitudinal section; 2 Skeletal striated muscle fibers in the oblique section; 3. Endomysium

CONCLUSIONS

Examining the 30 slides with the 4x, 10x, 40x and 100x objectives with the usual stains and H & E and Mallory, we were able to observe clear differences at the level of the chicken breasts from the two rearing systems, respectively the extensive system and the intensive chicken rearing system.

In conclusion, we can highlight that birds which have been raised in the extensive system, present many striations at the level of skeletal striated muscle fibers, the muscle fiber is better developed, the endomysium is hardly visible, and the adipose tissue is almost absent.

This was since the birds raised in the extensive system had enough space for movement, which led to a better development of the muscles.

For chicken raised in an intensive system, the muscle fiber is inadequately developed, with a reduced number of striations, the endomysium is rich in connective tissue, the sheath formed for each muscle fiber is thick, adipose tissue is abundant, the epimysium predominates in muscle fibers and all this indicates that the birds do not have enough space to move, which is due to the fact that the birds are raised in battery cages system.

REFERENCES

Chabault, M., Baéza, E., Gigaud, V., Chartrin, P., Chapuis, H., Boulay, M., Arnould, C., D'Abbadie, F., Berri, C., & Bihan-Duval, E. L. (2012). Analysis of a slow-growing line reveals wide genetic variability of carcass and meat quality-related traits. *BMC Genetics*, *13*(1).

Ismail, I., & Joo, S. (2017b). Poultry Meat Quality in Relation to Muscle Growth and Muscle Fiber Characteristics. *Korean Journal for Food Science of Animal Resources*, *37*(6), 873–883.

Joo, S. W., Kim, G. N., Hwang, Y., & Ryu, Y. (2013). Control of fresh meat quality through manipulation of muscle fiber characteristics. *Meat Science*, *95*(4), 828–836.

Roy, B. K., Oshima, I., Miyachi, H., Shiba, N.,

Nishimura, S., Tabata, S., & Iwamoto, H. (2006). Effects of nutritional level on muscle development, histochemical properties of myofibre and collagen architecture in the pectoralis muscle of male broilers. *British Poultry Science*, *47*(4), 433–442.

Verdiglione, R., & Cassandro, M. (2013). Characterization of muscle fiber type in the pectoralis major muscle of slow-growing local and commercial chicken strains. *Poultry Science*, *92*(9), 2433–2437.

Weng, K., Huo, W., Li, Y., Zhang, Y., Zhang, Y., & Xu, Q. (2022). Fiber characteristics and meat quality of different muscular tissues from slow- and fast-growing broilers. *Poultry Science*, *101*(1), 101537

Zotte, A. D., Gleeson, E., Franco, D., Cullere, M., & Lorenzo, J. M. (2020). Proximate Composition, Amino Acid Profile, and Oxidative Stability of Slow-Growing Indigenous Chickens Compared with Commercial Broiler Chickens. *Foods*, *9*(5), 546.

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

THE IMPACT OF AGE ON SOME BIOCHEMICAL PARAMETERS IN FELINES WITH CHRONIC KIDNEY DISEASE

Ioana-Nicole REU, Iuliana CODREANU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: ioanareu@yahoo.com

Abstract

According to specialty literature, the incidence of chronic kidney disease in felines had substantially risen in the recent years. This research is intended to show a correlation between the increasing age of felines (over 10 years) and the variations of some biochemical parameters (urea, creatinine, phosphorus and also symmetric dimethylarginine) in the progression of the chronic kidney disease. The research was performed over a period of two years at the University Emergency Hospital "Prof. univ. dr. Alin Bîrțoiu", Bucharest, on a total of 20 cases of felines suspected of chronic kidney disease, 10 of them older than 10 years were considered suitable for this study. One feline, an 11-year-old patient in uremic coma (n = 1) had the highest serum phosphorus, urea, creatinine and symmetric dimethylarginine levels. The other nine patients (n = 9) between 11 and 15 age had urea levels higher than 79.2 mg/dl, creatinine levels above 2 mg/dl and serum phosphorus levels higher than 7.35 mg/dl.

Key words*: felines, chronic kidney disease, biochemical parameters.*

INTRODUCTION

Chronic kidney disease (CKD) is the most frequent metabolic disease in domestic felines, especially in senior cats (>10 years old). In older felines, chronic kidney disease (CKD) is a common diagnosis (Reynolds & Lefebvre, 2013). The prevalence of chronic kidney disease is approximately 2-4% overall, in felines aged 1 and 2 years old and increases to 30-40% in felines older than 10 years. The prevalence of CKD in felines is higher than in dogs and the incidence of CKD diagnoses in felines has increased over the past few decades (Brown & Elliott, 2016; Markovich et al., 2015).

Additional risk factors for developing CKD, other than age have not been identified in felines, but weight loss or poor body condition, polyuria/polydipsia (PU/PD), higher creatinine concentrations, dehydration and potentially lower urine specific gravity (USG) may indicate the presence, or predict development of CKD (Bartlett P.C. et al., 2010).

The classical biomarker of chronic kidney disease (CKD) in felines - the serum creatinine concentration has significant limitations that reduce its value as an early CKD biomarker. New serum biomarkers, such as symmetric dimethylarginine (SDMA), could help in identifying felines with chronic kidney disease (CKD) before traditional indicators of glomerular filtration rate (GFR), such as urea and creatinine, would show significant increase, above the normal reference range (Syme & Elliot, 2016). Symmetric dimethylarginine (SDMA), also highlights the deviation from the normal functioning of the glomerular filtration rate, when 25% of the nephrons are damaged or destroyed. However, it is important to note that significant nephron loss has already occurred by the time, symmetric dimethylarginine (SDMA) is persistently above the reference range (Hall & Yerramilli, 2014).

It is possible to stage chronic kidney disease based on creatinine and SDMA levels. Therefore, in stage 1, creatinine will be 1.6 mg/dl and symmetric dimethylarginine <18 µg/dl; in stage 2, creatinine will be 1.6-2.8 mg/dl and symmetric dimethylarginine 18-25 µg/dl; in stage 3, creatinine will be 2.9-5 mg/dl and symmetric dimethylarginine 26-38 µg/dl and in the final stage, creatinine will be >5 mg/dl and symmetric dimethylarginine >38 µg/dl. Other researchers characterize the evolution and stage of the chronic kidney

disease just based on creatinine levels (Kidder & Chew, 2009).
Phosphorus retention during chronic renal failure has negative effects on renal function, renal histopathology and soft tissue mineralization in the kidneys (Syme & Elliot, 2016).

MATERIALS AND METHODS

This study was performed over a two-year period at the University Emergency Hospital "Prof. univ. dr. Alin Bîrțoiu" in Bucharest and at the Vietatis - The Vets Clinic of Bucharest, on a total of 20 felines suspected of chronic kidney disease; 10 of these felines, older than 10 years old were considered suitable for this research, all of them belonging to the European race. Each patient was clinical and paraclinical examined, by general methods and special methods, such as biochemical investigations (urea, creatinine, phosphorus, symmetric dimethylarginine).
Symmetric dimethylarginine test was performed in 5 of the 10 patients.
All study participants were in the third or fourth stage of chronic kidney disease.
Patients were classified into one of four CKD stages based on the creatinine and symmetric dimethylarginine levels, as well as creatinine levels independently.
The results obtained from the paraclinical examinations and the physiological interval values were specified in each individual case.
Skyla, IDEXX and Spotchem SP devices were used to perform the biochemical examinations.

RESULTS AND DISCUSSIONS

In this study, the biochemical examination results of 10 felines of varying ages are analysed: two patients aged 10 years, three aged 15 years, two aged 12 years, one feline aged 11 years, one aged 13 years and one feline aged 18 years. All of these patients had extremely elevated creatinine and urea levels compared to the physiological values for their species. Only one patient presented a serum phosphorus level within the physiological range; the rest presented elevated levels, which may be associated with chronic kidney disease. Regarding the symmetric dimethylarginine, which was performed on only half of the studied patients, it revealed a significant decrease in the glomerular filtration rate; this result was obtained before the other biochemical parameters were measured. The values of creatinine and symmetric dimethylarginine are highly correlated. The relationship between the two parameters is displayed in the table below (Table 1):

Table 1. The values of creatinine and symmetric dimethylarginine (SDMA) in patients classified in fourth stage of chronic kidney disease

Pacient's age	Creatinine (mg/dl)	SDMA (µg/dl)
15 years old	7.2	26
10 years old	19.01	35
18 years old	8.8	27
13 years old	10.2	28
12 years old	8.34	28

The normal range for creatinine is between 0.4 and 1.6 mg/dL and the normal range for symmetric dimethylarginine is less than 15 ug/mL. The only 10-year-old patient had a creatinine value of 19.01 mg/dl and a symmetric dimethylarginine value of 25 µg/dL and was presented in a uremic coma upon entering the clinic. Based on the values in Table 1, all five patients are classified as having stage 4 chronic kidney disease. Two additional 15-year-old patients were classified as being in the third stage of chronic kidney disease, despite having only creatinine determined and symmetric dimethylarginine not, their results are presented in Table 2:

Table 2. The values of creatinine in patients classified in third stage of chronic kidney disease

Patient's age	Creatinine mg/dl
15 years old	3.45
15 years old	3.64
11 years old	4.25

Having the lowest creatinine levels, these three patients had a more favourable evolution over a shorter period of time than the rest of the patients in the study, whose creatinine levels were well above the reference range (0.4-1.6 mg/dL).
All cats participating in this study had their levels of creatinine, urea and phosphorus

measured. These values varied according to age, with all patients being over 10 years old except for one who presented with uremic coma. The parameters' values are presented in Table 3.

Table 3. Biochemical parameters values (creatinine, urea and phosphorus) in all studied patients

Number of patient	Patient's age	Creatinine (mg/dl)	Urea (mg/dl)	Phosphorus (mg/dl)
1	10 years old	19.01	637	27.21
2	11 years old	4.26	168	8.2
3	12 years old	8.34	291	15.2
4	12 years old	5.5	188	7.8
5	13 years old	10.2	272	>18
6	15 years old	3.45	191	7.35
7	15 years old	3.64	134	6.76
8	15 years old	7.2	197	8.5
9	18 years old	8.8	290	10.7
10	18 years old	7.3	185	8.7

In this study, 10 female felines were examined; one was 10 years old one was 11 years old, two were 12 years old, one was 13 years old, three were 15 years old and two were 18 years old. Regarding the biochemical parameters, the highest values were recorded in the 10-year-old patient, who presented in a uremic coma (presented increased values above the limit that the analyser could register: creatinine - 19.01 mg/dl; urea - 637 mg/dl; phosphorus - 27.21 mg/dl and as symptoms presented: hypothermia, apathy, lethargy, depression, severe dehydration, uraemic breath, elevated serum urea nitrogen and creatinine concentrations and possibly seizures and coma prior to death) and in the 13-year-old patient (creatinine - 10.2 mg/dl; urea - 272 mg/dl; phosphorus - >18 mg/dl), who presented with severe hyperphosphatemia.

Patients classified as being in the third stage of chronic kidney disease, two cats aged 15 years (creatinine - 3.45 mg/dl; urea - 191 mg/dl; phosphorus - 7.35 mg/dl/ creatinine - 3.64 mg/dl; urea - 134 mg/dl; phosphorus - 6.76 mg/dl) and one aged 11 years (creatinine - 4.26 mg/dl; urea - 168mg/dl; phosphorus - 8.2 mg/dl), presented with urinary sediment and feline urological syndrome in the past and their diet consisted of special urinary care diet. They presented urinary sediment, which was analysed at a different clinic, where it was discovered to contain struvites, requiring specific treatment for dissolution. The two 18-year-old cats had high creatinine and urea levels (creatinine - 8.8 mg/dl; urea – 290 mg/dl/ creatinine - 7.3 mg/dl; urea - 185 mg/dl) due to a history of chronic kidney disease in their mother's lineage. However, they didn't present hyperphosphatemia (phosphorus - 10.7 mg/dl; phosphorus - 8.7mg/dl) and these levels could be decreased using the appropriate treatment.

The 12-year-old patient (creatinine - 5.5 mg/dl, urea -188 mg/dl, phosphorus - 7.8 mg/dl) is on the limit between the third and fourth stages of chronic kidney disease (creatinine values > 5 mg/dl are considered to be in the fourth stage), but had the best evolution of the general condition and implicitly of the creatinine and urea values, after the initiation of treatment. The second patient aged 12 years (creatinine - 8.34, urea - 291 mg/dl, phosphorus - 15.2 mg/dl) who was administered the same treatment showed decreases in values after a longer period of time than the first patient aged 12 years, also presenting hyperphosphatemia. Phosphate retention is a major contributor to the progression of chronic kidney disease in many species and it is well known that hyperphosphatemia is associated with a significant mortality risk in humans with end-stage renal disease.

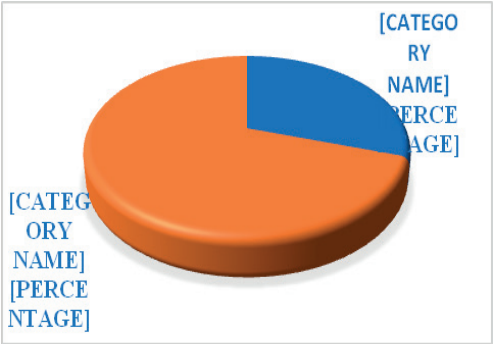


Figure 1. Identifying patients by gender

As concerning the gender, it can be observed in Figure 1 that more than half of the patients are

male (70%), with 50% of them being neutered (the scientific literature indicates that this could be a risk factor for developing chronic kidney disease), and 30% of the subjects being spayed females.
Also, the two felines aged 15 years and one aged 11 years that had previously presented feline urological syndrome and sediment were neutered males.

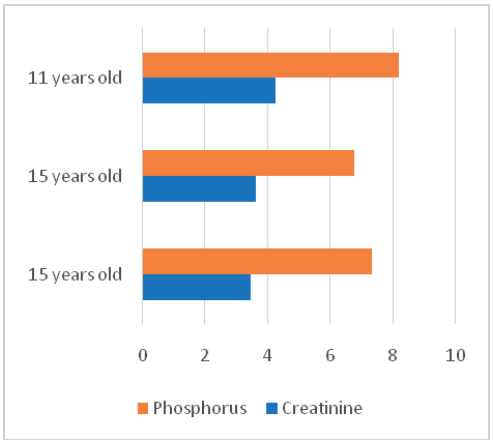


Figure 2. Creatinine and phosphorus levels in patients with third stage chronic kidney disease

Frequently, the third stage of chronic kidney disease is accompanied by hyperphosphatemia. The second 15-year-old patient presented a serum phosphorus value of 6.76 mg/dl (the reference range is 2.7-6.5 mg/dl), which cannot be considered hyperphosphatemia, unlike the first 15-year-old patient, who had a slightly elevated phosphorus level compared to the maximum value (7.35 mg/dl). Throughout their lives, the two cats consumed food with a low phosphorus content and high-quality protein, which played a crucial role in maintaining the phosphorus level within normal or close to normal limits.
The 11-year-old patient's phosphorus level was 8.2 mg/dl, also due to its diet that did not consist of high-quality food.

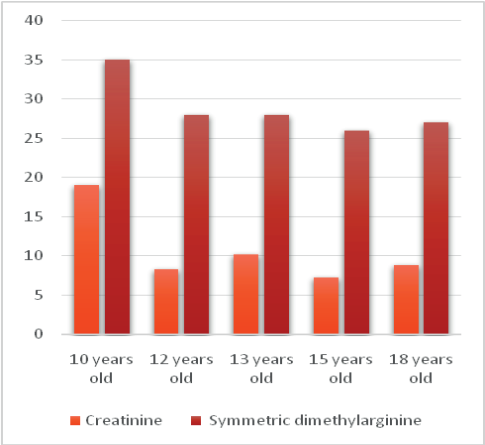


Figure 3. Creatinine and symmetric dimethylarginine values in patients with chronic kidney disease in the fourth stage

According to IRIS, based on the results of creatinine and SDMA in this study, 5 of the patients could be classified as being in the fourth stage of chronic kidney disease (CREA >5 mg/dl, SDMA >35 µg/dl). Although not all patients presented SDMA levels significantly above the normal range, they received the therapeutic protocol for stage 4 CKD, because they have creatinine levels that are significantly above the normal range.

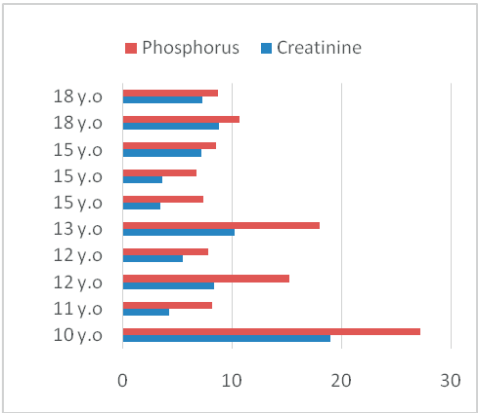


Figure 4. Graphic representation of creatinine and phosphorus levels, in all the studied patients

The highest serum phosphorus concentrations were found in patients aged 10 (27.21 mg/dl), 12 (15.2 mg/dl), 13 (>18 mg/dl) and 18 years, with concentrations indicating a severe hyperphosphatemia.
Shortly after reaching the clinic, the 10-year-old died of uremic coma.
However, with treatment, the phosphorus levels of the 12 and 13-year-olds were reduced to 7.5-8 mg/dL in two weeks. As concerning the 18-year-old patient with elevated phosphorus levels, it received long term (approximately one month) hyperphosphatemia treatment.

CONCLUSIONS

Age, particularly after 10 years, has a crucial role in the occurrence and progression of chronic kidney disease in felines, associated with food and other renal or urinary pathologies (feline urological syndrome).
The results of biochemical examination (creatinine, urea, phosphorus and symmetric dimethylarginine (SDMA) elevated levels were detected in three of the studied patients, indicating the extent of glomerular filtration damage.
These cats had a pathological history of feline urological syndrome and their diet consisted of low-quality protein and high phosphorus dry or canned food.

ACKNOWLEDGEMENTS

The research was carried out as part of an extensive study, the preliminary results being part of the PhD Thesis: "*Study on correlations between serum phosphorus level and different stages of feline chronic kidney disease"*.

REFERENCES

Bartlett P.C., Van Buren J.W., Bartlett Ad., et al. (2010). Case-control study of risk factors associated with feline and canine chronic kidney disease. *Vet Med Int*; 2010: 957570

Braff J., Obare E., Yerramilli M., Elliott J. (2014). Relationship between serum symmetric dimethylarginine concentration and glomerular filtration rate in cats. *J Vet Intern Med.*;28(6):1699-1701. doi:10.1111/jvim.12446

Brown, C.A., Elliott, J., Schmiedt, C.W., & Brown, S.A. (2016). Chronic Kidney Disease in Aged Cats: Clinical Features, Morphology, and Proposed Pathogeneses. *Veterinary pathology,* 53(2),309–326. https://doi.org/10.1177/0300985815622975

Caney Sm. (2017). An online survey of dietary and phosphate binder practices of owners of cats with chronic kidney disease. *Journal of Feline Medicine and Surgery,* 19(10):1040-1047. doi:10.1177/1098612X16672999

Hall J.A., Yerramilli M., Obare E., Jewell D.E. (2014) Comparison of serum concentrations of symmetric dimethylarginine and creatinine as kidney function biomarkers in cats with chronic kidney disease. *J Vet InternMed*.;28(6):1676-1683. doi:10.1111/jvim.12445

Jepson R.E., Brodbelt D., Vallance C., et al. (2009). Evaluation of predictors of the development of azotemia in cats. *J VetIntern Med*; 23: 806–813.

Kidder A.C., Chew D. (2009). Treatment Options for Hyperphosphatemia in Feline CKD: What's Out there? *Journal of Feline Medicine and Surgery.*;11(11):913-924. doi:10.1016/j.jfms.2009.09.01

Markovich J.E., Freeman L.M., Labato M.A., Heinze Cr. (2015). Survey of dietary and medication practices of owners of cats with chronic kidney disease. Journal of Feline Medicine and Surgery. 17(12):979-983. doi:10.1177/1098612X14563097

Reynolds, B.S., & Lefebvre, H.P. (2013). Feline CKD: Pathophysiology and risk factors--what do we know?. Journal of feline medicine and surgery, 15 Suppl 1, 3–14. https://doi.org/10.1177/1098612X13495234

Syme H.M., Elliott J. (2016). Risk factors for Development of Chronic Kidney Disease in Cats, Journal of veterinary internal medicine, 30(2), 602–610. https://doi.org/10.1111/jvim.13917

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

EVOLUTION OF THE REPRODUCTIVE SYSTEM MORPHOLOGY IN ROOSTERS FED ON VITAMIN A AND VITAMIN E SUPPLEMENTED DIETS

Diana ȘIPOTEANU, Mădălina CIOARIC, Ivona ZĂBAVĂ, Emilia CIOBOTARU-PÎRVU, Nicolae DOJANĂ

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: dianamogosh@yahoo.ro

Abstract

The paper presents the effects of long-term dietary supplementation with vitamin A and vitamin E on the testicle and epididymis morphology in roosters. The roosters were fed on diets enriched in vitamin A (600 IU/kg of diet) and/or vitamin E (60 IU/kg of diet) from 40 to 57 weeks of age. Histology and morphometry studies were performed on the testicle and on epididymis duct of the experimentally fed rooster. A better preservation of the seminiferous epithelium, refinement of seminiferous pericanalicular connective tissue, small islands of Leydig cells as well as the relative maintenance of the richness of the seminiferous pericanalicular blood vasculature are noted for vitamin supplemented groups versus control. Both vitamins diminished the ageing effects on the thickness and structure of the epididymis epithelium. Both vitamins prolonged the maintenance of Sertoli cell density ($P < 0.01$ versus control). The lumen epididymis fluid contains smaller amount of detached cytoplasmic fragments, cilia, and nuclei versus control. Vitamin A mainly protects the spermatogenesis line, while vitamin E mainly protects Sertoli and Leydig cells. No mutual inhibition or potentiating effects of the two vitamins were revealed.

Key words*: epididymis, rooster, testicle, vitamin A, vitamin E.*

INTRODUCTION

The use of antioxidant vitamins in poultry practice to improve the reproductive performance of roosters is well known. Among the most frequently used vitamins in this regard are the vitamin A and the vitamin E. Many scientific works are focused on the effects of these vitamins on some characteristics on semen biological properties such as ejaculate volume, semen density, semen motility, semen viability as well as on morphological characteristics of spermatozoa (Danikowski et al., 2002; Biswas et al., 2009; Baba & Asrol, 2017; Bălăceanu et al., 2019). Some scientific works are focused on the influence of antioxidant vitamins on the macro- and microscopic structure of the reproductive system of roosters. Thus, Sukmawati et al. (2019) studied the effect of vitamin E on testicle histology (in rats) and reported there is an ameliorative effect of vitamin E on testicle histology structure such as tubules diameter, epithelial thickness, Sertoli cell number, and antigen binding protein levels of rats. Saddein et al. (2019) reported the protective effect of vitamin E on the seminiferous epithelium in guinea pig males in experimental mancozeb intoxication. Yokota et al. (2018) reported a decrease in testicular volume in mice fed excess vitamin A (1000 IU/kg diet from 3 to 10 weeks of age), an effect qualified by the authors as toxic. Bosakowski et al. (1988) found no changes in testicular volume in rats fed diets containing twice the amount of vitamin A administered by Yokota et al. (2018). Triques et al. (2019) investigated the long-term effect (from hatching to 66 weeks of age) of other antioxidants (vitamin C, canthaxanthin and lycopene) on testis morphometry properties in roosters of different breeds and they found that supplementation with an antioxidant blend composed of canthaxanthin, vitamin C, and lycopene in roosters led to higher testicles (as length, thickness, width, and weight). Thus, the data regarding the effects of long-term treatments with vitamin A or E on testicular and epididymal morphology in roosters are rare. The purpose of this work was to determine the

effects of long-term vitamin A and vitamin E dietary supplementation on testicular morphometry, on the structure of the seminiferous epithelium and on the structure and epididymis morphometry in hybrid Cornish roosters.

MATERIALS AND METHODS

The experiment was carried out on 40-week-old Cornish hybrid roosters. A number of 64 animals were involved, divided in four groups (16 animals each one): a control group, an A group, an E group and an A+E group. The animals were housed and maintained in compliance with the technological norms of industrial breeding. All groups were fed on a commercial diet based on maize 35.6%, wheat 27.4%, soy extruded 21.3% and containing 15.43% crude protein, 3.89% calcium, 0.39% phosphorus, and 2,880 kcal/kg ME (as calculated values). Furthermore, group A diet was supplemented with 180 μg vitamin A (as retinal)/kg diet. Group E diet was supplemented with 270 mg vitamin E (as vDL-α-tocopherol acetate vitamer/kg diet. Group A+E diet was supplemented by both, A and E vitamins (same quantities). The basal commercial diet already contained 360 μg vitamin A/kg and 4.5 mg vitamin E/kg. The experimental feeding begun with the birds at 40 weeks and lasted 17 weeks. Five 40-week-old roosters were euthanized and then, five 57-week-old roosters from each group were euthanized at the end of the experimental feeding period. Testicle and epididymis tissues were immediately sampled and were histologically processed and stained with hematoxylin eosin (H-E), Malory and Malachite green according to the methods described by Cornilă & Manolescu (1996). The histological preparations were examined using an optical microscope at different magnifi-cations. Image captures were used for testicular and epididymal morphometric analysis (semini-ferous duct and epididymal duct diameter, and seminiferous and epididymal epithelium thickness) using the "ImageJ" program.

All procedures in this study were conducted in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes by Member States of the European Union. The experiment was approved by the ethic committee of the USAMV of Bucharest. Testicular and epididymal morphometry data were statistically processed and expressed as mean ± standard error of the mean. Differences between groups were analyzed based on the Student's *t* test, being considered significant for $P < 0.05$.

RESULTS AND DISCUSSIONS

Regarding the seminiferous tubular morpho-metry, in our experiences, it is found that the seminiferous tubules undergo a process of decreasing diameter and decreasing density, by age (Table 1). The decrease in seminiferous tubule diameter was made both by decreasing the thickness of the epithelium and by shrinking the tubular lumen.

Table 1. Morphometry of seminiferous ducts in rooster groups fed on vitamin A or vitamin E - enriched diets from 40 to 57 weeks of age versus control

	40-week-old roosters	57-week-old roosters			
		Control	Vitamin A	Vitamin E	Vitamin A+E
Duct diameter (mm)	332.3±98.5	254±36.3	294±22.4	279±66.6	288±12.0
Duct epithelium thickness (mm)	111.0$^{\#}$±12.3	65.5$^{\#;a;b}$±4.4	89.0^{a}±11.3	74.4±10.0	86.6±6.6
Density of seminiferous ducts[1]	233.3±45.4	190.7±33.0	213.3±16.6	209.9±33.0	211.0±8.5
Density of Sertoli cells[2]	22.2±1.9	11.0$^{a;b;c}$±2.2	14.4^{a}±3.2	16.2^{b}±3.6	13.3±6.5

[1]mean values on 10 microscopic fields (ob.10 x oc.10) taken randomly;
[2]mean values on 10 microscopic fields (ob. 100 x oc. 10) taken randomly;
Note: - values represent the average of the two testicles of the roosters;
- values represent the mean ± the standard error of the mean;
- n = 5 for 40-week-old roosters and n = 5 for 57-week-old roosters;
- values on the same line with the same superscripts differ significantly ($P < 0.05$).

Part of the diameter decrease was compensated by the increase in the thickness of the intertubular connective tissue, so there is no direct proportionality between the evolution of the diameter, density of the seminiferous ducts and the testicular volume. The fact oriented us

towards the analysis mainly the effect on the thickness of the wall of the seminiferous tubules. The results reveal a protective effect of both, vitamin A and E diet supplementation, on the thickness of the seminiferous tubules, maintaining its size. The protective effect was greater in the case of vitamin A. Thus, a physiological decrease, of 45% of the thickness was found for the epithelium of the seminiferous tubules from 40 to 57 weeks, in control roosters. The mean tubule thickness values are significantly higher in groups A compared to the control, revealing a protective effect of the vitamin A on spermatogenesis line. Vitamin E also showed a protective effect, but of a weaker amplitude. In this regard, according to Sarabia Frogoso et al. (2013), 44% of testicular weight in broiler hybrids can be lost between 36 and 55 weeks of age, and this is usually accompanied by a decrease in the diameter of the seminiferous tubules, a decrease that agrees with the results found in Cornish hybrid roosters of the present experiment.

Both vitamins A and E also led to the maintenance of Sertoli cell density (significant differences versus control, $P < 0.01$ in all supplemented groups). This fact explains the physiological effects of vitamin A predominantly on spermatogenesis line cells (finally reflected in semen density and spermatozoa count, in particular) and the predominant effect of vitamin E on the biological properties of sperm (motility, in particular). Our results are confirmed by other researchers who found that vitamin E can maintains the thickness of the seminiferous tubule epithelium, increasing the number of epithelial cells, with positive effects on testis weight and testosterone concentration in the rat (Kumar et al., 2004) and rabbit (Yousef, 2010).

The histological structure of the seminiferous epithelium of roosters fed on vitamin A-enriched diet is closer as structure, dimensions and component elements to that of the seminiferous epithelium of 40-week-old roosters compared to the control testicular seminiferous epithelium, denoting a protective effect of the vitamin A against the erosion process supported by seminiferous tubules, induced by the physiology ageing process.

Aspects of testicular tissue histology in roosters fed the vitamin E-supplemented diet are notable for maintaining a high density of seminiferous Sertoli cells and interstitial Leydig cells (Figure 1).

The results obtained in our research are in agreement with previous studies (Yokota et al., 2018) which demonstrated that vitamin A deficiency in rats induces a progressive loss of spermatogenic germ cells, ultimately leading to the appearance of seminiferous tubules containing only Sertoli cells and premeiotic germ cells, straight spermatogonia (Huang et al., 1988 cited by Yokota et al., 2018) and administration of vitamin A to vitamin A deficient rats resumes spermatogenesis, as a regulator of gene expression (Yokota et al., 2018).

Manson and Mauer (1974) revealed a remarkable regenerative response in hamsters experimentally deficient in vitamin E: when the degeneration had reached quite advanced stages, the restoration of the germinal epithelium in most tubules was good as a result of vitamin E administration, but a variable number of seminiferous tubules showed only limited repair.

Similarly, in rats, Bensoussan et al. (1998) showed that reintroducing dietary vitamin E to deficient rats restored a normal appearance to the structure of the testis and epididymis, indicating that the effects on these tissues are reversible.

Taken together, these data indicate that vitamin E plays an important role in maintaining the viability of the spermatid population, allowing epididymal epithelial cells to acquire their appearance.

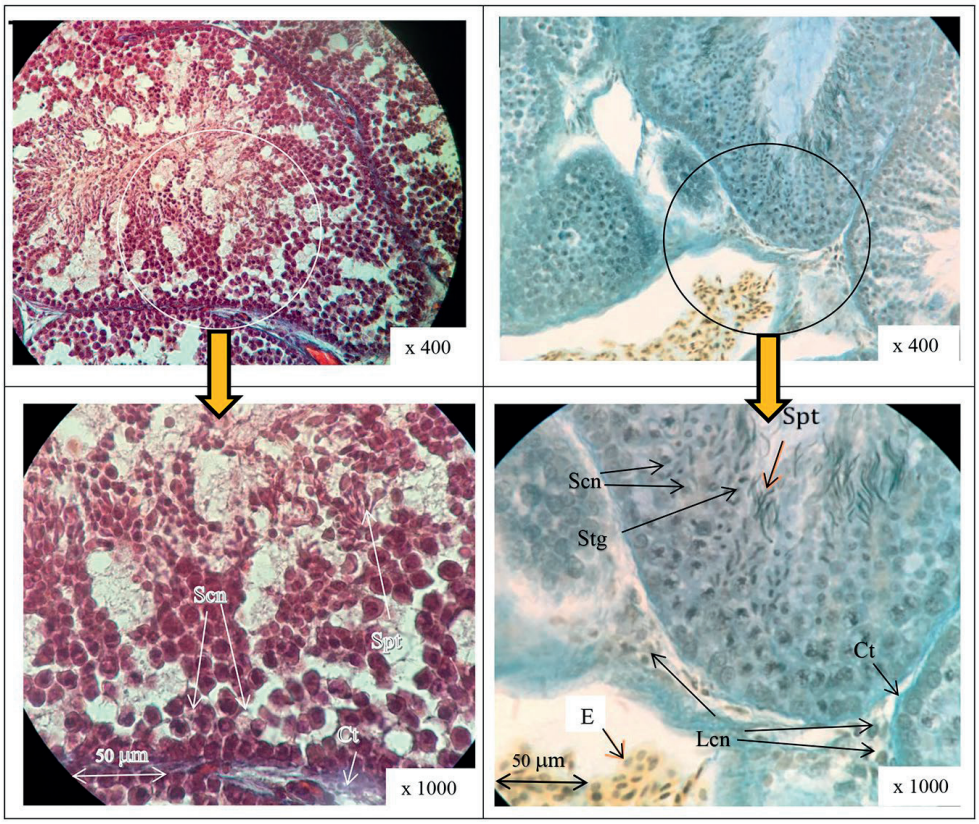


Figure 1. *Left:* testis of a 57-week-old control rooster. Disorganization of the seminiferous structure is noted, with the appearance of numerous vacuoles. Above, thick sheets of connective tissue can be seen, surrounding the seminiferous tubules. At the bottom, numerous spermatides (Spd) can be seen, still retaining a high mass of residual cytoplasm. Scn = Sertoli cell nuclei; Spr - spermatids; Ct -connective tissue. Mallory staining
Right: testis of 57-week-old rooster fed the vitamin A-enriched diet for 17 weeks. The preservation of the full development of the seminiferous epithelium, the fine lamellae of seminiferous pericanalicular connective tissue, small islands of Leydig cells as well as the relative maintenance of the richness of the seminiferous pericanalicular blood vasculature are noted. Green Malachite staining.
E - erythrocytes (with pink-yellow colored cytoplasm); Spt - spermatozoa; Stg - spermatogonia; Lcn - Leydig cell nuclei; Scn - Sertoli cell nuclei; Ct- connective tissue

During the aging process, from 40 to 57 weeks, there was a 36% decrease in the thickness of the epididymis epithelium in the control (Figure 2). In contrast, in groups supplemented with vitamin A, this decrease in epididymis epithelial thickness was less (only 18% in group A), suggesting a higher protective effect of vitamin A on this structure in the long-term vitamin treatment, the differences being significant compared to the control group ($P < 0.01$) and 33% in group E.

A similar situation was identified in the case of the diameter of the epididymis duct, the created space being replaced by connective tissue.

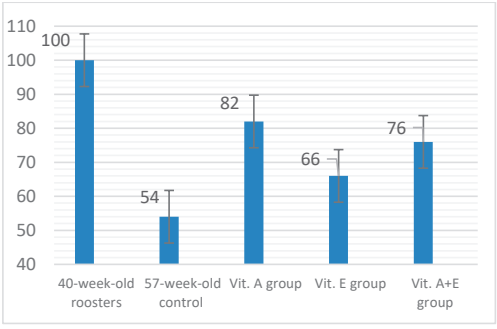


Figure 2. Long-term evolution of the vitamin A or E enriched diets on the thickness of the epididymis epithelium in roosters (% from values at 40 weeks of age)

With regard to the histological structure and the content of the epididymis and deferent duct lumen (Figure 3), in 57-week-old control roosters, spermatogonia, spermatocytes and fragments of basophilic or eosinophilic cytoplasmic mass originating from Setoli cells or epididymal epithelial cells appear among the spermatozoa (Figure 3, A).

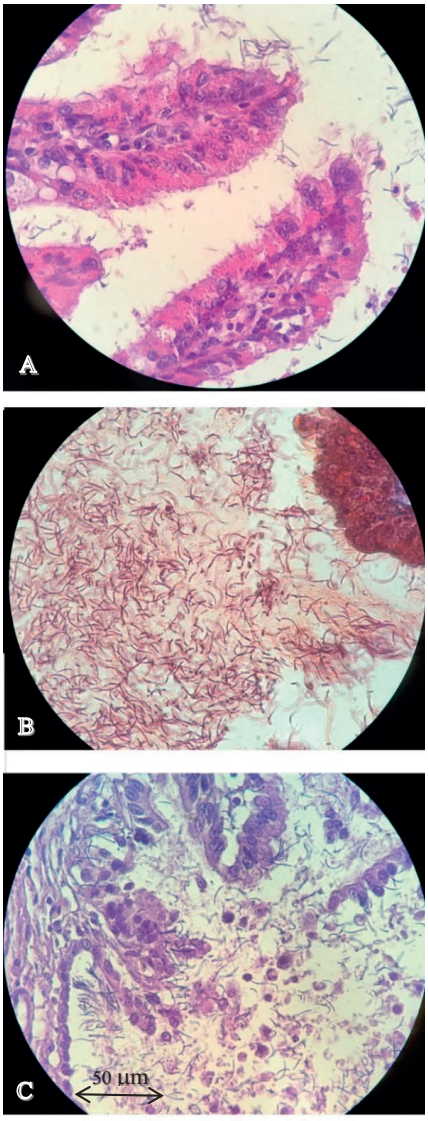


Figure 3. Comparative structural aspects of the epididymis epithelium and epididymis luminal content in 57-week-old roosters, following 17 weeks of experimental feeding with vitamin A (midle) or E (down) enriched diets versus a control (up) (see detailed explanations in the text). H-E (A and C) and Malory (B) staining (x1000)

Much reduced amount of eosinophilic cytoplasmic mass and spermatogonia were found in 57-week-old roosters fed for 17 weeks on diets enriched in vitamin A. (Stereo)epididymal cilia are well developed (Figure 3, B). In 57-week-old roosters fed for 17 weeks with diets enriched in vitamin E, the epididymal epithelium is eroded, reduced in some places to a single row of cells (Figure 3, C). In the lumen, numerous large spermatogonia, spermatocytes and fragments of cytoplasmic mass from Sertoli cells or from epididymis epithelium cells are identified. Data from the literature regarding the protective effects of antioxidant vitamins on the epididymal epithelium confirm our results: protective effects of vitamins (ascorbic acid) on the epididymis structure (affected by the experimental toxic action) were reported by Chitra et al. (2003) in the rat.

Similarly, Krishnamoorthy et al. (2007) demonstrated the protective effect of vitamin E on epididymal spermatozoa, explained by improving the biochemistry of epididymal secretions, also in rats. Again, Bensoussan et al. (1998) provide, as shown, a more edifying example of the protective role of vitamin E on the epididymis in rats after five weeks of feeding them a diet deficient in vitamin E compared to animals fed on a commercial diet. All the effects of the researched vitamins must be interpreted in the context of their antioxidant effect on the enzymes involved in redox processes in tissues (Escorcia et al., 2020).

CONCLUSIONS

Long-term feeding (17 weeks) on diets enriched in vitamin A or E of hybrid Cornish roosters allowed the identification of specific protective effects of the two vitamins on the structure of the seminiferous epithelium and the epididymal one. Vitamin A mainly protects the spermatogenesis line, while vitamin E mainly protects Sertoli cells and Leydig cells. Both vitamins have a protective effect on the epididymal epithelium against the physiology ageing process, especially vitamin A. No mutual inhibition or mutual enhancement of the two studied vitamins were identified. The effects of the vitamin A and vitamin E diet supplements on the testicular morphology will be reflected in the

improvement of the biological properties of the semen, allowing the extension of the economic exploitation of Cornish hybrid roosters over a longer period of time than that foreseen in the commercial technologies.

REFERENCES

Baba A.R., & Asrol, M.A. (2017). Effect of vitamin E supplementation on semen quantity and quality of Local Kampong roosters. *Mal. J. Anim. Sci*. 20(1): 37-43.

Bălăceanu, R.A., Sima, L., & Dojană, N. (2019). The effect of different level of vitamin E diet supplementation on the sperm in adult cocks raised in industrial system. *Scientific Papers Veterinary Medicine Timisoara*, ISSN 1221-5295.

Bensoussan K., C R Morales C.R., & Hermo L. (1998). Vitamin E deficiency causes incomplete spermatogenesis and affects the structural differentiation of epithelial cells of the epididymis in the rat. *J. Androl*. 19(3):266-88.

Biswas A., Mohan J., & Sastry K.V.H. (2009). Effect of higher dietary vitamin E concentrations on physical and biochemical characteristics of semen in Kadaknath cockerels, *British Poultry Science*, 50:6, 733-738. DOI: 10.1080/00071660903264369

Bosakowski, T., Levi, A.A., & Edgcomb, J.H. (1988). Studies onthe testicular effects of vitamin A palmitate in the Sprague dawley rat. *Food Chem. Toxicol*., 26, 767-773.

Chitra,, K.C., Ramachandra Rao K., & Mathur, P.P. (2003). Effect of bisphenol A and co-administration of bisphenol A and vitamin C on epididymis of adult rats: a histological and biochemical study. *Asian J Androl.* 5(3):203-8.

Cornilă, N., & Manolescu, N. (1996). *Structura şi ultrastructura organelor la animalele domestice*. Editura Ceres, Bucureşti.

Danikowski, S., Halle, I., & Flachowsky, G. (2002). Influence of high levels of vitamin E on semen parameters of cocks. *Journal of Animal Physiology and Animal Nutrition.* 86: 11-12, 376-382.

Escorcia, L.M., Sánchez-Godoy, F., Ramos-Vidales D., Medina-Campos, O.N., & Pedraza-Chaverri, J. (2020). Effect of the Age and Body Weight of the Broiler Breeders Male on the Presentation of Oxidative Stress and Its Correlation with the Quality of Testicular Parenchyma and Physiological Antioxidant. *Vet. Sci*. 7, 69.

Krishnamoorthy, G., Venkataraman, P., Arunkumar, A., Vignesh, R.C., Aruldhas, M. M., & Arunakaran, J. (2007). Ameliorative effect of vitamins (alpha-tocopherol and ascorbic acid) on PCB (Aroclor 1254) induced oxidative stress in rat epididymal sperm. *Reprod Toxicol.* 23(2):239-45. doi: 10.1016/j.reprotox.2006.12.004. Epub 2006 Dec 22.

Kumar, R., Prakash, S., Kushwah, A., & Vijayan, V. (2004). Effects of vitamin C and E induced oxidative stress, androgen binding protein and lactate in rat Sertoli cells. *Reprod Toxicol*. 19:201–8; https://doi.org/10.1016/j.reprotox.2004.08.001.

Manson, K.E., & Mauer, S.I. (1974). Reversible Testis Injury in the Vitamin E-deficient Hamster. *J. Nutr*. 105(4):484-90. doi: 10.1093/jn/105.4.48.

Saddein E., Haghpanah, T., Nematollahi-Mahani S., Seyedi, F., & Ezzatabadipour, M., (2019). Preventative Effects of Vitamin E on Testicular Damage and Sperm Parameters in the First-Generation Mice Pups due to Pre- and Postnatal Mancozeb Exposure. *Journal of Toxicology*. https://doi.org/10.1155/2019/4763684.

Sarabia Fragoso, J., Pizarro Díaz, M., Abad Moreno, J.C., Casanovas Infesta, P., Rodriguez-Bertos, A., & Barger, K., (2013). Relationships between fertility and some parameters in male broiler breeders (body and testicular weight, histology and immunohistochemistry of testes, spermatogenesis and hormonal levels). *Reprod. Domest. Anim*., 48, pp. 345-352

Sukmawati, Y., Arisanty, D., Tofrizal, A., & Amir A. (2019). Vitamin E ameliorates testicular histological features and androgen binding protein levels in testicle of rats induced by allethrin. *Journal of Advanced Veterinary and Animal Research*. 6: 4, 486–491.

Triques, G.E., De Cristo, A.B., Canevese, M., Santos Marques, P.F., Burin, A.M., & Fernandez, J.I.M. (2022). Effect of antioxidant supplementation in diets of roosters during the post-peak phase on reproduction and production characteristics of offspring. *Zootechnia*. C*ienc. anim. bras*. 20, 1-13, e-43072.

Yokota, S., Shirahata, T., & Yusa, J. (2018). Long-term dietary intake of excessive vitamin A impairs spermatogenesis in mice. *J. Toxicol. Sci*., 44:4, 257-271.

Yousef, M.I. (2010). Vitamin E modulates reproductive toxicity of pyrethroid lambda-cyhalothrin in male rabbits. *Food Chem. Toxicol. Elsevier*. 48(5):1152–9; https://doi.org/10.1016/j.fct.2010.02.002.

CLINICAL SCIENCES

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

BOVINE HERPES VIRUS QUANTIFICATION BY qPCR IN THE BLOOD OF ASIMPTOMATIC LATE-TERM COWS

Nicolae Tiberiu CONSTANTIN[1, 2], Florin Petrișor POSASTIUC[1], Crina Raluca ANDREI[1], Ovidiu GEICU[1], Florin IORDACHE[1], Loredana STANCA[1], Liviu BÎLTEANU[1], Andreea Iren ȘERBAN[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independenței, 050097, District 5, Bucharest, Romania
[2]Research and Development Institute for Bovine Balotești, Balotești, București-Ploiești km 21, 077015, Ilfov, Romania

Corresponding author email: tiberiu.constantin@fmvb.usamv.ro

Abstract

Bovine herpesvirus-1(BoHV-1) infections can be asymptomatic nonetheless it can also cause systemic illness in young calves and several diseases in adult cattle, including infectious bovine rhinotracheitis, infectious pustular vulvovaginitis and abortions. BoHV-1 can also establish recurrent life-long latent infections after primary infection. For viral load detection and quantification, we analysed blood samples from 19 asymptomatic pregnant cows belonging to three different breeds (Montbéliard, Holstein and Romanian Black Spotted). Viral DNA extraction from plasma was performed using the Nucleic Acid Extraction or Purification Reagent Kit (Medicalsystem Biotechnology Co., Ltd. Ningbo, China), and the Auto-Pure 32A automatic extractor (Allsheng Instruments Co., Ltd. Hangzhou). The BHV-1 putative fibronectin binding protein Genesig Advanced Kit (Primerdesign Ltd, UK) was used for qPCR amplification. The qPCR reactions were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). After analysis of the amplification curves, only one sample out of 19 was positive with a viral load of 4×10^3 copies/mL of blood. The affected animal was asymptomatic, which emphasizes the need for recurrent testing of transmissible infectious pathogens, and increasing biovigilance for minimizing eventual economical losses.

Key words: *Bovine herpesvirus-1, cow, quantitative PCR, late-term pregnancy, detection.*

INTRODUCTION

Bovine herpesvirus-1 (BoHV-1) infection is a common occurrence in ruminants' populations worldwide (Lopes et al., 2019). According to the International Committee on Taxonomy of Viruses (2023), BoHV-1 is a member of *Varicellovirus* genus, included Herpesviridae family with three subtypes BoHV-1.1, BoHV-1.2a and BoHV-1.2b. Being a double-stranded DNA virus spread to Europe in the late 1960s and early 1970s (Graham, 2013), BoHV-1 causes infectious bovine rhinotracheitis (IBR) in both domestic and wild ruminants, affecting the respiratory and reproductive systems (Oberto et al., 2023). On the genital tract, this virus is the causative agent for infectious pustular vulvovaginitis and balanopostitis, temporary infertility, embryonic death, abortions and generalized disease in newborn calves (Murkan, 2019). Furthermore, the virus is known to induce immunological suppression, conjunctivitis, encephalitis, and a decrease in milk production (Marin et al., 2016).

BoHV-1 establishes latency in the trigeminal ganglion or pharyngeal tonsils following primary infection, or in the sacral ganglia following genital infection causing the animals to remain carriers and potential disseminators for the rest of their lives (Muylkens et al., 2007; Ostler, 2023). Stress linked with parturition, transport, animal movement and mixing, inclement weather, simultaneous infection, poor husbandry or food, overcrowding, or following corticosteroid treatment can all cause reactivation (Raaperi et al., 2014; Narayan et al., 2018). Additionally, the number of animals in the latent infection phase is much higher than the number of animals exhibiting clinical signs, this fact making it easy to be transmitted and difficult to be controlled or eradicated (Lopes et al., 2019). All of these problems

associated with BoHV-1 made it one of the most economically damaging pathogens. Thus, Can et al. (2016) registered a financial loss of 509 USD, due to the high abortion rate as a result of this infection and the average cost of infection was estimated at about 379 USD.

Most of BoHV-1.1 strains were isolated from respiratory tract infections or abortion cases, while BoHV-1.2 strains were commonly identified in genital organ lesions (Ostler, 2023). Cell cultures, histopathological examinations, serological testing, polymerase chain reaction (PCR), immunohistochemistry (IHC) and immunofluorescence (IF), Western blot, enzyme linked immunosorbent assay (ELISA), and electron microscopy were all used to diagnose BoHV-1 infections, the only accurate distinguishing criterion being viral DNA analysis by restriction endonuclease fingerprinting (Muylkens et al., 2007). Quantitative polymerase chain reaction (Q-PCR) is a method by which the amount of the PCR product can be determined, in real-time, and is very useful for investigating gene expression (Narayan et al., 2018). Based on this, the current study aimed to trace the presence of the virus in cows' blood as a source of BoHV-1, in order to obtain a germfree plasma required for the transfusion of newborn calves in need.

MATERIALS AND METHODS

Ethical statement

The authors of this study respected all rights of animals' welfare in correlation to European Union and National legislation (Directive 2010/63/UE; Law 34/2014), and none of them suffered during any of the implied procedures.

Sample collection

Blood samples were collected via coccygeal vein from 19 asymptomatic pregnant cows from three different breeds (Montbéliard n = 7, Holstein n = 4, and Romanian Black Spotted n = 8) belonging to 3 commercial dairy farms located in Ilfov county, Romania, using 18 G needles and BD Vacutainer K2 EDTA (Plymouth, UK) collection tubes. Furtherly, the specimens were centrifuged at 1500× *g* for 15 min at 4°C, and plasma was harvested and frozen at −80°C until DNA extractions.

DNA extraction from plasma

Viral DNA extraction from plasma was performed using the Nucleic Acid Extraction or Purification Reagent Kit (Medicalsystem Biotechnology Co., Ltd. Ningbo, China), and the Auto-Pure 32A automatic extractor (Allsheng Instruments Co., Ltd. Hangzhou) following manufacturer's guidelines.

Detection and quantification of BoHV-1 by qPCR

For qPCR amplification the Bovine herpesvirus 1 putative fibronectin binding protein Genesig Advanced Kit (Primerdesign Ltd, UK) based on TaqMan principle was used. The amplification reactions were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with CFX Maestro Software.

Amplification of the target pathogen was detected using specific primers and a FAM fluorochrome-labelled probe complementary to the pathogen target gene. Also, to confirm a valid DNA extraction, the kit included a mixture of primer and a FAM fluorochrome-labelled probe that detects an endogenous gene. Therefore, it wasn't possible to multiplex with BoHV-1 detection.

In order to carry out the PCR reactions, two reaction mixtures are prepared, one containingprimers and probes complementary with the pathogen target gene and the second, containing primers and probes complementary with the endogenous control (a specific gene in the bovine genome) according to Table 1.

Table 1. PCR reaction components

Reagents	Per reaction (µl)
2xSsoFast Advanced Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA)	10
Target gene/endogenous control primer and probe	1
Nuclease-free water	4
DNA sample	5

For quantitative analysis, a calibration curve was performed by serial dilutions (1, 10, 10^2, 10^3, 10^4 and 10^5 DNA copies/µL) using the positive control template provided by the kit.

The qPCR run protocol settings are shown in Table 2.

Table 2. qPCR time and temperature steps

Stage	Time	Temperature	Cycle	Signal scan
Enzyme activation	1 min	95°C	1	-
Denaturation	10 sec	95°C	50	-
Annealing/ elongation	60 sec	60°C		FAM
Final elongation	5 min	60°C	-	-

The CFX Maestro Software performed automatically the calibration curve and calculated the concentration of the samples in DNA copies/µL.

RESULTS AND DISCUSSIONS

Regarding both domestic and wild ruminants, BoHV-1 has generally been considered a serious threat to the upper respiratory system and reproductive function. Therefore, it is crucial to streamline this pathogen's diagnosis in order to higher in-farm eradication chances.
In the current investigation, we used the qPCR approach on a total of 19 cows belonging to three different commercial farms.
The amplification profile curves for BoHV-1 DNA standards with concentrations of 10^5, 10^4, 10^3, 10^2, 10, and 1 copies/µL are shown in Figure 1, and the calibration curve used for the quantification of the number of BoHV-1 DNA copies/µL in the samples is shown in Figure 2.

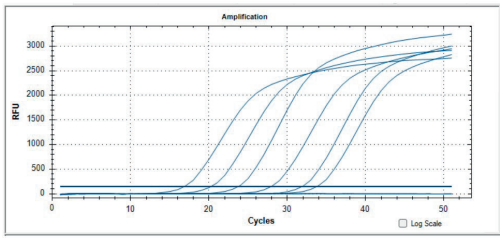


Figure 1. The amplification profile curves corresponding to 10^5, 10^4, 10^3, 10^2, 10, and 1 BoHV-1 DNA copies/µL standards

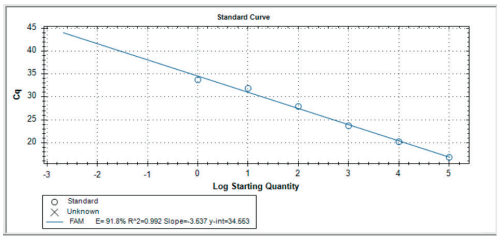


Figure 2. The calibration curve $Cq = f(log(\,copies\ DNA/\mu L))$, where Cq represents the quantification cycle

The amplification profile curves of the examined samples are displayed in Figure 3. Only one sample belonging to a Holstein female, which had a viral load of $4x10^3$ copies/mL of blood, was positive, as it can be observed in Figure 3.

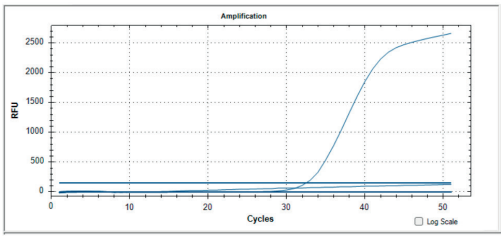


Figure 3. The amplification profile curves of the 19 examinated blood samples using primers and FAM fluorochrome-labelled probe complementary to the putative fibronectin binding protein gene of BoHV-1 genome

The Figure 4 displays the amplification profile curves of the 19 examinated blood samples using primers and FAM fluorochrome-labelled probe complementary to endogenous control, showing that all examined samples' extracted DNA are of high quality and are enough for pathogen detection.

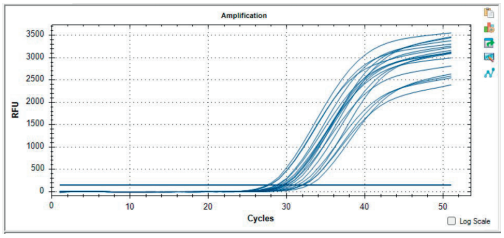


Figure 4. The amplification profile curves for blood samples evaluated using complementary primers and probes to the endogenous control

Various molecular techniques have been developed in recent years for the differentiation of bovine alphaherpesviruses that are closely related to BoHV-1. Numerous multiplex PCR techniques, including traditional and real-time techniques, have been created.
Thus, BoHV-1 can be isolated not just from blood samples (Jithin et al., 2019), but also from sperm, uterine lavage, aborted foetus (Malla et al., 2018) or respiratory tract (Marin et al., 2016).
Relatively recent, Hanna Ferreira et al. (2018) demonstrated that milk seems to be a suitable

sample for the viral nucleic acid detection, as a more sensitive test compared to the serological method, for the latent BoHV-1 infection diagnosis has been described.

In order to distinguish BoHV-1 DNA, Oliveira et al. (2009) presented a nested PCR that amplified BoHV-1 DNA in the first round before running two type-specific PCRs. The authors have nonetheless shown that some samples may have viral DNA quantities below the PCR detection limit. In addition, this method is a nested PCR that necessitates the use of agarose gels in a manner similar to all other conventional PCRs, and additional precautions must be made to avoid cross-contamination.

Comparatively to traditional PCR, real-time qPCR significantly decreased the chance of contamination. Being more sensitive than traditional PCR, it provides a quick, trustworthy, and quantitative testing approach (Diallo et al., 2011).

To check for false-negative results brought on by unsuccessful nucleic acid extraction or the presence of inhibitory components in the reaction, it is crucial to utilize an internal/endogen control. By employing such an internal/endogen control, it was ensured that DNA extracts from "difficult" samples were PCR-competent, preventing the reporting of false-negative findings and boosting assay robustness.

The investigations on the Balkan region are not very thorough, although BoVH-1 or related pathogens from blood samples were highlighted in Romania (Aniță et al., 2017) and Bulgaria (Peshev, 2021) with the addition of one Serbian paper which referred to nasal swabs specimens (Nišavić et al., 2018). Therefore, reported incidence rates were variable and method-dependent 63.60% (Aniță et al., 2017), 37.5% (Peshev, 2021), 3.6% (Nišavić et al., 2018). Nevertheless, the latter cited research papers used less sophisticated techniques (ELISA and classical PCR).

Based on their research efforts, Lopes et al., (2019) deliberated that the uterus is a viral replication target. Additionally, detecting BoHV-1 in the uterus, oviduct and ovaries of tissue samples obtained from cows, they mentioned that the placenta and uterine tissue may be the source of the spread, leading to foetal infection and abortion.

El-Mohamady et al. (2020), after three successive passages for BoHV-1 isolation, showed a clear cytopathic effect in 8 (20%) out of 40 sperm samples. One year later, using the same sample type , Untari et al. (2021) indicated that there is no infection in the semen of 27 bulls while using PCR detection in Indonesia.

Recently, El-Mayet et al. (2022) used the qPCR proved that BoHV-1 latent infections in female calves can be reactivated by stress after progesterone exposure.

In the present work, the viral DNA extraction and the real time qPCR assay protocols were validated. This assay was highly sensitive and could detect the BoHV-1 genome even when DNA from only 10^3 viral particles/mL blood was used.

Regarding host bloodstream invasion, little is known about the behaviours of several alpha-herpesviruses. Further testing will be done on this protocol capacity to identify and quantification BoHV-1 in blood, milk and sperm samples taken from different dairy farms.

Bovine alpha-herpesvirus DNA can be quickly, sensitively, and specifically detected using real-time qPCR. It can also be helpful for the simultaneous detection of different bovine alpha-herpesvirus variants. This method is a great resource for identifying these viruses in cattle for both research reasons or epidemiological surveys.

CONSLUSIONS

The qPCR method demonstrates golden standard potential for identifying BoHV-1 positive animals despite the small sample size of this investigation.

From the standpoint of financial losses, early detection of BoHV-1 positive individuals is essential for dairy farm management. More than that, it is crucial to carry out screening programmes using the qPCR approach, especially if a vaccination program is established in the farm.

ACKNOWLEDGMENTS

This work was financially supported by University of Agronomic Sciences and Veterinary Medicine of Bucharest (UASVM Bucharest) by an Internal Research Project, Contract no. 1061/15.06.2022 COD 2022-0019 – *"The development of eco-innovative therapies for the treatment and prophylaxis of calves' neonatal enteritis, in order to reduce the consumption of antimicrobials and ensure animal health"*.

REFERENCES

Aniță, D., Răileanu, C., Oșlobanu, L., & Crivei, L. A. (2017). Detection of BHV-1 antibodies in bovine raised in rural households from Iași County. *Lucrări Științifice U.S.A.M.V. „Ion Ionescu de La Brad" Iași*, *60*(4), 459–462.

Can, M. F., Ataseven, V. S., & Yalçın, C. (2016). *Estimation of production and reproductive performance losses in dairy cattle due to bovine herpesvirus 1 (BoHV-1) infection*. Veterinarski Arhiv, 86 (4), 499-513.

Diallo, I. S., Corney, B. G., & Rodwell, B. J. (2011). Detection and differentiation of bovine herpesvirus 1 and 5 using a multiplex real-time polymerase chain reaction. *Journal of Virological Methods*, 175(1), 46–52.

El-mayet, F. S., Toomer, G., Ostler, J. B., Harrison, K. S., Santos, V. C., Wijesekera, N., Stayton, E., Ritchey, J., & Jones, C. (2022). Progesterone Sporadically Induces Reactivation from Latency in Female Calves but Pro fi ciently Stimulates Bovine Herpesvirus 1 Productive Infection. *Journal of Virology*, 96(5), 1–14.

El-mohamady, R. S., Behour, T. S., & Rawash, Z. M. (2020). Concurrent detection of bovine viral diarrhoea virus and bovine herpesvirus-1 in bulls ' semen and their effect on semen quality. *International Journal of Veterinary Science and Medicine*, *8*(1), 106–114.

Ferreira, C., Campos, M. G., Marcus, P., Vidigal, P., Santos, M. R., Carvalho, O. V. D. E., Bressan, G. C., Lopes, J., Fietto, R., Paulino, E., Almeida, M. R., & Júnior, A. S. (2018). Latent bovine herpesvirus 1 and 5 in milk from naturally infected dairy cattle. *J. Vet. Med. Sci*., 80(11):1787–1790.

Graham, D. A. (2013). Bovine herpes virus-1 (BoHV-1) in cattle - a review with emphasis on reproductive impacts and the emergence of infection in Ireland and the United Kingdom Bovine herpes virus-1 (BoHV-1) in cattle - a review with emphasis on reproductive impacts and the emergence of infection in Ireland and the United Kingdom. *Irish Veterinary Journal*, 2013, 66:15.

Jithin, P. M., Sankar, S., Mani, B. K., Simon, S., Reji, R. M., Bosewell, A., & Mini, M. (2019). Serological and molecular detection of Bovine Herpes Virus 1 infection in cattle by enzyme linked immunosorbent assay and polymerase chain reaction. *Indian J. Anim. Res*., 53(12) 2019:1661-1665.

Lopes, V., Queiroz-castro, D., Paulino, E., Teles, V., Santos, M. R., Fernando, L., Souza, L. De, Gomides, C., Toledo, R., & Silva-júnior, A. (2019). Detection of bovine herpesvirus 1 in genital organs of naturally infected cows. *World Vet J*, 11(4): 709-712.

Malla, J., Chakravarti, S., Gupta, V., & Chander, V. (2018). Novel Polymerase Spiral Reaction (PSR) for rapid visual detection of Bovine Herpesvirus 1 genomic DNA from aborted bovine fetus and semen. *Gene*, 644, 107–112.

Marin, M. S., Quintana, S., Leunda, M. R., Recavarren, M., Pagnuco, I., Späth, E., Pérez, S., & Odeón, A. (2016). A new method for simultaneous detection and discrimination of Bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) using real time PCR with high resolution melting (HRM) analysis. *Journal of Virological Methods*, 227, 14–22.

Murkan, M. Ö. T. İ. (2019). *Comparative detection of bovine herpesvirus-1 using antigen ELISA , immunohistochemistry and immunofluorescence methods in cattle with pneumonia*. *43*(3).

Muylkens, B., Thiry, J., Kirten, P., Schynts, F., Muylkens, B., Thiry, J., Kirten, P., Schynts, F., & Thiry, E. (2007). Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. *Vet. Res.*, 38 (2007) 181–209.

Narayan, L., Thodangala, N., Kumar, S., Sri, K., Leela, N., Venkata, R., Reddy, C., & Putla, B. (2018). Evaluation of a specialized fi lter-paper matrix for transportation of extended bovine semen to screen for bovine herpesvirus-1 by real-time PCR. *Journal of Virological Methods*, 257, 1–6.

Nišavić, J., Knezevic, A., Stanojevic, M., & Radalj, A. (2018). Molecular detection of bovine herpesvirus 1 (BoHV-1) in cattle in Serbia. *Revue Méd. Vét.,* 2018, 169, 7-9, 180-184.

Oberto, F., Carella, E., Caruso, C., Acutis, P. L., Lelli, D., Bertolotti, L., Masoero, L., & Peletto, S. (2023). A Qualitative PCR Assay for the Discrimination of Bubaline Herpesvirus 1, Bovine Herpesvirus 1 and Bovine Herpesvirus 5. *Microorganisms*, 2023, 11, 577, 1–11.

Oliveira, M. T., Silva, A. D., Esteves, P. A., Campos, F. S., Franco, A. C., Hu, S. O., Roehe, P. M., & Rijsewijk, F. A. M. (2009). High prevalence of co-infections with bovine herpesvirus 1 and 5 found in cattle in southern Brazil. *Veterinary Microbiology*, 139, 67–73.

Ostler, J. B. (2023). The Bovine Herpesvirus 1 Latency-Reactivation Cycle, a Chronic Problem in the Cattle Industry. *Viruses*, 15(552), 1–16.

Peshev, R. (2021). Investigations on bovine herpesvirus 4 infections in cattle in Bulgaria. *Bulgarian Journal of Veterinary Medicine*.

Raaperi, K., Orro, T., & Viltrop, A. (2014). Epidemiology and control of bovine herpesvirus 1 infection in Europe. *Veterinary Journal*, 201(3), 249–256.

Taxonomy, C. I. (2023), https://ictv.global/

Untari, T., Kristianingrum, Y. P., & Kusumawati, A. (2021). Detection of Bovine Herpesvirus 1 from Semen by Real-time PCR to Prevent the Spread of Infectious Bovine Rhinotracheitis Infection. *World Vet J*, 11(51304), 709–712.

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

DERMAL MELANOMAS IN A GREY HORSE: CASE STUDY

Alexandra Mihaela CRISTIAN, Niculae TUDOR, Mario CODREANU, Ana DOBRIN, George NICOLAE

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăşti Blvd, District 1, 011464, Bucharest, Romania, Phone: +4021.318.25.64, Fax: + 4021.318.25.67,

Corresponding author email: alexandrapopa613@gmail.com

Abstract

Melanoma is a relatively common type of cancer in horses, particularly in those with gray or white coats. As horses age, the likelihood of developing melanoma increases, and it is estimated that around 80% of aged populations of gray horses will develop the condition. A 20 year-old grey Standardbred female was referred to the Faculty of Veterinary Medicine Bucharest presenting with insidious weight loss over the past year, depression, inappetance, cutaneous masses and pigmented peri-anal masses likely melanomas. Clinical examination revealed normal respiratory rate, cardiac frequency în normal parameters. Different firm well-circumscribed masses were also palpable in the various locations. The X-ray revealed the absence of pulmonary masses or pathogenetic consequences of primary tumor, without modifications of the pulmonary area and the absence of specific pattern lung. The ultrasonographic examination revealed the presence of inhomogeneities with areas of hypoechogenicity, well delimited by a hyperechogenic capsule. The cytological aspects characteristic of the diagnosis of melanoma were presented and identified regardless of the collection site.

Key words: dermal melanomas, horse.

INTRODUCTION

Melanoma is a relatively common type of cancer in horses, particularly in those with gray or white coats. As horses age, the likelihood of developing melanoma increases, and it is estimated that around 80% of aged populations of gray horses will develop the condition.

Melanoma in horses can manifest as variably pigmented and infiltrative tumors that often present in advanced stages as a multicentric malignancy. These tumors can be located in various parts of the body, including the skin, eyes, and internal organs. While some horses may develop only a few small melanomas that do not cause significant problems, others may develop large or aggressive tumors that can cause pain, discomfort, and other health issues.

It's important to note that the potential for equine melanomas to become malignant can vary based on a number of factors, including the location and size of the tumor, as well as the individual horse's age and overall health.

While some older studies have suggested a relatively high incidence of malignancy among equine melanomas, more recent clinicopathological studies have provided varying results. As noted, one study reported a 14% incidence of malignant dermal melanomas, while another study of Lipizzaner horses found a 50% incidence of melanomas but no clinical evidence of malignancy.

It appears that in the veterinary literature, melanocytic tumors have often been categorized as either benign or malignant melanomas. However, there are actually four distinct clinical syndromes that are recognized: melanocytic nevus, dermal melanoma, dermal melanomatosis, and anaplastic melanoma.

Dermal melanomas and dermal melanomatosis are histologically similar lesions, and they are differentiated based on their clinical features. Dermal melanomas present as discrete masses, while dermal melanomatosis involves multiple cutaneous masses, with at least one of the masses presenting in a typical location. As mentioned earlier, these typical sites include the undersurface of the tail, anal, perianal and genital regions, perineum, and lip commissures.

Anaplastic melanomas, on the other hand, are considered the most aggressive form of melanocytic tumors and are characterized by rapidly growing masses that are invasive and have a high metastatic potential. Melanocytic nevi, on the other hand, are typically benign

and do not tend to progress into malignant forms.
It's worth mentioning that not all melanomas in horses will become malignant, and some may remain benign throughout the horse's lifetime. However, given the potential for these tumors to become malignant and cause health issues, it's important for horse owners and veterinarians to monitor any melanomas that develop and seek appropriate treatment if necessary. This may include surgical removal, radiation therapy, or other interventions depending on the specifics of the case.

MATERIALS AND METHODS

Case history and clinical findings

A 20 year-old grey Standardbred female was referred to the Faculty of Veterinary Medicine Bucharest presenting with insidious weight loss over the past year, depression, inappetance, cutaneous masses and pigmented peri-anal masses likely melanomas. Clinical examination revealed normal respiratory rate, cardiac frequency în normal parameters. Different firm well-circumscribed masses were also palpable in the various locations.
Additional diagnostic testing included bloodwork, thoracic radiographs, ultrasound, and medullogram (Figures 1-7).

Figure 1. Large confluence of nodular and plaque-like melanomas on the ventral tail and perineum

Figure 2. Diffuse firm mass at the level of the olecranon

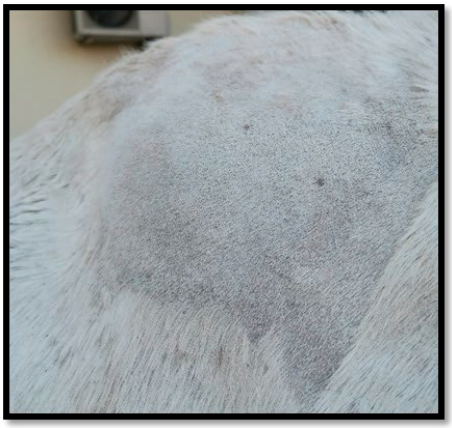


Figure 3. Diffuse mass at the level of the whiters

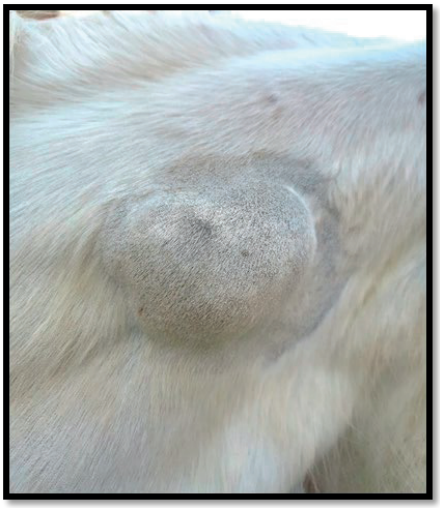


Figure 4. Localized firm and well-circumsed mass at the neck level

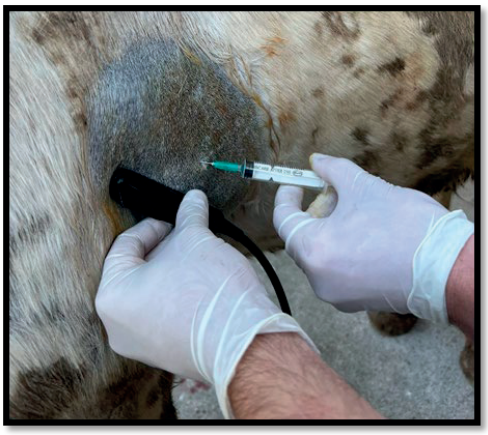


Figure 5. Ultrasound guided puncture from the mass for cytology exam

Figure 6. Puncture from the anal mass for citology exam

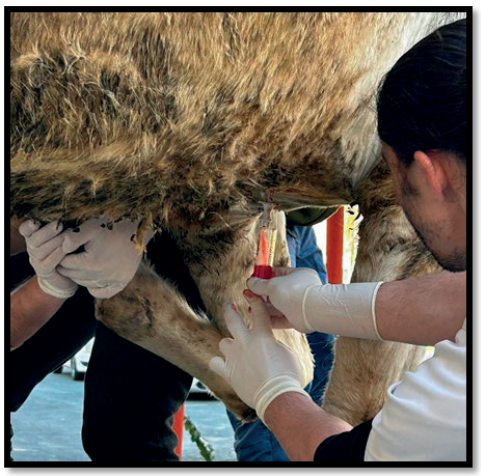


Figure 7. Sternal Puncture for medullogram

For the cytological examination, the slides obtained by fine-needle aspiration were stained with May Grunwald-Giemsa stain.

RESULTS AND DISCUSSIONS

In regard of the hematological examination the parameters identified were within the normal values being a great indicator of a an systemic complications on the organism (Figure 8).

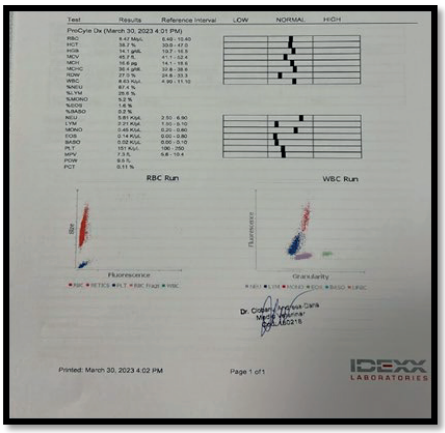


Figure 8. Result of hematological examination, horse, standardbred

The X-ray revealed the absence of pulmonary masses or pathogenetic consequences of primary tumor, without modifications of the pulmonary area and the absence of specific pattern lung (Figures 9, 10).

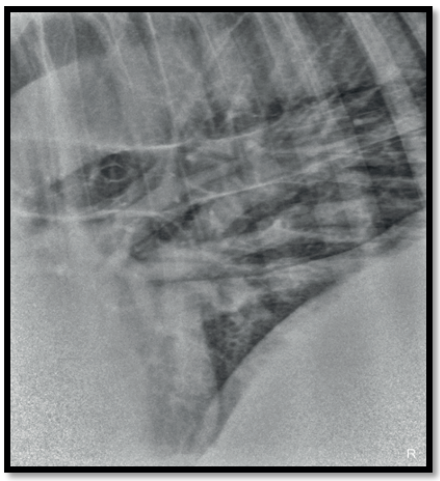


Figure 9. X-ray cardiac pulmonary lobe - Normal X-ray image

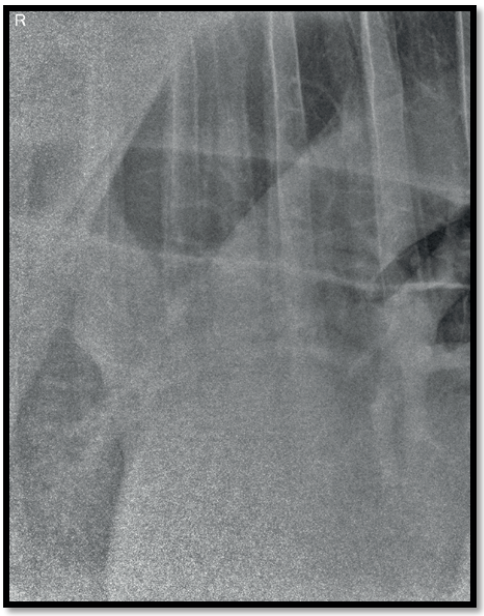


Figure 10. X-ray apical pulmonary lobe - Normal X-ray image

The ultrasonographic examination revealed the presence of inhomogeneities with areas of hypoechogenicity, well delimited by a hyperechogenic capsule (Figures 11, 12).

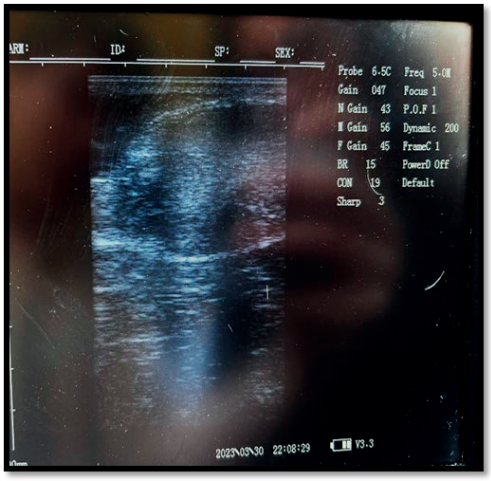


Figure 11. Ultrasound exam on the well-circumsed mass at the neck level

Dermal melanomatosis is most commonly observed in horses that are over 15 years old, and the present study included a female with age above 15 years old. Small melanocytic tumors typically do not cause any clinical signs and may only be noticed as a cosmetic blemish. However, larger tumors can cause physical obstruction of the anal sphincter, penis and prepuce, or vulvar commissure, leading to issues such as dyschezia (difficulty defecating), dysuria (difficulty urinating), and difficulties with coitus and parturition. In the present study, the most common clinical complaint reported was difficulty defecating, which is consistent with the obstructive effects of the perianal tumors.

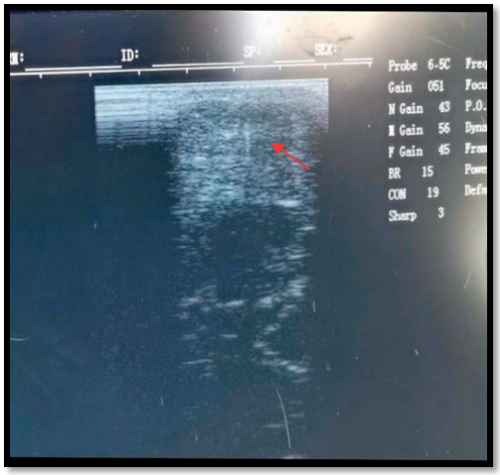


Figure 12. Ultrasound exam on the well-circumsed mass at the neck level (arrow - needle)

Gross description of the masses

The grey-coated examined mare presented multiple pigmented cutaneous-subcutaneous masses. The first tumor was located at the ventral part of the tail base, extending for approximately 25-30 cm of the tail's length, poorly defined, pigmented, involving both the perineal and the perianal areas. The skin was thick, firm, showing a large plaque-like mass consisting of multiple dark pigmented nodules, 1-3 cm in diameter, located on the tail and several partially pigmented (maculated aspect) 2-5 cm nodules on the perineum and perianal region. Two other subcutaneous masses were noted on the right side, one being localised lateral to the withers, 10-15 cm in diameter, poorly defined, and another one on the lateral cervical region, in the cranial half, well-circumscribed, spherical to irregular shaped, firm, 5 cm in diameter. Furthermore, in the right parotid area, between the base of the

external ear and the ramus of the mandible, there was observed another 5 cm firm pigmented subcutaneous mass. Two other pigmented lesions were located bilaterally in the axillary region, having approximately 5 cm diameter in the right side and 7-8 cm in the left side, both being represented by multiple subcutaneous coalescing firm irregular-shaped 1-2 cm nodules. Fine needle aspiration was performed on all the masses and slides were submitted for cytological examination.

Cytological examination

All masses showed similar cytological aspects (Figures 13, 14, 15). The slides were of high cellularity represented by well-differentiated round to oval melanocytes with variable amounts of intracytoplasmic melanin pigment granules showing mild to moderate anisocytosis and anisokaryosis. The nuclear: cytoplasm ratio is increased and variable sized prominent nucleoli can be encountered. No mitotic figures were noted in the examined slides. Also, melanophages with vacuolated cytoplasm were occasionally encountered. Slides background was mostly represented by melanin pigment from ruptured cells and blood contamination.

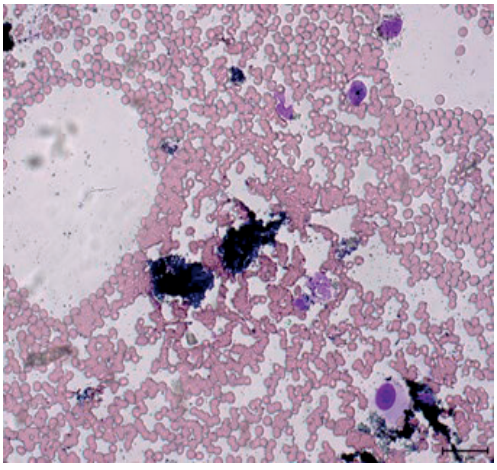


Figure 13. Cytological aspects of the lateral cervical mass, 400x, M.G.G. - Several melanocytes with mild anisokaryosis and anisocytosis and one vacuolized macrophage are noted

There are acknowledged four major types of equine melanocytic tumors: melanocytic nevus, dermal melanoma, dermal melanomatosis and anaplastic malignant melanoma. Dermal equine melanoma can be benign or malignant, being grossly described as isolated masses located on the perineum and the ventral tail area, that can affect young or older horses.

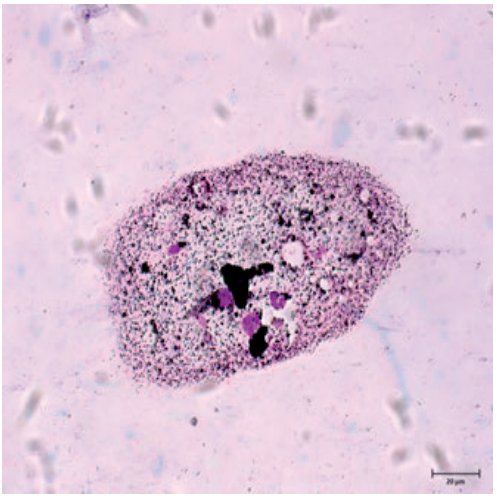


Figure 14. Cytological aspects of the tail plague-like mass, 400x, M.G.G. - Anisocytosis and anisokaryosis are observed. Numerous pigment granules are seen in the background

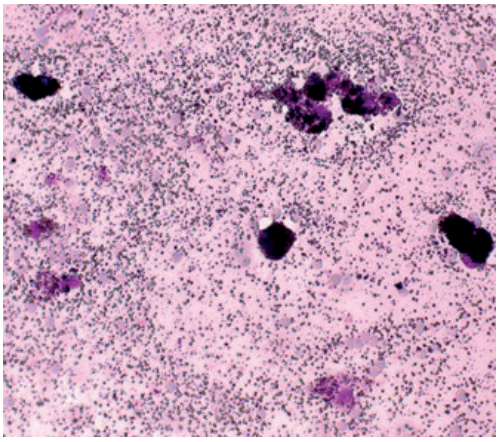


Figure 15. Cytological aspects of the axillary region mass, left side, 400x, M.G.G. - Neoplastic cells contain abundant amounts of melanin pigment and the background is marked by large amount of black pigment granules

On the other hand, melanomatosis is distinguished by multiple irregular coalescenting lesions disseminated on the ventral tail (93.3% cases), perianal (43% cases) perineal, crest and parotid salivary gland regions and other external or internal locations,

being frequently encountered in older than 15 years grey horses Many authors describe dermal melanomatosis having a high metastatic rate, considering this form a malignant stage of dermal melanoma. Thus, cytological aspects correlated with gross lesions features and clinical aspects sustain the provisional diagnosis of dermal melanomatosis.
For further evaluation of the tumors, biopsy samples from the masses should be provided for histopathological examination.

Dermal melanomatosis is a condition characterized by the presence of multiple cutaneous masses, with at least one of the masses presenting in a typical location. These typical sites include the undersurface of the tail, anal, perianal and genital regions, perineum, and lip commissures. This condition is most frequently seen in mature horses, and it is associated with a slightly older average age of 17 years. However, studies have shown that the mean age of horses with dermal melanomatosis was less than 10 years.
Unfortunately, this condition is not amenable to surgical resection, and it is likely to be associated with visceral metastasis.

CONCLUSIONS

The accumulation of data from anamnesis, clinical examinations and paraclinical investigations led to a definite diagnosis of melanoma. The X-ray revealed the absence of visceral metastasis, being in accordance with the literature. The ultrasonographic examination revealed the presence of inhomogeneities with areas of hypoechogenicity, well delimited by a hyperechogenic capsule. The cytological aspects characteristic of the diagnosis of melanoma were presented and identified regardless of the collection site.

REFERENCES

Adams W.J., Morris D.L. (1994). Short-course cimetidine and survival with colo-rectal cancer. *Lancet.*; 344:1768–1769.

Argyle D.J. (2010). *New developments in immunotherapy.* Proceedings of 35th World Small Animal Veterinary Congress. Geneva.

Finocchiaro LME, Riveros M.D., Glikin G.C. (2009). Cytokine-enhanced vaccine and suicide gene therapy as adjuvant treatments of metastatic melanoma in a horse. *Vet Rec.* ;164:278–279.

Fleury C., Berard F., Balme B., Thomas L. (2000). The study of cutaneous melanomas in Camargue-type gray-skinned horses (1): Clinical-pathological characterization. *Pigment Cell Res.*; 13: 39–46.

Goetz T.E., Ogilvie G.K., Keegan K.G., Johnson P.J. Cimetidine for treatment of melanomas in three horses. *J Am Vet Med Ass.* 1990; 196: 449–452.

Gorham S, Robl M. Melanoma in the grey horse: the darker side of equine ageing. *Vet Med.* 1986; 81: 446–448.

Heinzerling L.M., Feige K., Rieder S., Akens M.K., Dummer R., Stranzinger G., Moelling K. Tumor regression induced by intratumoral injection of DNA coding for human interleukin 12 into melanoma metastases in gray horses. *J Mol Med.* 2001; 78: 692–702.

Hewes C.A., Sullins K.E. Use of cisplatin-containing biodegradable beads for treatment of cutaneous neoplasia in equidae: 59 cases (2000-2004). *J Am Vet Med Ass.* 2006; 229: 1617–1622.

Jeglum K.A. In: *Current Therapy in Equine Medicine.* 4. Robinson N.E., editor. Pennsylvania: WB Saunders Company; 1999. Melanomas; pp. 399–400.

Kinnunen R.E., Tallberg T., Stenback H., Sarna S. Equine sarcoid tumour treated by autogenous tumour vaccine. *Anticancer Res.* 1999; 19: 3367–3374.

Knottenbelt D.C. *The non-surgical management of the equine sarcoid.* 2009 (Proceedings of the Annual Journal of the Association of French Veterinarians).

Knottenbelt, D.C., Patterson-Kane, J.C., Snalune, K.L. (2015). *Clinical Equine Oncology*. Elsevier.

Kubecova M., Kolostova K., Pinterova D., Kacprzak G., Bobek V. Cimetidine: an anticancer drug? *Eur J Pharm Sci.* 2011; 42: 439–444.

Laus F., Cerquetella M. Evaluation of cimetidine as a therapy for dermal melanomatosis in grey horse. *Israel J Vet Med.* 2010;65:48–52.

Littlewood, J.D., Lloyd, D.H., Craig, J.M. (2022). *Practical Equine Dermatology*. Oxford, UK: John Wiley & Sons, Inc.

MacGillivray K.C., Sweeney R.W., Del Piero F. Metastatic melanoma in horses. *J Vet Intern Med.* 2002; 16: 452–456. doi: 10.1111/j.1939-1676.2002.tb01264.x.

Metcalfe, L.V., O'Brien, P.J., Papakonstantinou, S., Cahalan, S.D., McAllister, H., Duggan, V.E. (2013). Malignant melanoma in a grey horse: case presentation and review of equine melanoma treatment options. *Irish Veterinary Journal*, 66(1). 22.

Millikan L.E., Hook R.R., Manning P.J. Immunobiology of melanoma. Gross and ultrastructural studies in a new melanoma model: the sinclair swine. *Yale J Biol Med.* 1973; 46: 631–645.

Milner R, Salute M, Farese J. *Immune response to Melanoma Surface Antigen (GD3) Vaccine in normal dogs.* Chicago; 2006. (Proceedings of 4th Annual Canine Cancer Conference).

Moriarty C.M., Stucky J.L., Hamburger K.W., Patil K.D., Foley J.F., Koefoot R.R. Blood histamine and solid malignant tumours. *J Cancer Res Clin Oncol.* 1988; 114: 588–592.

Muller J.V., Feige K., Wunderlin P., Heinzerling L.M. Double-blind placebo-controlled study with interleukin-18 and interleukin-12-encoding plasmid DNA shows antitumor effect in metastatic melanoma in gray horses. *J Immunother.* 2011; 34: 58–64.

Patterson-Kane J.C., Sanchez L.C., Uhl E.W., Edens L.M. Disseminated metastatic intramedullary melanoma in an aged grey horse. *J Comp Path.* 2001; 125: 204–207. doi: 10.1053/jcpa.2001.0481.

Pimenta, J., Prada, J., Cotovio, M. (2023). Equine Melanocytic Tumors: A Narrative Review. *Animals*, 13. 247.

Pulley L.T., Stannard A.A. In: *Tumors in Domestic Animals.* 3. Moulton J.E., editor. Iowa: University of California Press; 1990. Tumors of the skin and soft tissues; pp. 75–82.

Rieder S., Stricker C., Joerg H., Dummer R., Stranzinger G. A comparative genetic approach for the investigation of ageing grey horse melanoma. *J. anim Breed Genet.* 2000; 117: 73–82.

Rosengre P.G., Golovko A., Sundstrom E. et al. A cis-acting regulatory mutation causes premature hair graying and susceptibility to melanoma in the horse. *Nat Genet.* 2008; 40: 1004–1009.

Scott D.W., Miller W.H. *Equine Dermatology.* St. Louis: Saunders Elsevier; 2003.

Seltenhammer M.H., Simhofer H., Scherzer S. Equine melanoma in a population of 296 grey Lippizaner horses. *Equine Vet J.* 2003; 35: 153–157.

Theon A.P., Wilson W.D., Magdesian K.G., Pusterla N., Snyder J.R., Galuppo L.D. Long-term outcome associated with intratumoral chemotherapy with cisplatin for cutaneous tumors in equidae: 573 cases (1995-2004) *J Am Vet Med Ass.* 2007; 230: 1506–1512.

Valentine B.A. Equine melanotic tumors: a retrospective study of 53 horses (1988-1991). *J Vet Intern Med.* 1995; 9: 291–297.

Walton, R.M., Cowell, R.L., Valenciano, A.C. (2021). *Equine Hematology, Cytology, and Clinical Chemistry*. Hoboken, USA: John Wiley & Sons, Inc.

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

COMPLEX INVESTIGATIONS IN DIAGNOSIS OF MAREK'S DISEASE IN POULTRY

Georgeta DINESCU, Elvira GAGNIUC, Gabriel TOMESCU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: elvira.gagniuc@fmvb.usamv.ro

Abstract

Marek`s Disease is an oncogenic disease that affects both commercial and backyard poultry, caused by Alphaherpesvirus. Lymphoproliferative syndromes are characterized by lymphoma and are most commonly represented by T lymphocyte proliferation, with involvement of several visceral organs. Six poultry from the same population, aged between 1-12 years, non-vaccinated, have been examined after death. The following investigations have been done: gross examination, histopathology, immunohistochemistry (anti-CD3 antibody for T-lymphocytes and anti-PAX5 antibody for B-lymphocytes), and real-time polymerase chain reaction (RT-PCR). Macroscopically, all the poultry presented hepatic tumour nodules, along with tumoral enlargement of the sciatic nerve. Inconsistently, neoplasms in other organs, such as the spleen, heart, pharynx, and ovary, have been observed. The histopathological findings on the tumour mass showed a proliferation of small to large lymphocytes. Tumour cells were characterized by large pleomorphic nuclei with prominent nucleoli. The immunophenotype of transformed cells was identified as CD3 positive by immunohistochemistry; in contrast, PAX5 was negative. Virus presence confirmation was achieved through PCR. In conclusion, Marek`s Disease can manifest in chronic form, at any age, being characterized by pleomorphic lymphocytic infiltration on a systemic involvement.

Key words*: Marek's Disease, T lymphocytes, anti-CD3 antibody, systemic.*

INTRODUCTION

Marek's Disease (MD) is a poultry contagious disease, with tumoral character, caused by *Gallidalphaherpesvirus 2* (GaHV-2), a DNA virus, from the *Mardivirus* genus, *Herpesviridae* family, *Alphaherpesvirinae* subfamily. It was first described in 1907 by the Hungarian veterinarian József Marek (Sharma & Sharma, 2020). It is an ubiquitous virus, resistant for long periods in the environment, especially in dust and shelters, since chickens spread the virus through their skin and feathers. There are three serotypes, the first of which has the highest virulence and oncogenic capacity. At present, there are only specific and nonspecific measures that can be taken against the disease, but no curative ones (Stiube, 2005; Boodhoo et al., 2016).

The infection is transmitted by the respiratory route, subsequently, the GaHV-2is spread via a haematogenic way, infecting the lymphocytes. The virus replicates in the lymphoid line cells, attaching itself to their nucleus, and causing B cell cytolysis, simultaneously with activation of T lymphoid cells and reticulocyte hyperplasia. Secondary, it is distributed on a systemic level; the main lesion is a consequence of T lymphocyte proliferation, which determines the pleomorphic infiltrate in tissues and organs, represented by lymphocytes in various differentiated cellular stages (Izumiya et al., 2019). Clinically, chickens present motor disorders, weakness, and high mortality (Graham, 2016; Brash et al., 2012).

This research had its starting point in the clinical suspicion of the disease in a poultry population grown in a household system, with ages between 1 and 12 years, in which a mortality rate of 90% was noted. The same manifestations of the disease have been noted in household systems from the same geographic area. The affected poultries had no contact with other birds and were not vaccinated or dewormed. Although in the affected households ducks were present, they did not manifest any symptoms, which strengthened the suspicion of a poultry infectious disease.

The study aims to present gross and microscopic lesions specific to GaHV-2 infection and to demonstrate the importance of modern methods

of investigation in establishing a certain diagnosis of Marek disease.

MATERIALS AND METHODS

Six birds were examined, a rooster and five hens, of common breed, with ages between 1 and 12 years of age. The region in which the cases were reported is the Neajlov riverbed, Giurgiu, Romania.

Establishing a diagnosis was in stages, ante-mortem and post-mortem, such as a clinical exam, necropsy, cytopathology and histopathological examination, immuno-histochemistry, and real-time polymerase chain reaction (RT-PCR).

The clinical exam aimed to evaluate constitution, behaviour, posture, exterior appearance, major clinical signs (digestive, respiratory, and nervous), and productivity (laying and fattening degree).

The necropsy focused on exterior appearance, with special attention being given to the feather follicle aspect. In opening the celomic cavity, the focus was aimed on the macroscopic appearance of the viscera. The examination continued with the skeletal muscle system, peripheral nervous system, and osteoarticular system (Dolz & Majo, 2019).

For cytopathology examination slides were prepared from the liver, spleen, gonads, and kidneys. The slides were prepared through scraping and smearing, stained with the May-Grünwald Giemsa method and examined with an optical microscope Olympus BX41. Samples of cutaneous tissue, brachial plexus and sciatic nerves, skeletal muscles, myocardium, lungs, spleen, kidneys, gonads, oviduct, liver, digestive system, cerebrum, cerebellum and eyes were sampled for histological examination. The samples were fixed in a 10% neutral buffer formalin solution, prepared following a paraffin embedding protocol, and routinely stained with haematoxylin-eosin (HE).

Additionally, in order to identify T or B cell markers of the tumour, immunohistochemical analysis (IHC) were performed on formalin-fixed, paraffin-embedded tissue, from nervous and liver tissue. We used CD3 (anti-CD3 (2GV6) Rabbit Monoclonal Primary Antibody) and an antibody against PAX5 (anti-PAX5 (SP34) Rabbit Monoclonal Primary Antibody) that identify the T-lymphocytes (cytoplasmic region of the CD3ε-chain) and B-lymphocytes (PAX-5 encodes for transcription factor B-cell-specific activator protein - BSAP). These antibodies were previously used by other authors (Stamilla et al., 2020) and proved cross-reactivity with chicken T- and B-lymphocytes. All histological and immunohistochemical slides were analysed using an Olympus BX41 microscope and digital micrographs were acquired using an Olympus DP25 digital camera.

For the PCR examination, samples of feathers, cloaca swabs (sterile swabs without medium), and nervous, genital, and lymph-hematopoietic tissue were sampled from case #4. Feathers were stored in a sterile container, and tissue samples (brain, ovary, and spleen) were fixed in 70% ethyl alcohol and preserved in tightly sealed sterile containers. The PCR test was performed in an external laboratory and the sample collection, fixation, and transport were done accordingly to the laboratory's recommendations.

RESULTS AND DISCUSSIONS

General clinical signs in the examined poultry were represented by lethargy, weight loss to emaciation, haemorrhagic diarrhoea, losses of equilibrium, paralysis, and death. The intensity of these manifestations was different for each individual, but the order of appearance was similar, spreading over a few weeks period. Firstly, progressive weight losses, without appetite alteration and with egging decrease, followed by progressive emaciation of the muscles and lethargy. Furthermore, cutaneous/subcutaneous proliferative changes have been noticed periocular, which determined an asymmetric deformation of the head (Figure 1). The final stages of the disease have shown nervous signs, such as abnormal vocalisations, loss of equilibrium, ataxia, incoordination, and abnormal positioning of wings and legs, followed by paralysis. The death occurred in a few days after instalment of nervous signs.

Exterior examination of the carcass, in the majority of cases (4/6), feathers were molted. The skin was scaled, especially in the pectoral and internal side of the legs area (Figure 2), and

the feather follicles presented hypertrophy, especially in the pectoral area (Figure 3).
After skinning, a significant loss of subcutaneous conjunctive adipose tissue was observed, as well as generalized muscle atrophy, without noticing proliferative masses of tumoral lesions.

Figure 1. Periocular tumoral mass on the left side

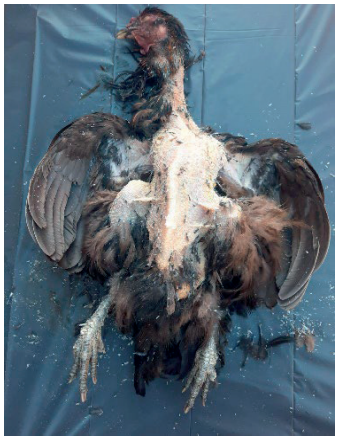


Figure 2. Cachexia and severe skin scaling

Figure 3. Skin from the pectoral area, with hypertrophied feather follicle

It was aimed to bring forth the peripheral nervous plexus (brachial and sciatic) to identify the possible morphologic changes. These nervous structures inconstantly presented size changes, with bilateral evolution, especially in the younger subjects (1-2 years of age). Macroscopically, the enlarged growth was noticed, with matting and grey-yellow colour (Figures 4 and 5).

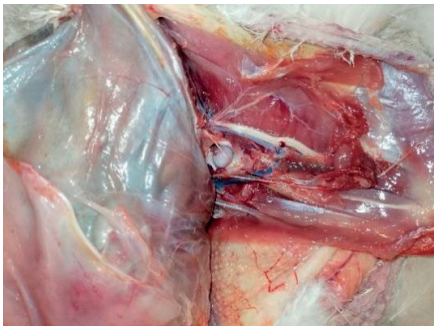


Figure 4. Left leg. Sciatic nerve with enlarged growth, matted and of grey-yellow colour

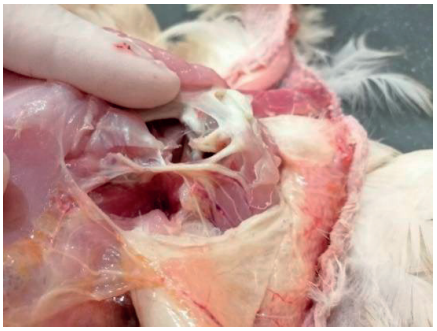


Figure 5. Left-wing. Brachial plexus - enlarged, matted, grey-yellow colour

The cerebrum, cerebellum, and brain stem were eviscerated as one piece (Figure 6) and were examined on the surface as well as in the section; no gross lesions were identified.

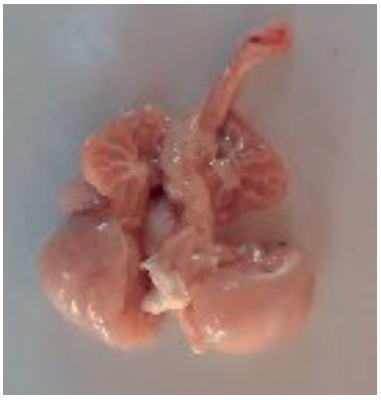


Figure 6. Cerebral tissue on a longitudinal section

In all cases examined, the liver resented hepatomegaly and on the visceral side and free margins of the lobes white, fatty, compact in-section nodules were observed (Figure 7).

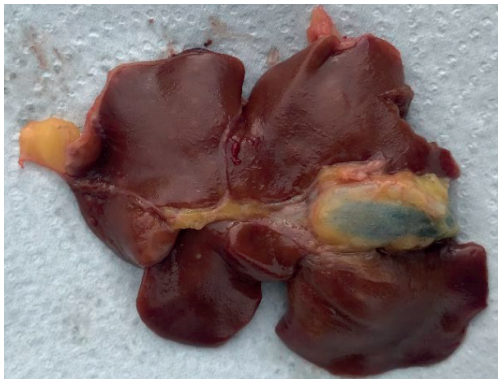


Figure 7. Liver. Multifocal white proliferations

In cytology examination, abnormal morphologic changes were observed only in the slides from the liver at hepatocytes cells, expressed by intracytoplasmic vacuolization, erythrocytes, and moderate number of tumoral cells - lymphoblasts (Figure 8). In all other slides, no observed the malignant lymphoid cells.

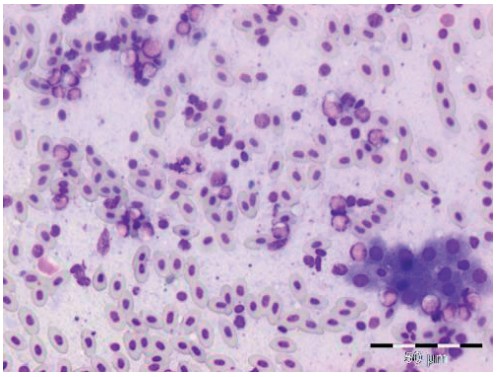


Figure 8. Liver cytology. Hepatocytes with intracytoplasmic vacuolization, erythrocytes, and a moderate number of tumoral lymphoblasts (x400, MGG)

At cutaneous tissue, we observed changes in dermal architecture and feather follicles with a pleomorphic lymphocytic infiltrate, disposed of diffuse or nodular. In the *dermal papillae*, a severe lymphocytic infiltrate can be observed. Different structures of the feather follicle can't be differentiated (*dermal papillae*, pulp, *stratum germinativum*, and *epidermal collar*) (Bacha & Bacha, 2012), and follicular *stratum corneum* and the muscle fibres of the feather follicle appear hypertrophic/hyperplasic (Figure 9). Furthermore, barbs stems appear infiltrated with pleomorphic lymphocytes (Figure 10).

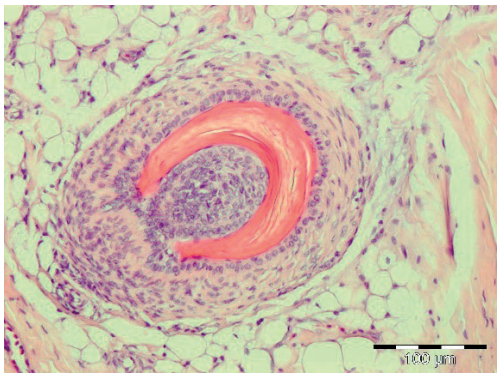


Figure 9. Feather follicle with altered histologic structure, only the follicular *stratum corneum* can be differentiated. Abundant pleomorphic lymphocytic infiltrates the *dermal papillae* and pulp, also hypertrophy of follicular muscle fibres (x200, HE)

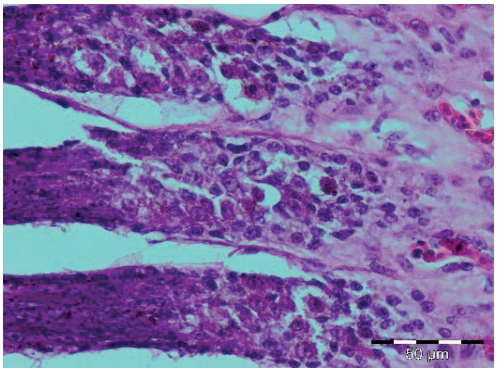


Figure 10. Barbs stems with tumoral lymphocyte pleomorphic infiltrate (x400, HE)

In peripheral nervous tissues, on histopathologic examination, we observed dilacerations of the nerve fibres with oedema can be observed of abundant pleomorphic tumoral lymphocytic infiltrate. Also, the cellular infiltrate was found in perivascular cuffing appearance, as well as diffuse infiltrate (Figure 11).

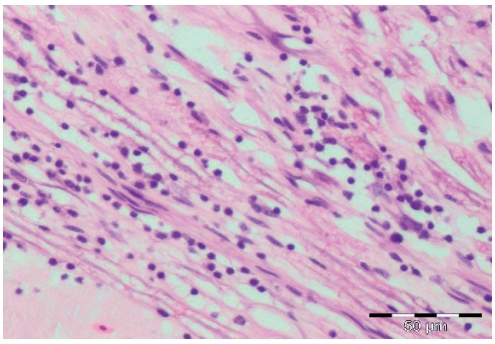


Figure 11. Peripheral nerve, longitudinal section. Mononuclear infiltrate that dilacerates the nervous fibres (x400, HE)

In the skeletal muscles (Figures 12 and 13) similar lesions to those described in the nervous tissue have been found, noticeable being the severe dilacerations of the muscle fibres, perivascular cuffing, and diffuse appearance of the pleomorphic lymphocytic infiltrate, as well as the necrosis of the muscle cells.

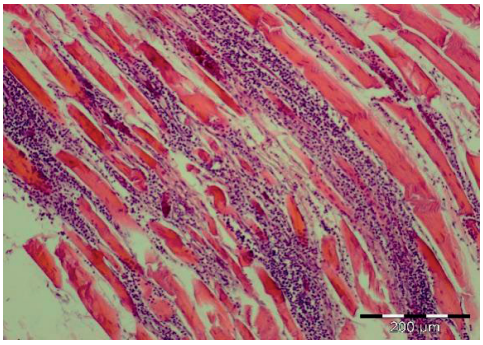


Figure 12. Skeletal muscle, longitudinal section. Abundant pleomorphic lymphocytic infiltrate, with severe dilacerations of the muscle fibres and oedema (x100, HE)

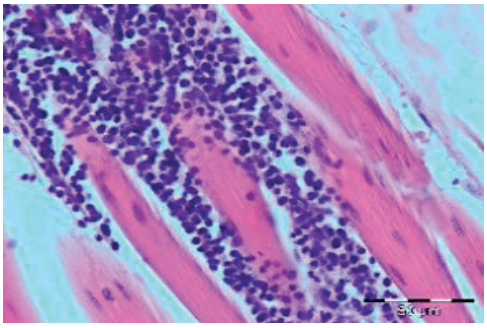


Figure 13. Skeletal muscle, longitudinal section. Pleomorphic lymphocytic infiltrate disposed of diffuse, with muscle fibre necrosis (x400, HE)

Histopathologic examination of the cerebellum brought forth the presence of tumoral infiltrate in its layers - molecular, Purkinje neurons, and granular (Figure 14).

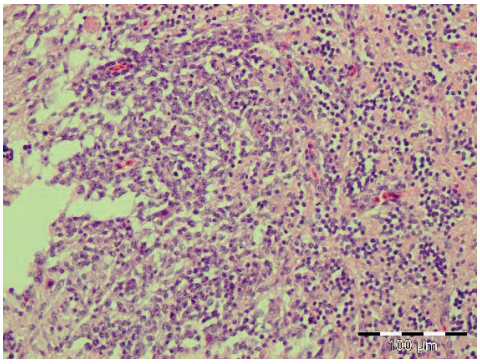


Figure 14. Cerebellum. Microscopic lesion of tumour mass, the uniform proliferation of tumoral lymphoblast and small to medium lymphocyte (x200, HE)

A perineuronal oedema can be observed, as well as pleomorphic lymphocytic infiltrate disposed of diffuse or perivascular and perineuronal cuffing. The Purkinje cells layer is marked by neuronal necrosis and satellitosis, from place to place the Purkinje cells arereplaced by neoplastic cells (Figure 15). These histologic aspects explain the severity and evolution of the neurological clinical signs in the late stages of the disease.

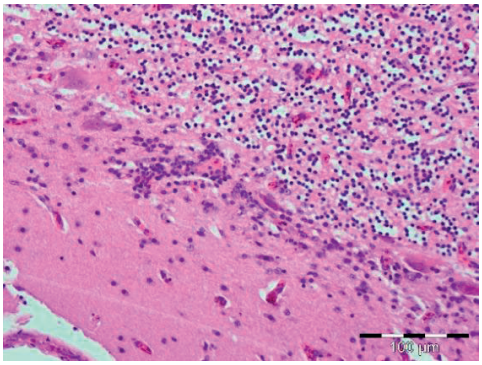


Figure 15. Cerebellum. Severe pleomorphic lymphocytes infiltration of the Purkinje layer with neuronal necrosis, satelitosis and perivascular cuffing (x200, HE)

The pleomorphic lymphocytic infiltrate presented the highest degree of proliferation in the central nervous tissue, with lesions being extended, and extremely severe (Figures 16 and 17).

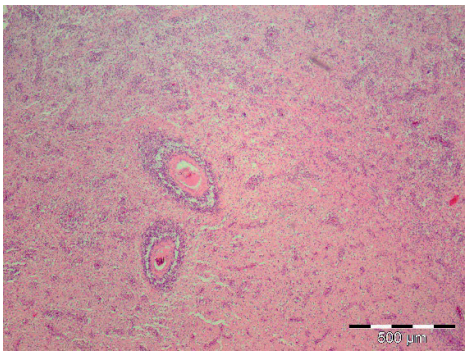


Figure 16. Cerebrum. Overview, neuropil with diffuse pleomorphic lymphocytic infiltrate and perivascular cuffing and oedema (x40, HE)

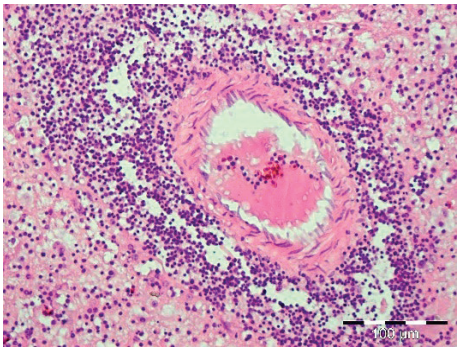


Figure 17. Cerebrum. Arterial wall with severe infiltrate in the *tunica adventitia*. Lymphocytic population with extension tendency in the neuropil, generating perivascular oedema (x200, HE)

In the cerebrum, the same changes have been noticed, expressed by severe perivascular cellular infiltrate associated with oedema in the Virchow-Robin space. Furthermore, neuronal necrosis, gliosis, and satellitosis can be identified (Figure 18).

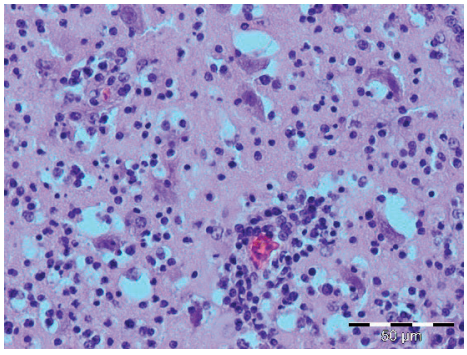


Figure 18. Cerebrum. Pleomorphic lymphoblasts, oedema in the Virchow-Robin space, satelitosis, and gliosis (x400, HE)

Severe histopathologic lesions were found in the liver, with the destruction of the vascular parietal structure due to the abundant presence of the pleomorphic lymphocytic population. The same tumoral infiltrate was observed around bile ducts, but also in their lumen (Figure 19). Neoplastic cells infiltrated Disse spaces, causing oedema (Figure 20).
Histopathological examination of the other viscera (myocardium, lungs, spleen, kidneys, gonads, oviduct, digestive system) revealed similar aspects as those described in the liver.

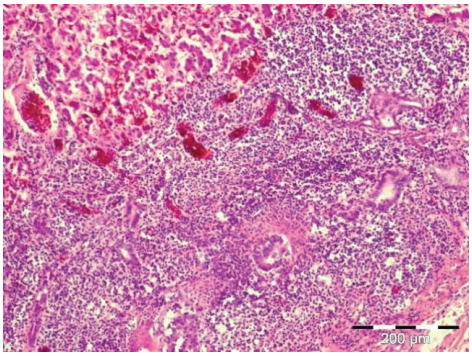


Figure 19. Liver. Diffuse pleomorphic lymphocytic infiltrate in the hepatic parenchyma, with the destruction of tissue architecture. Hepatocytes necrosis can be observed, congestion and bile ducts surrounded by neoplastic cells (x100, HE)

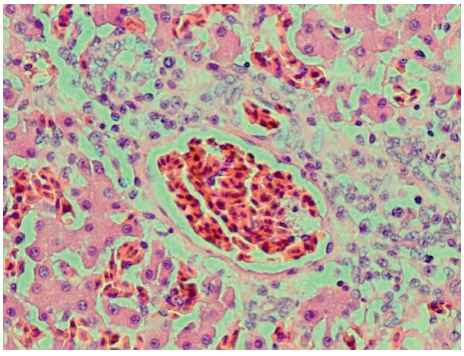


Figure 20. Liver. Tumoral cells re-infiltrated around the centrilobular veins and Disse spaces (x400, HE)

The Cluster Differentiation 3 (CD3) is a protein complex and T cell co-receptor that is involved in activating both the cytotoxic T cell (CD8+ native T cell) and T helper cells (CD4+ native T cells). In the samples, most of these cells resulted in CD3 stained in a form of membrane precipitate, brown coloured in the IHC analysis, as reported in Figures 21 and 22.

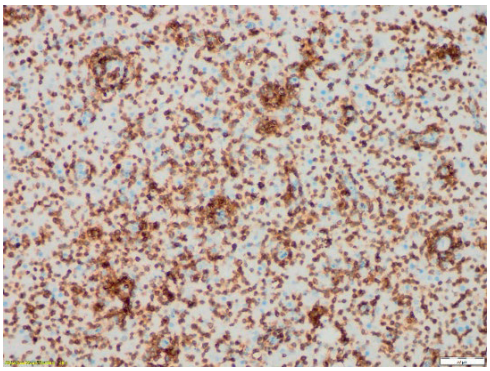


Figure 21. Cerebrum. IHC CD3, diffuse positive staining (x200)

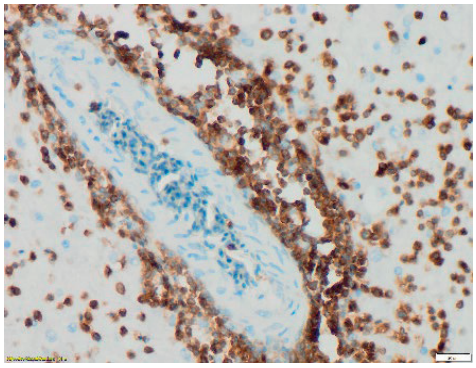


Figure 22. Cerebrum. Blood vessel CD3, diffuse positive staining (x400)

The immunophenotype of transformed cells was identified as CD3 positive by immunohistochemistry; in contrast, PAX5 (B cell marker) was negative.

The T lymphocytes in the liver were marked positive for anti-CD3 antibodies, confirming the tumoral cells population (Figure 23).

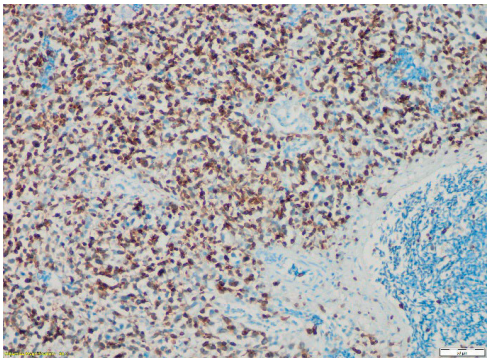


Figure 23. Liver. IHC CD3, diffuse positive staining (x200)

The present study highlights the importance of complex investigation in establishing a diagnosis of Marek's Disease. This subject was less approached nationally in the last years; the last study conducted presented the evolution of an outbreak of Marek's in broiler chickens and in layer youth raised in an intensive system in western Romania (Fodor et al., 2009).

The present study reports for the first time the presence of GaHV-2 naturally infected layer adults of different ages (1-12 years), raised in the household system, in Romania. Thus, comparison of the results of this study with those of other recent existing studies is limited for this age category. In the study above mentioned (Fodor et al., 2009), disease evolution was acute, and there were no macroscopic changes noted in the nerves, viscera, and skin, but histologic findings were similar to those described in our study, respectively the presence of a pleomorphic lymphocytic population, appearance diffuse or perivascular cuffing in different tissues and organs.

A few international scientific studies (Mete et al., 2016; Dunn et al., 2020; Abreu et al., 2016) described the disease evolution in chickens with ages up to 3 years, but in this study, one of the subjects was 12 years old. Since in the Romanian household system, it is rare to find chickens above 5 years of age, we consider that bringing forth the fact that GaHV-2 infection and specific lesions in poultry over 10 years represent a novelty of this paper. Even though the histopathological findings described are similar to those exposed in other scientific papers (Das et al, 2018; Dunn et al., 2020; Abdul-Aziz et al., 2016; Suma et al., 2018), the present study shows the existence of microscopic lesions specific for MD constantly, in all examined subjects.

Currently, confirmation of the disease through IHC and RT-PCR is constantly used (Stamilla et al., 2020; Wilson et al., 2022; Mete et al., 2016).

The complex investigations in this study allowed the differential diagnosis compared to other morbid entities in which MD can be confused.

CONCLUSIONS

General clinical signs in the subjects examined were unspecific: lethargy, weight loss to emaciation, haemorrhagic diarrhoea, equilibrium loss, paralysis, and death, aspects that can be encountered in various poultry diseases.

Results of gross and microscopic examinations described a complex overview of Marek Disease, expressed by infiltration of a pleomorphic lymphocytic population in the skin, central and peripheral nervous system, skeletal muscle tissue, and liver.

Infiltration of T tumoral lymphocytes in cutaneous tissue and feather follicles is specific to the diseases once the dust generated by skin scaling and feathers from infected poultry are virus reservoirs.

Oedema associated with the pleomorphic lymphocytic perivascular infiltrate and cellular necrosis in the skeletal muscle system and nervous system explains the neuromotor clinical signs of the disease.

Positive response in immunophenotyping cells with CD3 allowed the highlighting of T lymphocytes specific to MD proliferation. Confirming Marek's disease diagnosis, by identifying the virus, was done through RT-PCR.

REFERENCES

Abdul-Aziz, T., Barnes, H., Fletcher, O., & Swayne, D. (2016). Nervous System. In T. Abdul-Aziz, H. Barnes, & O. Fletcher, *Avian Histopathology Fourth Edition* (pp. 469-519). Florida, USA: The American Association of Avian Pathologists, Inc.

Abreu, D., Santos, F., Jose, D., Tortelly, R., Pereira, V., & Nascimento, E. (2016). Pathological Aspects of a Subclinical Marek's Disease. Case in Free-Range Chickens. *Brasilian Journal of Poultry Science*, 198.

Bacha, L., & Bacha, W. (2012). *Color Atlas of Veterinary Histology Third Edition.* Chichester, UK: John Wiley & Sons, Inc.

Boodhoo, N., Gurug, A., Sharif, S., & Behboudi, S. (2016). Marek's disease in chickens: a review with focus on immunology. *BioMed Central*, 1-2. doi:10.1186/s13567-016-0404-3

Brash, M., Ojkic, M., & Shivaprasad, H. (2012). Viral Diseases. In M. Boulianne, *Avian Disease Manual* (pp. 30-31). Florida, USA: The American Association of Avian Pathologists, Inc.

Das, S., Das, D., Panda, S. K., Sagarika, S., & Jena, B. (2018). Clinico-Pathological Studies of Marek's Disease in Chickens. *International Journal of Livestock Research, 8*, 207-217.

Dolz, R., & Majo, N. (2019). *Atlas of Avian Necropsy, Macroscopic Diagnosis Sampling.* Zaragoza, Spain: Grupo Asis Biomeda, SL.

Dunn, J., Gimeo, I., & Nair, V. (2020). Marek's Disease. In D. Swayne, *Diseases of Poultry, 14th Etidion* (pp. 562-564). Hoboken, USA: John Wiley & Sons, Inc.

Fodor, I., Coman, M., & Catana, N. (2009). An Outbreak of Marek's Disease in Broiler Chickens: Epidemiological, Clinical and Anatomopathological Aspects. *Lucrari Stiintifice Medicina Veterinara, XLII (1):*1-4. Timisoara: Faculty of Veterinary Medicine Timisoara.

Graham, J. (2016). Viral Neoplasms: MD, LL and RE. In J. Graham, *Blackwell's Five –Minute Veterinary Consult, Avian* (pp. 302-304). Massachusetts, USA: John Wiley & Sons, Inc.

Izumiya, Y., Jin, D., Lupiani, B., Liao, Y., & Reddy, S. (2019). Marek's Disease Virus. In S. Samal, *Avian Virology, Current Research and Future Trends* (pp. 345-364). Norfolk, UK: Caister Academic Press.

Mete, A., Gharpure, R., Piresky, M., Famini, D., Sverlow, K., & Dunn, J. (2016). Marek's Disease in Backyard Chickens, A Study of Pathologic Findings and Viral Loads in Tumorous and Nontumorous Birds. *Avian Diseases, 60*, 826-836.

Sharma, R., & Sharma, N. (2020). Marek's Disease. In N. Sharma, & R. Sharma, *Avian Pathology, A Colour Handbook* (pp. 159-165). New Delhi, India: New India Publishing Agency.

Stamilla, A., Messina, A., Condorelli, L., Licitra, F., Antoci, F., Lanza, M., . Puleio, R. (2020). Morphological and Immunohistochemical Examination of Lymphoproliferative Lesions Caused by Marek's Disease Virus in Breeder Chickens. *Animals: an open acces journal from MDPI*, 10(8), 1280.

Stiube, P. (2005). Boala lui Marek. In R. Manzat, *Boli Virotice si Prionice ale Animalelor* (pp. 106-166). Timisoara, Romania: Brumar.

Suma, U., Rahman, M., Nooruzzman, M., Chowdhury, E. H., & Islam, M. R. (2018, december). Pathology of Marek's disease in layer chickens in Bangladesh. *Bangladesh Veterinarian*, 34(2):35-41. doi:10.3329/bvet.v34i2.49886

Wilson, L., Lewis, M., Baigent, S., Abate, V., Dolega, B., Morrison, L., Walker, D. (2022). Marek's Disease in an Indian Peafowl (*Pavo cristatus*) with Clinical Ocular Disease and Paraparesis. *Journal of Comparative Pathology, 195*, 7-11.

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

FIELD CASTRATION OF TEN STALLIONS: ANESTHESIA AND RECOVERY MONITORING

Ioana ENE[1], Ovidiu ROȘU[2], Mario CODREANU[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest,
59 Marasti Blvd, District 1, Bucharest, Romania
[2]Animal Rescue and Care Association (ARCA), 61-63 Ion Ionescu de la Brad Blvd,
District 1, Bucharest, Romania

Corresponding author email: ene.m.ioana@gmail.com

Abstract

This study evaluates an anesthetic protocol for 10 mixed-breed horses (2-22 years old) that have undergone field surgical castration. Premedication was administered intravenously in the left jugular vein with Detomidine ($\bar{X}$ = 0.02 mg/kg). Induction was achieved with a combination of Ketamine ($\bar{X}$ = 2.38 mg/kg) and Midazolam ($\bar{X}$ = 0.038 mg/kg), administered intravenously, in the same syringe. Heart rate ($\bar{X}$ = 43.46 bpm), respiratory rate ($\bar{X}$ = 20.65 bpm), capillary refill time ($\bar{X}$ = 1.26 sec), Oxygen saturation ($\bar{X}$ = 85.73), and rectal temperature ($\bar{X}$ = 37.15 °C) were measured from induction until full recovery. We evaluated the time between premedication and induction (T0 - $\bar{X}$ = 5.5 minutes), from induction until lateral recumbency (T1 - $\bar{X}$ = 1.4 minutes), surgery duration (T2 - $\bar{X}$ = 10.5 minutes), time from the surgery until recovery in standing position (T3- $\bar{X}$ = 22.9 minutes). All animals required assistance until complete recovery and were ataxic while standing/walking for T4- $\bar{X}$ = 12.3 minutes. The anesthetic protocol provided good analgesia and muscle relaxation. All horses recovered well and no postoperative complications were seen.

Key words*: anesthesia, equine, castration, midazolam, recovery.*

INTRODUCTION

Complications associated with castration of stallions can be mild but also life-threatening, their appropriate management diminishes or even helps prevent some risks that lead to complications (hemorrhage, post-operative swelling, seroma formation, infection, eventration, peritonitis, penile damage, continued stallion-like behavior and hydrocele formation (Kilcoyne, 2013).

The recovery phase is always the hardest part of anesthesia with horses and has one of the biggest mortality rates (Gozalo-Marcilla et al., 2021). Monitoring of anesthesia (Muir et al., 2009) and the recovery time were evaluated to assess the level of postoperative complications during field conditions. Equine assisted recovery in the field and its limitations with the elected protocol of anesthesia are evaluated.

MATERIALS AND METHODS

The study took place between October and November 2021, in Ploiești county. Ambient temperatures ranged from 16ºC to 20ºC during the day with an average of 18ºC), with an average and a maximum wind speed of 3.2 m/s. No castrations were performed during bad weather conditions. The study's main purpose was to castrate ten fractious stallions without any complications. Ten abused male equines originated from different environments and were rescued by a horse sanctuary and had to be castrated to prevent hormonal-driven activities such as conspecific aggressivity and undesired female mountings (Siegal et al., 1996). Horses were difficult to approach and at least one caretaker was always present. Food and water were restricted and restrained on the day of surgery (Costea, 2017).

The weight was measured with a measuring tape and according to the established formula: weight (kg) = (heart girth × heart girth × body length)/(11,880 cm^3). Protocols were decided, and respectively dosages were adjusted according to the temperament and age of each stallion (Wagner et al., 2011). The anesthesia protocol implies a premedication with Detomidine (D) 0.015-0.025 mg/kg, followed

by induction (administered intravenously) with Ketamine (K) 2.2-2.4 mg/kg and Midazolam (M) 0.03-0.04 mg/kg.
As a part of a multimodal approach, a loco-regional bilateral intratesticular block with Procaine hydrochloride (X=3.5-4.5 mg/kg) completed the protocol. After induction, horses achieved the anesthetic plane necessary for the surgical procedure and were immediately castrated.
The elected surgical technique was the scrotal closed castration in lateral recumbency with the Henderson equine castrating instrument. (McKinnon et al., 2011). The patient was monitored during anesthesia and the time in minutes between premedication and induction (T0), the time from induction until lateral recumbency (T1), the duration of surgery (T2), time from the end of surgery until standing position (T3) and the time of ataxia while standing/walking until complete recovery (T4).
During anesthesia, heart rate, pulse rate and oxygen hemoglobin saturation SpO_2 were evaluated using a stethoscope and with the pulseoximeter, Nonin® 2500 A; Minneapolis, USA with a probe attached to the upper lip or tongue, and respiratory rate was evaluated using a stethoscope or by observing the thoracic movements, rectal temperature via a digital thermometer and capillary refill was taken, every five to ten minutes from induction until complete recovery (Figure 1).
The stallions needed to be assisted during recovery. The head blanket was gently removed and they were guided from lateral recumbency to sternal recumbency, then to a standing position (either by themselves or supporting the head and the tail to prevent rolling over or falling again).

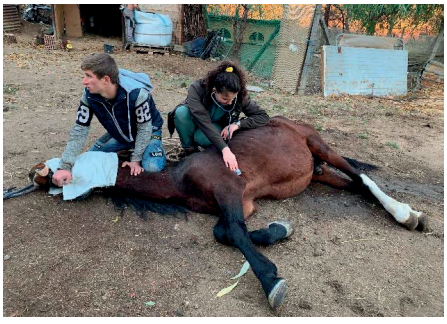


Figure 1. Monitoring the stallion in lateral recumbency

As a standard protocol, from T3 (moment of lateral recumbency) until T4, the time of complete recovery is assessed, as a key aspect of a smooth recovery in the field. The standard protocol was applied to all recumbent stallions.
Postoperatively, they received the following protocol: Flunixin meglumine 1.1 mg/kg administered intravenously, Amoxicillin trihydrate 8 mg/kg intramuscular, Gentamicin 6 mg/kg administered intravenously (subcutaneous route of administration) as a standard dose of 6000 UI/horse.

RESULTS AND DISCUSSIONS

Each horse received a halter before the administration of premedication (if this wasn't possible because the stallion was fractious, it was put on after induction) that helped with guiding his head, along with the rump (by pulling the tail) and keeping it in standing position as long as it was needed (Figure 2).
The time between premedication and induction (T0 - $\bar{X}$ = 5.5 minutes), induction until lateral recumbency (T1 - $\bar{X}$ = 1.4 minutes), surgery duration (T2 - $\bar{X}$ = 10.5 minutes), time from the end of surgery until standing position (T3) and the time of ataxia while standing/walking until complete recovery (T4), were assessed and recorded.

Figure 2. Holding the stallion in a standing position to facilitate a smoother recovery and marking the key aspects of complete recovery without complications

All animals required assistance until complete recovery and were ataxic while standing or walking for T4- $\bar{X}$ = 12.3 minutes. All horses presented ataxia, from mild to moderate, and were assisted during recovery. Safety for the handler and the stallion were key attributes evaluated on the ground at all times.

Chemical immobilizations such as the standard remote combination of Ketamine-Medetomidine administered through a dart gun (Roşu et al., 2021), were always taken into consideration but this study was achieved by administering the substances via the intravenous route to all ten intractable stallions.

Table 1. Field anesthesia doses for intractable horses (calculated after weight measurement) with the meaning of N as the number of horses that took part in the study, A as the age of the horses in years, W = weight after applying the formula and obtaining a value in kilograms, D = Detomidine, dose in mg/kg, K = Ketamine, dose in mg/kg and M = Midazolam, dose in mg/kg

N	A (years)	W (kg)	D (mg/k)	K (mg/k)	M (mg/kg)
1.	8	350	0.037	2.2	0.028
2.	4	300	0.023	2.66	0.033
3.	21	350	0.017	2.28	0.042
4.	4	400	0.015	2.25	0.0375
5.	3	325	0.018	2.46	0.038
6.	20	250	0.02	2.8	0.04
7.	15	350	0.017	2.28	0.042
8.	22	250	0.016	2.4	0.04
9.	2	320	0.015	2.18	0.039
10.	3	300	0.023	2.33	0.041

Premedication was administered intravenously in the left jugular vein with Detomidine ($\bar{X}$ = 0.02 mg/kg). Induction was achieved with a combination of Ketamine ($\bar{X}$ = 2.38 mg/kg) and Midazolam ($\bar{X}$ = 0.038 mg/kg), administered intravenously in the left jugular vein, in the same syringe. All stallions received an intratesticular block with Procaine hydrochloride ($\bar{X}$ = 4.25 mg/kg). For the entire protocol, from T0 until Heart rate ($\bar{X}$ = 43.46 bpm,), respiratory rate ($\bar{X}$ = 20.65 rpm, capillary refill time ($\bar{X}$ = 1.26 sec), SpO_2 ($\bar{X}$ = 85.73), and rectal temperature ($\bar{X}$ = 37.15°C) were evaluated from induction until full recovery.

Table 2. Recorded mean values of all stallions from T1 until T3. Legend: N = number of horses, HR = heart rate, RR = respiratory rate, CRT = capillary refill time

N	HR	RR	SpO_2	CRT
1	47.5	30.25	87.25	1
2	34	21	77	1.5
3	48.75	21.75	86.25	1.375
4	45	19.5	84.5	1
5	46.75	25.25	90.5	1
6	40.3	10.66	73.6	1.5
7	47.5	18.25	91.25	1.25
8	40.5	21.75	79.66	2
9	37.6	20.33	97	1
10	46.75	17.75	90.33	1

The physiological measurements (heart rate, respiratory rate, oxygenation, and capillary refill time) were assessed, in line with the recorded times of anesthesia and recovery. One out of ten stallions had the HR of 65 beats/minute (first recorded tachycardia). Stallion number 1 started with a HR of 65 that lowered to 36 until T2 finished, others recorded with normal HR. Stallion number 10 started with a HR of 58 beats/minute (bpm) in T1 and the last value, recorded in T3, was 38 bpm in T4. All heart rates were in normal ranges in the recovery phase (all the values were monitored in T3, as part of recovery), with one exception (horse 3) that had a 54 bpm in T3. All increasing HR (horses 1, 3 and 10) have been linked to increased stress due to manipulation (horses 1 and 10) as the horses were fractious, and due to a normal HR specific to individual (horse 3), as it was increased in every recording, and the gut motility was normal at auscultation (done only for horse 3 with diagnosis purpose).

The two stallions had the longest surgery duration (T2 increased, 16 and 20 minutes). This did not affect the anesthetic plane during the surgery and after.

All stallions remained in lateral recumbency for at least 30 minutes after induction (with the blanket over the eyes, and nostrils exposed), calculating T2 and T3 with a mean of 33.4 minutes. They had to be supervised, otherwise, they would try to stand after the surgery but could not do it on their own, not even with assisted recovery. The transition from lateral recumbency to standing position was well coordinated and mild ataxia was present in all stallions afterward. The stallions have been through light to medium plane of anesthesia, turning to light again towards the end of anesthesia (acknowledging the end as the time of removal of the blanket from the head and revealing visual and hearing stimuli). Palpebral and corneal reflexes were present at all times. No stallion showed signs of pain (muscle tremors, movements, sounds) (Muir et al., 2009).

Recovery was well coordinated, assisted in all ten horses. The stallions were evaluated after a week and they were assessed as clinically healthy with all signs leading to a normal behaviour (the usual eating and drinking habits,

normal social interaction and no physiological or musculoskeletal weakness).

Table 3. Recovery times (monitored in minutes) with N = number of horse evaluated, T0 as the time in minutes between premedication and induction, T1 as the time from induction until lateral recumbency, the duration of surgery (T2), time from induction until recovery - from lateral recumbency to standing position (T3) and the time of ataxia while standing/walking until complete recovery (T4)

N	T0 (min)	T1 (min)	T2 (min)	T3 (min)	T4 (min)
1.	13	2	7	30	5
2.	2	1	20	14	8
3.	7	1	12	26	7
4.	2	2	8	22	8
5.	6	2	16	17	6
6.	3	2	9	25	5
7.	5	1	7	27	30
8.	4	1	6	28	15
9.	5	1	10	20	5
10.	8	1	10	20	34

CONCLUSIONS

The used combination of Detomidine in premedication and Ketamine - Midazolam in induction proved to be reliable for the field castration of intractable stallions reducing the risk of postoperative complications.

Mild ataxia was present with the elected protocol but can be significantly reduced by prolonging the time the stallion remains in lateral recumbency for at least 30 minutes after induction (calculating T2 and T3) and has no visual stimuli (with the blanket over the eyes, and nostrils exposed). Prolonging the time that the horse stays in lateral recumbence (maintenance of anesthesia) reduces the time the horse remains ataxic in a standing position before walking carelessly.

All recoveries were assisted, proving that there should be a team of at least two people monitoring the horse for safety reasons. An assisted recovery is always recommended.

The authors recommend the possibility of supplemental Oxygen in field conditions that was unavailable for this study.

REFERENCES

Clarke, K. W., & Trim, C. M. (2013). Veterinary Anaesthesia E-Book. Elsevier Health Sciences. Chapter 11 - Anaesthesia of the horse

Costea R., Rosu O., Ene I. (2021). Evaluation of An Anaesthesia Protocol Following Translocation Of Feral Horses Outside The Letea Forest. *Scientific Works. Series C. Veterinary Medicine*, Vol. LXVII, Issue 1, ISSN 2065-1295, 59-62.

Costea Ruxandra (2017). Anesteziologie. Printech, 101-102.

Gozalo-Marcilla, M., & Ringer, S. K. (2021). Recovery after General Anaesthesia in Adult Horses: A Structured Summary of the Literature. *Animals,* 11(6), 1777. doi:10.3390/ani11061777

Kilcoyne, Isabelle (2013). Equine castration: A review of techniques, complications and their management. Equine Veterinary Education. 25. 10.1111/eve.12063

McKinnon, A. O., Squires, E. L., Vaala, W. E., & Varner, D. D. (Eds.) (2011). Equine reproduction. John Wiley & Sons.

Muir, W. W., & Hubbell, J. A. E. (2009). Equine anesthesia: Monitoring and emergency therapy. St. Louis, Mo: Saunders/Elsevier.

Roşu, O., Melega, I., Evans, A. L., Arnemo, J. M., & Küker, S. (2021). Evaluation of Medetomidine-Ketamine for Immobilization of Feral Horses in Romania. Frontiers in Veterinary Science, 8.

Siegal, M., Barlough, J. E., & University of California, Davis. (1996). UC Davis book of horses: A complete medical reference guide for horses and foals. New York: HarperCollinsPublishers.

Stover, B. C., & Caulkett, N. A. (2021). Anesthesia techniques used for field castration of 10 intractable horses. The Canadian veterinary journal = *La revue veterinaire canadienne,* 62(5), 501–504.

Wagner Elizabeth L. PhD, Patricia J.Tyler MS (2011). A Comparison of Weight Estimation Methods in Adult Horses. *Journal of Equine Veterinary Science,* 31(12): 706-710.

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

COMPARATIVE LEVELS OF ANTIBIOTIC RESISTANCE IN PIGS RAISED UNDER DIFFERENT TECHNOLOGIES

Anca GOCAN (SECARĂ)[1], Carmen Dana ȘANDRU [1, 2], Diana OLAH[1], Gheorghiță DUCA[2], Constantin CERBU[1], Aurel VASIU[1], Emoke PALL[1, 2], Cristian VĂLEANU[1], Marina SPÎNU[1, 2]

[1]University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Veterinary Medicine, 3-5 Mănăștur Street, 400372, Cluj-Napoca, Romania;
[2] Institute of Research and Development for Montanology Cristian, 147 Street XIII, Cristian, Sibiu, Romania

Corresponding author: marina.spinu@gmail.com

Abstract

Swine are considered one of the most important species of food animals worldwide, the majority of meat for human consumption in numerous countries being represented by pork. Sometimes heavily treated with antibiotics to maintain herd health, swine could be considered a sentinel species for humans in respect to antibiotic resistance.The research compared the dynamics of antibiotic resistance by distance between an intensive farm, where antibiotic use is considerable, and small households, with no antibiotic use, at 5 (A) and 10 (B) kilometers from the farm. Twenty-eight clinically healthy pigs (n = 16, farm, n = 6 A, n = 6 B) were sampled. Standard microbiological techniques, identification of the strains by Vitek 2 system and Kirby Bauer test to assess the antimicrobial resistance were applied. Of the total bacterial species identified, E. coli (72%) dominated while 10% were Gram+ bacilli and 10% Gram - unidentified coco-bacilli. The highest MAR (multiple antibiotic resistance) index was calculated in E. coli (MAR = 0.88, A and B) and also two other strains from the farm (MAR = 0.77). The high MAR indices stand for the presence of antibiotic resistance in untreated animals, urging for a more accurate surveillance of the phenomenon.

Key words*: swine, farming technology, antibiotic resistance, spatial dynamics.*

INTRODUCTION

Swine are considered to be one of the most important farmed species of animals worldwide, owing it to their large-scale exploitation by which a partial coverage of the growing global meat demand - up to 13% increase by 2030 - is managed (OECD/FAO, 2021). Moreover, due to their anatomophisiology, sharing numerous similarities to that of humans, swine elicit an increasing interest in human medicine a donor model of various organs.

Antimicrobial resistance of pathogenic agents, a continuously expanding phaenomenon, originated in the exposure to antibiotics, either following therapy or horizontally, by simple exposure to bacteria hosting such genes (Munita and Arias, 2016). The spread of non-discriminating, extended and lengthy antibiotic use in farmed animals increased the prejudice to human health both through direct contact and food of animal origin (Peng et al., 2022). This also increased very much the farming costs (Founou et al., 2017).

Given the extended farming of swine communities, disease transmission poses a severe risk to animal health and welfare, therefore antibiotics represent a fast resourse to alleviate the symptoms and control microbial agents, being critically important in veterinary medicine (Holmer et al., 2019; Pyörälä et al., 2014). Thus, swine could be considered as a sentinel species for humans, hence the antibiotic resistance found in swine must also produce strong alarm signals in human medicine (Neil, 2015).

E. coli, a member of *Enterobacteriaceae,* inhabits the intestine and is considered an indicator of fecal pollution of the environment (Pholwat et al., 2020). It ubiquitous presence makes *E. coli* a perfect candidate for investigations on its antimicrobial resistance, as a measure of antimicrobial resistance in both

hosts and habitat and aslo provides important information on the spread of MAR

Nevertheless, there are few studies on the multiple antibiotic resistance (MAR) or multi-drug resistance (MDR) and their spread on large farms, but also in the neighbouring premises and their broader environment.

Therefore, the aim of the work was to compare the level of antibiotic resistance of bacterial strains isolated from pigs reared on an intensive farm, representing an environment with extended use of antibiotics, with those isolated from pigs reared in households, at 5 and 10 kilometers distance from the farm, with much lesser if any, use of antibiotics.

MATERIALS AND METHODS

The isolation and identification of pathogenic and conditionally pathogenic bacteria and the evaluation of their antibiotic resistance were pursued, both in pigs belonging to an intensive.

For that, fecal samples were collected by use of sterile swabs from the rectum of clinically healthy pigs of which 16 from the farm, and 6 from each A and B villages, located 5 and 10 km away from the farm, respectively.

All samples were transferred to simple broth, cultivated for 24 h at 37°C and then spread to nutritious agar to obtain isolated colonies. After the expiration of the incubation time (24 h at 37°C), the morphology of the colonies allowed the first stage identification. Further, the samples were subjected to identification on chromogenic McConkey and Chapman agar media. The final identification was performed by the use of Vitek2 Compact system (BioMerieux, France) and 64 wells for biochemical testing.

The sensitivity to antibiotics in the isolated strains was tested against: oxytetracycline, tulathromycin, methicillin, cefquinome, enrofloxacin, neomycin, amoxicillin and clavulanic acid, colistin and chloramphenicol by the Kirby Bauer diffusion method and diametres of the inhibitions zones were measured. Due to its importance in human and animal pathology, *E. coli* was chosen in this experiment to exemplify the presence and spread of antimicrobial resistance.

The data were statistically analysed for significance of the treatment effect by using Microsoft Excel software.

RESULTS AND DISCUSSIONS

Of the total bacterial species identified, 72% were represented by *E. coli*, 10% by Gram-positive bacilli, 10% totaled Gram-negative coccobacilli unidentified following Vitek testing. Only 4% represented *Staphylococcus sciuri*, respectively *Citrobacter amalonaticus*.

The interpretation of the diameters of the inhibition zones of the strains taken in the study allowed the assessment of the effectiveness of each antibiotic used. Thus, the most effective antibiotics proved to be cefquinome, from the class of cephalosporins and neomycin, from the class of aminoglycosides, and the most ineffective was methicillin, an antibiotic from the class of penicillins. Out of 29 antibiograms performed, 24 of the tests showed total resistance to methicillin. The presence of resistant colonies was most frequently identified against chloramphenicol.

All studied strains, both from the farm and from households, showed total methicillin resistance, except for one *E. coli* strain isolated from farm pigs, one *E. coli* strain and one *Staphylococcus sciuri* strain isolated from in two distinct individuals from household B. The same two microorganisms showed sensitivity to all antibiotics.

In *E. coli* strains (n = 14) isolated from farmed pigs, the highest resistance was recorded to oxytetracycline, followed by amoxicillin/clavulanic acid, respectively methicillin.

No resistance was observed to tulathromycin and cefquinome (Figure 1).

In the *E. coli* strains (n = 5) isolated from pigs in household A, resistance to amoxicillin, methicillin and oxytetracycline was observed in the same proportion. No resistance to tulathromycin was noted (Figure 2).

In *E. coli* strains (n = 2) isolated from pigs in household B, the highest resistance was noted to oxytetracycline, followed by amoxicillin. No resistance to colistin was noted (Figure 3).

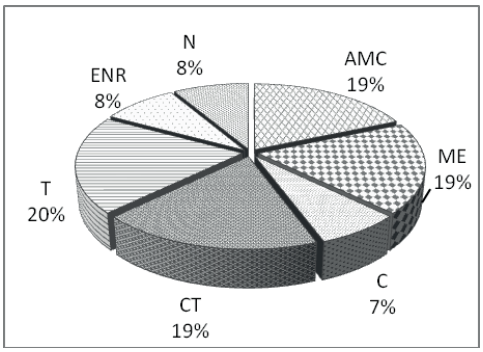


Figure 1. Resistance of *E. coli* strains isolated from the farmed swine

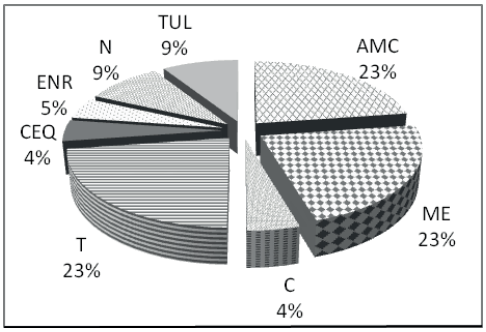


Figure 2. Resistance of *E. coli* strains isolated from household A

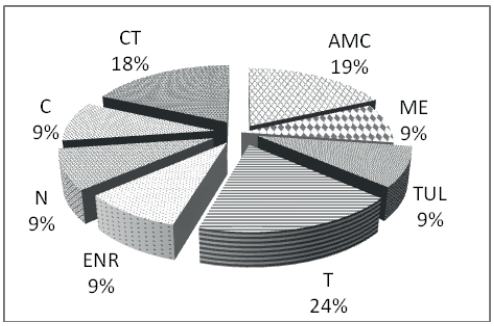


Figure 3. Resistance of *E. coli* strains isolated from household B

The MAR index is defined as the ratio of the number of antibiotics to which the strain was found to be resistant to the number of antibiotics to which the bacterial strain was exposed (Sandhu et al., 2016).

A MAR index > 0.2 indicates the existence of a multidrug-resistant bacterial strain from a source where antibiotics have been intensively used, while an index < = 0.2 suggests that the strain originates from a source where antibiotic treatment it was less often applied (Adenaike et al., 2016).

The highest MAR index was recorded in strains of *E. coli* from households A and B (MAR = 0.88) (Figure 5), followed by another strain of *E. coli* isolated from the farm (MAR = 0.77) (Figure 4).

Bacteria that showed a MAR index of 0 were represented by an *E. coli* strain from household B, another *E. coli* strain from the farm and *Staphyloccocus sciuri*, isolated from household B (Figure 6).

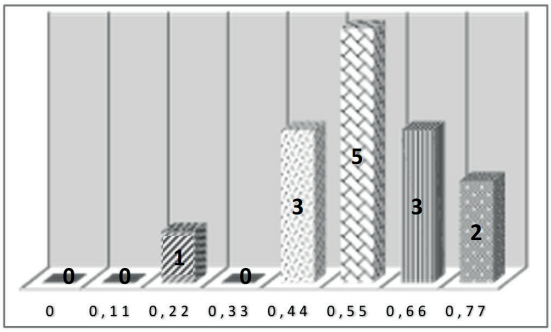


Figure 4. Correspondence between the number of resistant strains and their MAR index on the farm

Escherichia coli is commonly found in the digestive tract of both humans and animals. It is also isolated from soil, water or food, as a result of contamination through faeces or during the slaughter of animals for consumption. Shiga-toxin-producing *E. coli* (STEC) O157 emerged as a public health threat following its initial identification as a pathogen in a 1982 episode of illness associated with consumption of raw beef (Etcheverría and Padola, 2013). *E. coli* strains O157:H7 and O157:NM (non-motile) are recognized as major etiological agents in hemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) in humans (Bruyand et al., 2018).

The US Centers for Disease Control and Prevention estimates that *E. coli* O157:H7 causes approximately 73,400 illnesses and 60 deaths each year in the United States. Recent reports indicate that antimicrobial resistance of *E. coli* O157 is increasing (Abebe et al., 2023). However, the extent to which different antimicrobial use practices have contributed to the rise of antimicrobial resistance is not yet fully known (Zheng et al., 2012).

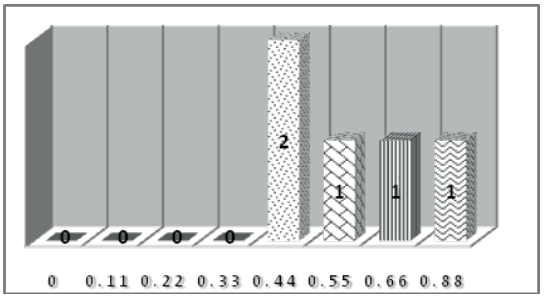


Figure 5. Correspondence between the number of resistant strains and their MAR index in household A

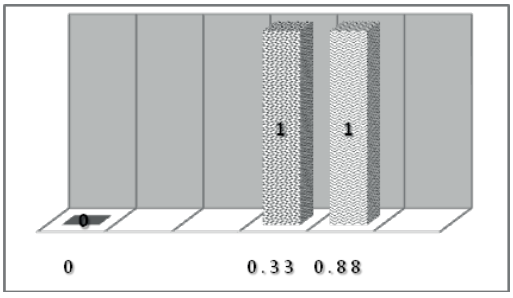


Figure 6. Correspondence between the number of resistant strains and their MAR index in household B

E. coli is one of the most common causes of infection in humans and animals worldwide. *E. coli* is associated with a wide variety of diseases, but there is a high degree of host and disease specificity for different serotypes. Thus, certain pathogenic *E. coli* serotypes dominate as causes of intestinal infections in humans and various animal species. In contrast, extraintestinal infections are caused by types of *E. coli* that are found in the normal intestinal flora (Chan et al., 2014).

Few studies describe antimicrobial susceptibility patterns among *E. coli* O157 isolates from humans and animals. Until near the end of the 20th century, most strains demonstrated susceptibility to antibiotic action, with resistance to ampicillin, streptomycin, sulfonamides and tetracycline being observed (Wegener et al., 1999). In poultry litter, it was found that probably the *groEL* gene fundamented *E. coli* resistance to ampicillin, colistin, tetracycline, sulphonamides, or cephalothin (Khong et al., 2023) A study conducted between 1984 and 1987 notes susceptibility of all 56 strains of *E. coli* isolated from human cases to all antibiotics tested (amoxicillin with clavulanic acid, ampicillin, ceftazidime, ceftriaxone, cefuroxime, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, streptomycin, sulfisoxazole, tetracycline, ticarcillin, tobramycin and trimethoprim-sulfamethoxazole). Of the 176 strains isolated between 1989 and 1991, 13 (7.4%) were resistant to streptomycin, sulfisoxazole, and tetracycline (Kim and Cha, 2021).

CONCLUSIONS

The results obtained in this study supported the initial working hypothesis, according to the book it was expected to quantify an increased MAR index in the farm (0.77), which decreases with increasing distance from it (0.55 at 5 km and 0.44 at 10 km from the farm) .

The very high values of the MAR indices, which correspond to the *E. coli* strains identified on the farm, confirm the hypothesis that the use of antibiotics at this level was intense.

Although the maximum number of strains with an intermediate MAR index were identified on the farm, strains with a MAR index of 0 were also isolated here, similar to households located at a distance of 10 km from it, which suggests the more frequent use of certain categories of antibiotics and avoidance of others in intensive rearing.

The existence of antibiotic resistance even at relatively large distances from the farm may indicate other mechanisms of installation of the phenomenon than strictly treating sick animals.

MAR indices with high values support the need for increased vigilance of the veterinarian and even corrective measures from the perspective of antibiotic use.

AUTHORS' CONTRIBUTION

All authors had equal contribution in the study design, sampling, sample processing, data processing and writing the paper.

REFERENCES

Abebe, E., Gugsa, G., Ahmed, M., Awol, N., Tefera, Y., Abegaz, S., & Sisay, T. (2023). Occurrence and antimicrobial resistance pattern of *E. coli* O157:H7 isolated from foods of Bovine origin in Dessie and Kombolcha towns, Ethiopia. *PLoS neglected tropical diseases*, *17*(1), e0010706.

Adenaike, O.D. (2016). Multidrug Resistance and Multiple Antibiotic Resistance Index of *Escherichia coli* Strains Isolated from Retailed Smoked Fish. *Journal of Natural Sciences Research, 6*, 7-10.

Bruyand M., Mariani-Kurkdjian P., Gouali M., de Valk H., King L.A., Le Hello S., Bonacorsi S., Loirat C., 2018, Hemolytic uremic syndrome due to Shiga toxin-producing *Escherichia coli* infection, Médecine et Maladies Infectieuses, 48:3,167-174

Etcheverría, A. I., Padola, N. L. (2013). Shiga toxin-producing *Escherichia coli*: factors involved in virulence and cattle colonization. *Virulence*, *4*(5), 366–372.

Founou R. C., Founou L. L., Essack S. Y. (2017). Clinical and economic impact of antibiotic resistance in developing countries: a systematic review and meta-analysis. *PLoS One* 12:e0189621.

Holmer, I., Salomonsen, C.M., Jorsal, S.E. , Astrup L. B., Jensen V. F., Borck Høg B., Pedersen K. (2019) Antibiotic resistance in porcine pathogenic bacteria and relation to antibiotic usage. *BMC Vet Res* 15, 449

Munita J. M., Arias C. A. (2016). Mechanisms of antibiotic resistance. *Microbiol. Spectr.* 4 1–24.

Neill J. O. (2015). Antimicrobials in agriculture and the environment: reducing unnecessary use and waste. *Rev. Antimicrob. Resist.* 1–40.

OECD/FAO (2021). OECD-FAO Agricultural Outlook 2021-2030, OECD Publishing, Paris, https://doi.org/10.1787/19428846-en.

Peng Z., Hu Z., Li Z., Zhang X., Jia C., Li T., Dai M., Tan C., Xu Z., Wu B., Chen H., Wang X. (2022). Antimicrobial resistance and population genomics of multidrug-resistant *Escherichia coli* in pig farms in mainland China. *Nat Commun* **13**, 1116

Pholwat, S., Pongpan, T., Chinli, R., Rogawski McQuade, E. T., Thaipisuttikul, I., Ratanakorn, P., Liu, J., Taniuchi, M., Houpt, E. R., & Foongladda, S. (2020). Antimicrobial Resistance in Swine Fecal Specimens Across Different Farm Management Systems. *Frontiers in microbiology*, *11*, 1238. https://doi.org/10.3389/fmicb.2020.01238

Pyörälä, S., Baptiste, K. E., Catry, B., van Duijkeren, E., Greko, C., Moreno, M. A., Pomba, M. C., Rantala, M., Ružauskas, M., Sanders, P., Threlfall, E. J., Torren-Edo, J., & Törneke, K. (2014). Macrolides and lincosamides in cattle and pigs: use and development of antimicrobial resistance. *Veterinary journal (London, England : 1997)*, *200*(2), 230–239.

Zheng F., Lin G., Zhou J., Cao X., Gong X., Wang G., Qiu C. (2012). Discovery and characterization of gene cassettes-containing integrons in clinical strains of *Riemerella anatipestifer*, *Veterinary Microbiology*, 156:3–4, 434-438

Chan, J., Lo, W. U., Chow, K. H., Lai, E. L., Law, P. Y., Ho, P. L. (2014). Clonal diversity of Escherichia coli isolates carrying plasmid-mediated fosfomycin resistance gene fosA3 from livestock and other animals. *Antimicrobial agents and chemotherapy*, *58*(9), 5638–5639.

Wegener, H. C., Aarestrup, F. M., Jensen, L. B., Hammerum, A. M., & Bager, F. (1999). Use of antimicrobial growth promoters in food animals and *Enterococcus faecium* resistance to therapeutic antimicrobial drugs in Europe. *Emerging infectious diseases*, *5*(3), 329–335.

Rasheed, M. U., Thajuddin, N., Ahamed, P., Teklemariam, Z., Jamil, K. (2014). Antimicrobial drug resistance in strains of Escherichia coli isolated from food sources. *Revista do Instituto de Medicina Tropical de Sao Paulo*, *56*(4), 341–346.

Kim, D. W., Cha, C. J. (2021). Antibiotic resistome from the One-Health perspective: understanding and controlling antimicrobial resistance transmission. *Experimental & molecular medicine*, *53*(3), 301–309.

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

USE OF VETSHIELD®/SOFTSHIELD® COLLAGEN CONTACT LENSES IN MELTING CORNEAL ULCERS IN DOGS: 342 CASES (2013-2022)

Iuliana IONASCU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: iuliana.ionascu@usamv.ro

Abstract

Melting corneal ulcers in dogs develop secondary to the imbalance between proteinases and proteinase inhibitors in the healing process of corneal wounds. Common complications of melting corneal ulcers in dogs are descemetocele, staphyloma and uveitis, which can lead loss of vision. Medical records of 342 dogs diagnosed with melting corneal ulcers from May 2013 to November 2022 treated surgically using VetShield® and SoftShield® collagen bandage lenses and third eyelid flap. Dogs included in the study had a clinical diagnosis of melting corneal ulcer without evidence of retinal detachment or lens luxation confirmed by ocular ultrasonography. 204/342 cases (59.65%) of treated dogs regained their vision and corneal transparency; 113/342 cases (33.04%) had corneal fibrosis and pigmentation with improved vision; 17/342 cases (4.97%) had lost vision due to corneal scarring and 8/342 (2.34%) underwent intrascleral prosthesis due to secondary glaucoma as a complication. Placement of bandage collagen lenses and third eyelid flap in melting corneal ulcers in dogs is an easy, straightforward surgical procedure which can be performed by any veterinarian practitioner with good results.

Key words*: bandage collagen lens, corneal scar, melting corneal ulcer, third eyelid flap.*

INTRODUCTION

Melting ulcerative keratitis in dogs is a serious condition with a high risk of corneal perforation and blindness (Famose, 2014). Maintenance and repair of corneal stromal extracellular matrix (ECM) requires a tightly coordinated balance of ECM synthesis, degradation and remodeling in which proteolytic enzymes (proteinases) perform important functions (Ollivier et al., 2007; Gellat ,2013). Ulcerative keratitis in dog is associated with initially high levels of tear film proteolytic activity, which decrease as the ulcer heals. The proteinase levels in melting ulcers remain elevated leading to rapid progression of the ulcers (Ollivier et al., 2007). The most common bacterial species associated with canine keratomalacia are *Pseudomonas aeruginosa* and β-hemolytic *Streptococcus* sp. (Tsvetanova et al., 2021).

Melting corneal ulcers usually have an underlying infectious cause, but non-infectious causes are also possible. Risk factors for the development of corneal melting ulcers include ocular trauma and ocular exposure particularly in brachycephalic breeds due to lagophthalmos. Quantitative or qualitative tear film deficiencies are predisposing factors and play an important role in the imbalance between proteolytic enzymes and protease inhibitors.

Depending on the depth of the stromal defect, treatment of melting corneal ulcers can be medical or surgical (Williams et al., 2017).

When the melting process progresses despite the medical management, surgery is indicated to avoid corneal perforation and permanent blindness. Surgical stabilisation in response to progression of stromal loss was required in less than half of the cases in one study (Guyonnet et al., 2022.

Reported surgical techniques in melting corneal ulcers include: corneoconjunctival transposition (CCT), conjunctival grafts, porcine small intestinal submucosal (SIS) graft (Vanore et al., 2007) (ACellVet®) porcine urinary bladder submucosa (Chow et al., 2015), (ACell®) bioscaffolding matrix (Keenan et al., 2020) (Tutopach®) bovine pericardium graft (Dulaurent et al., 2014), amniotic membrane transplantation (Gimenez et al., 2015; Ion et al., 2016; Costa et al., 2019) (BioCorneoVet®) porcine corneal stroma xenograft (Sanitillo et al., 2021) and Vetrix BioSIS plus® (Barachetti et al., 2020). A newer treatment modality used in human and veterinary medicine is the corneal

collagen cross-linking (CXL), which uses riboflavin and UV-A irradiation to increase corneal stability (Williams et al., 2017). The aim of this study is to report the use of VetShield® and Softshield® collagen bandage lenses as a treatment option in the management of melting corneal ulcers in dog. To the authors' knowledge, this is the first report of its use in melting corneal ulcers in dogs evaluating the long-term postoperative outcome in regards to corneal transparency, scarring, integrity, and maintenance of vision.

MATERIALS AND METHODS

Medical records of 342 dogs diagnosed with melting corneal ulceration from May 2013 to November 2022, that underwent VetShield® or Softshield® collagen bandage lens placement, followed by a third eyelid flap, were reviewed.
All dogs underwent complete ophthalmic and physical examination. Signs of blepharospasm, mucopurulent discharge, corneal edema, stromal dissolution (Figure 1), corneal vascularization (Figure 2), conjunctivitis and uveitis (Figure 3) were recorded.

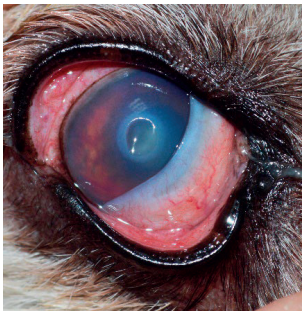


Figure 1. OD Melting corneal ulcer with corneal edema and dissolution in a 7 year- old Crossbred

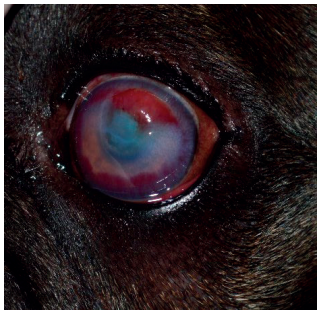


Figure 2. OS Melting corneal ulcer with corneal vascularization in a 3 year-old French Bulldog

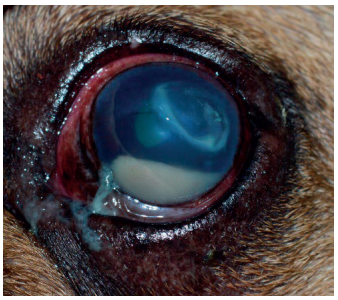


Figure 3. OS Melting corneal ulcer, secondary uveitis in a 4 year-old French Bulldog

Dogs were included in the study only if there was no retinal detachment or lens luxation as confirmed by ocular ultrasound (Codreanu et al., 2022). Prior to surgery, additional diagnostic tests, such as complete blood count and serum biochemistry were performed in every case.
Dogs were placed under general anesthesia after pre-medication with dexmedetomidine (Dexdomitor 0.1 mg/ml, Orion Pharma) 15 mcg/kg, ketamine (Ketamidor 100 mg/ml, Richter Pharma, Austria) 5 mg/kg and butorphanol (Butomidor 10 mg/ml, Richter Pharma, Austria) 0.2 mg/kg IM. Anaesthesia was induced with propofol (Propofol Lipuro 10 mg/ml, Braun Germany) 2-4 mg/kg IV. The patients were intubated, maintained on oxygen and isoflurane 1.5-2% (Anesteran, Rompharm S.A., Romania).
The cornea was flushed using saline solution and a drop of tropicamide (Tropicamida®, S.C. Rompharm Company SRL, Ilfov, Romania) was applied for pharmacologic mydriasis.
After placement of the eyelid speculum, antibiotic eye drops were instilled (ofloxacin, Floxal®, Bausch & Lomb Rochester, NY, SUA or tobramycin, Tobrom® S.C. Rompharm Company SRL, Ilfov, Romania).

Figure 4. Softshield® collagen bandage lenses, original packaging

For tectonic support of the cornea, VetShield® bandage collagen lenses (Vetshield Collagen Corneal Shield 72 hr, Oasis Medical Inc, USA) or Softshield® (Figure 4) bandage collagen lenses (Softshield Collagen Corneal Shield 72 hr, Oasis Medical Inc, USA) were used.
Preparation of the cornea was carried out by removing the corneal epithelium using a sterile cotton swab. The collagen bandage lens was hydrated with saline (Figure 5) and inverted.

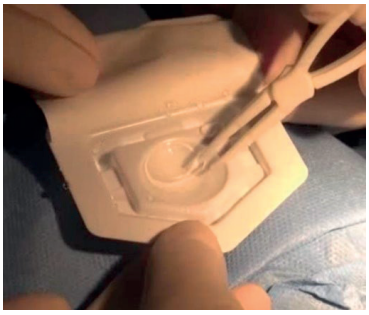


Figure 5. The collagen lens is hydrated using saline

The antibiotic eyedrops were instilled in the concave surface of the collagen lens (Figure 6) and placed on the corneal surface (Figure 7).

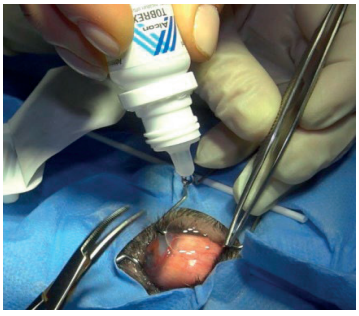


Figure 6. The antibiotic eyedrop sollution is instilled in the concave surface of the collagen lens

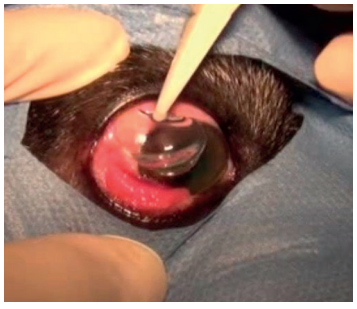


Figure 7. Placement of the collagen lens on the corneal surface

The air bubbles were removed from under the bandage collagen lens by applying gentle pressure using sterile cotton tipped applicators. Subsequently, the collagen lens remains well-adhered to the corneal surface.
A complete third eyelid flap was placed using a simple interrupted suture (Vicryl 3/0, Ethicon, Johnson & Johnson, Germany) in order to mantain the collagen lens on the corneal surface (Figure 8).

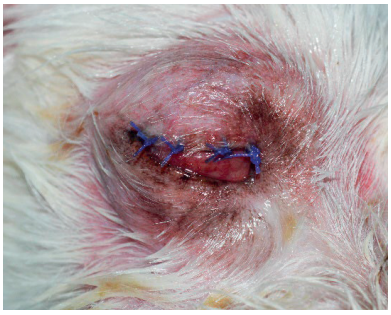


Figure 8. Clinical appearance of the third eyelid flap

In brachycephalic dogs, the third eyelid flap was placed between the third eyelid margins and the superior conjunctival fornix owing to a short nictitating membrane and shallow orbit. The short third eyelid appears to be a characteristic in these breeds (Figure 9).

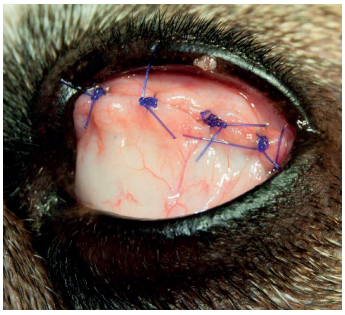


Figure 9. Sutures placed between the third eyelid leading edge and the superior conjunctival fornix

Postoperatively, the use of Elizabethan collar was mandatory (Figure 10). The sutures were removed after 21 days.
Postoperative medications included doxicycline (Ronaxan® 20 mg, Boehringer Ingelheim, Germany) 10 mg/kg SID for 14 days; probiotics (Synbiotic D-C®, ADM Protexin Limited, GB) for 14 days; local antibiotics (ofloxacin, Floxal®, Bausch & Lomb Rochester, NY, SUA)

for 21 days and kanamycin ointment applied BID on the third eyelid sutures (Kanamicina®, SC Antibiotice SA, Iasi, Romania) for 21 days.

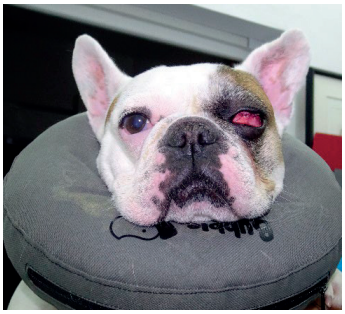


Figure 10. The use of Elizabethan collar is mandatory after the surgery

Postoperatively, dogs were re-evaluated weekly and the ocular ultrasound was performed to evaluate the diameter of the eye globe, the corneal healing process and to detect possible complications that can occur such as anterior and posterior synechiae, pupillary seclusion, descemetocele or retinal detachment. After sutures' removal at 21 days, dogs were re-evaluated at 2 and 6 months.

The melting corneal ulcers were considered healed when the fluorescein test was negative, cornea was transparent, with minimal scarring and dogs regained vision.

RESULTS AND DISCUSSIONS

The melting corneal ulcers had been treated by referring veterinarians for a median of 5 days (range: 1–10 days) prior to enrolment in this study. The patients received medical treatment consisted of topical and systemic antibiotics and artificial tears with hyaluronic acid.

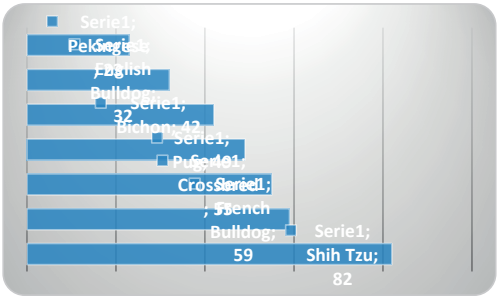
All the surgeries were performed by the same clinician (Iuliana Ionascu) at the Ophthalmology Department of the Faculty of Veterinary Medicine, Bucharest.

The dog breeds included in the study (table 1) were Shih Tzu (n=82), French Bulldog (n=59), Crossbred (n=55), Pug (n=49), Bichon (n=42), English Bulldog (n=32) and Pekingese (n=23).

The median age was 7 years, with a range between 7 months and 13 years.

The Schirmer Tear Test had low values in all cases (0 mm/min to 10 mm/min). The intraocular pressures were within the normal range in the early stage of the melting corneal ulceration and were lower in dogs with secondary uveitis.

Table 1. Dog breeds included in the study



Of all dogs (n=342), included in this study (Table 2), 160 dogs (46.78%) presented with 25% of the cornea affected (Figure 11, Figure 12); 131 dogs (38.31%) had 50% of the cornea affected (Figure 13, Figure 14) and 51 dogs (14.91%) had 100% of the cornea affected by ulceration (Figure 15, Figure 16).

Visual acuity was decreased in patients from groups A and B and absent in patients from group C.

Table 2. The size (%) of the corneal surface affected, highlighted by the fluorescein test

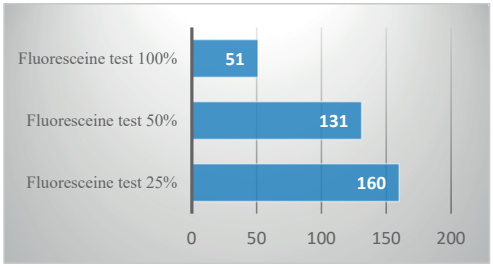


Figure 11. OD Melting corneal ulcer. The fluorescein test is positive on less than 25% of the corneal surface. (7 year-old Crossbred)

Figure 12. OS Melting corneal ulcer. The fluorescein test is positive over25% of the corneal surface. (4 year-oldEnglish Bulldog)

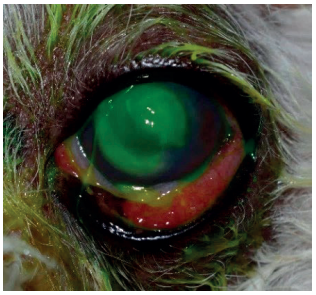


Figure 13. OS Melting corneal ulcer. The fluorescein test is positive over 50% of the corneal surface. (11 year-old Caniche)

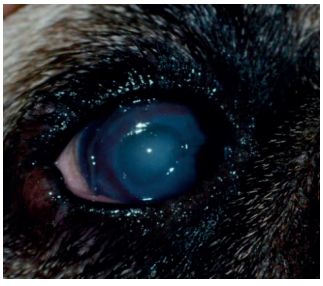


Figure 14. OD Melting corneal ulcer. Note the malacic appearance of 50% of the corneal surface. (2 year-old Pug)

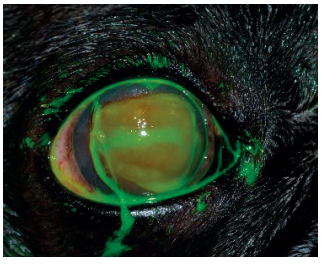


Figure 15. OD Melting corneal ulcer. Corneal vascularization is bordering the ulcer and the fluorescein test is positive over 100% of the corneal surface. (3 year-old French Bulldog)

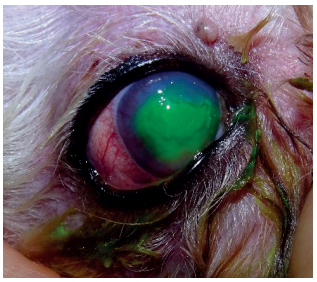


Figure 16. OD Melting corneal ulcer. Note the malacic appearance and the positive fluorescein test over 100% of the corneal surface. (10 year-old Bichon)

Microbiology analysis was not performed, however according to the studies on bacterial flora (McKeever et al., 2021) in dogs with melting corneal ulcers (*S. pseudintermedius, ß-hemolytic Streptoccocus spp., and P. aeruginosa*) ceftriaxone (Cefort®, S.C. Antibiotice, S.A., Iasi, Romania) was administered intraoperatively 20 mg/kg IV.

Moreover, the pharmacokinetics study (Bowden et al., 2022) of extended release parenteral ceftiofur (Excede®) in canine tear film compared with minimal inhibitory concentrations (MICs) of ceftiofur against common ocular pathogens in dog is extremely low compare to concentration after a single injection (up to 10 days in tear compartment), we used in our study one single dose of ceftriaxone intraoperatively.

Collagen bandage lenses should be placed on the corneal surface immediately after hydration.

Between two types of bandage collagen lenses used in this study, there are differences related to the texture after their hydration using saline.

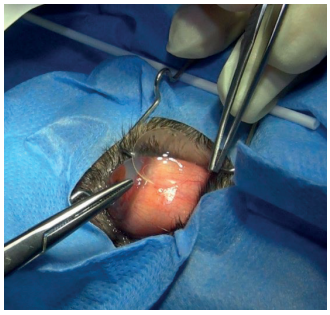


Figure 17. The stable shape of the VetShield® collagen lens after hydration

VetShield® bandage collagen lenses remain in a stable shape after hydration and are easy to

mobilize and place on the corneal surface (Figure 17).
The SoftShield® bandage collagen lens tends to fold over itself after hydration (Figure 18) and is more difficult to place on the corneal surface. After application and fixation, both types of collagen lenses remain firmly attached to the corneal surface.

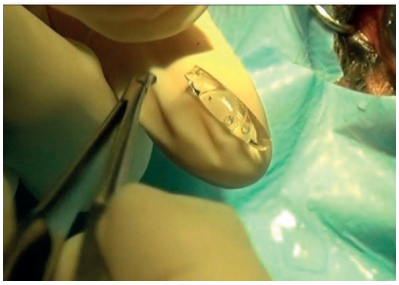


Figure 18. The SoftShield® collagen lens folded over itself after hidration

In 96 brachycephalic patients (28%), the third eyelid flap was placed between the leading edge of the nictitating membrane and the conjunctiva of the superior conjunctival fornix. In this breed, limited third eyelid excursion is a common characteristic.
204/342 dogs (59.65%) in group A and B, had minimal corneal scarring or transparent cornea at 21 days after the surgery, when the sutures were removed and had a negative fluorescein test. Furthermore they were visual and continued long term treatment with 1.2% hyaluronic acid and aminoacids (an-Hypro®, An-Vision, Germany). Re-evaluations at 2 and 6 months after the surgery revealed normal Schirmer Tear Test readings and transparent cornea (Figure 19, Figure 20 and Figure 21) or with minimal scarring.

Figure 19. Case from Figure 2, clinical appearance 2 months after surgery. Transparent cornea and negative fluorescein test.
(3 year-old French Bulldog)

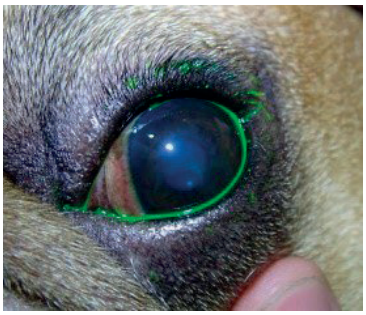


Figure 20. Case from Figure 3, clinical appearance 2 months after surgery revealing cornealtransparency and negative fluorescein test
(4 year-old French Bulldog)

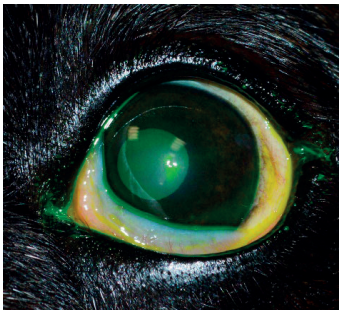


Figure 21. Case from Figure 3, clinical appearance 2 months after surgery. The dog regained vision, the cornea maintained itss transparency and was fluorescein negative (4 year-old English Bulldog)

In 87/342 of dogs in group A and B (25.44%), corneal vascularisation was reported, 21 days later, when the sutures were removed. The blood vessels were reaching the periphery of the bandage collagen lens with negative fluorescein test (Figure 22).

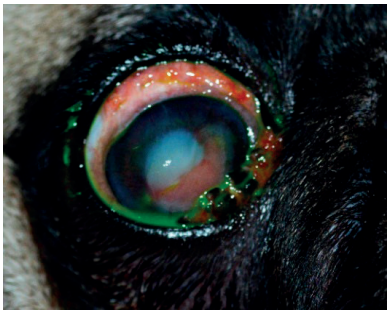


Figure 22. Case from Figure 13, clinical appearance 21 days postoperatively (2 year-old Pug)

These cases were prescribed long-term 1.2% hyaluronic acid and amino acid drops (an-Hypro®, An-Vision, Germany) and healing eye

drops (ii-2018) containing sodium hyaluronat, acetylcysteine and insulin (Ionascu et al., 2020) twice a day. A follow-up performed 2 months later revealed minimal corneal scarring (Figure 24) or corneal vascularisarion reaching the centre of the opaque cornea but the dogs were visual (Figure 23).

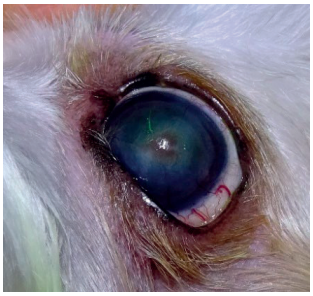


Figure 23. Case from Figure 12, clinical appearance 2 months after surgery.Corneal scarring and vascularization are noted (11 year-old Caniche)

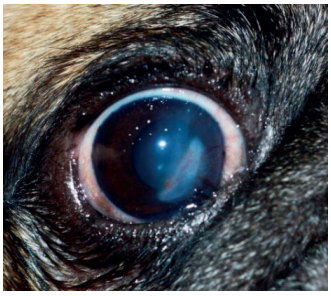


Figure 24. Case from Figure 21, clinical appearance 2 months after surgery. Corneal scarring is noted (2 year-old Pug)

The dogs included in group C (51 cases) were presented with the entire cornea affected and for which postoperative outcome showed the greatest variability.

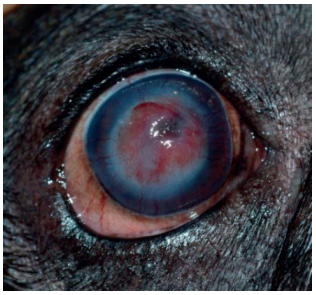


Figure 25. Case from Figure 15, clinical appearance 2 months after surgery. Note the central corneal vascularization (3 year-old French Bulldog)

19/51 dogs from group C (5.55%) had corneal ulcers that were considered healed within 2 months with corneal opacity and persistent focal central vascularization (Figure 25) or with corneal opacity and diffuse persistent vascularization (Figure 26).

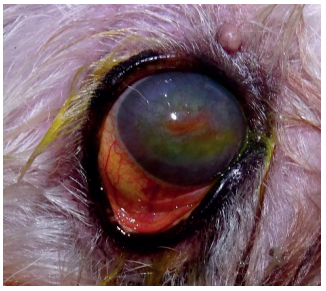


Figure 26. Case from Figure 16, clinical appearance 2 months after surgery. Note the opaque cornea and diffuse corneal vascularization (10 year-old Bichon)

Six months after surgery, the corneal scarring reduced in size and 19/51 patients regained their vision (Figure 27).

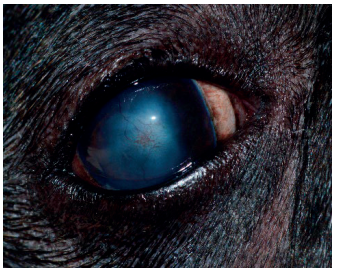


Figure 27. Case from figure 25, clinical aspect 6 months after the surgery. Minimal corneal scarring (3 years old French Bulldog)

17/55 dogs from group C (4.97%) healed with a significant corneal scarring (Figure 28, Figure 29) or pigmentation (Figure 30) and visual loss.

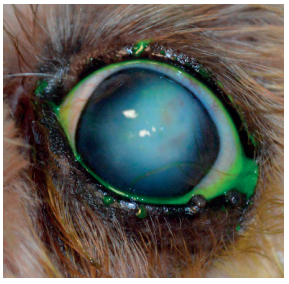


Figure 28. OS Corneal scarring, clinical appearance 6 months after surgery. Visual reflexes were absent. (9 year-old Crossbred)

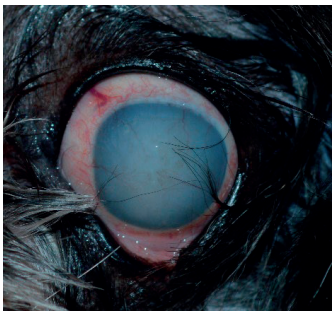


Figure 29. OS Corneal scarring, clinical appearance 6 months after surgery. Visual reflexes were absent (1 year-old Shih Tzu)

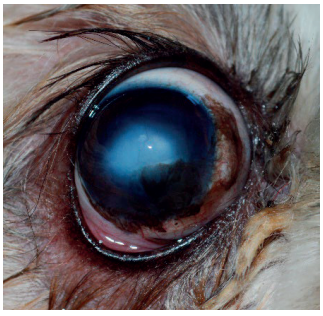


Figure 30. OD Corneal scarring and pigmentation, clinical appearance 6 months after surgery. Visual reflexes were absent (7 year-old Shih Tzu)

7/51 dogs in group C (2.05%) underwent a second surgery (placement of SoftShield® collagen lens and third eyelid flap) owing to third eyelid flap sutures breakdown at seven days postoperatively (Figure 31, Figure 32).
Studies in animals with corneal epithelial defects and keratectomy wounds have shown that SoftShield® collagen lenses have a significant role in re-epithelialisation and reduction of stromal inflammation and edema (Eshar et al., 2011; Ion et al., 2016; Willoughby et al., 2002).
Softshield® lenses have a dissolution time of 72 hours, however the sutures were only removed at 21 days to allow a complete corneal healing. According to the author's experience (Unpublished data), when the sutures were prematurely removed before the 21 days cut-off, for example at 7 days after the surgery, the cornea is most often not healed, has stromal edema, and moderate vascularization (Figure 32).
Therefore, in those cases, dogs underwent a second procedure to have the contact lens and third eyelid flap replaced.

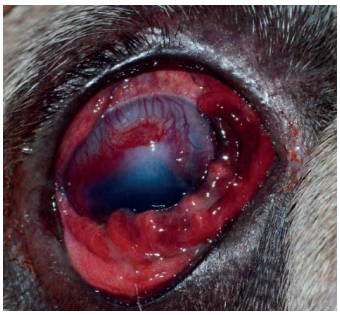


Figure 31. Clinical appearance 7 days after the first surgery. Note the corneal vascularization and opacity (7 year-old English Bulldog)

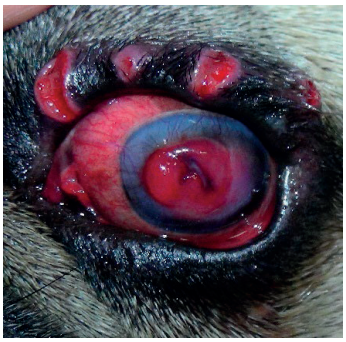


Figure 32. Clinical appearance 7 days after the first surgery. Note the moderate corneal vascularization (1 year-old Pug)

Clinical appearance 6 months after the second surgery in 7/51 dogs shown improved corneal vascularization (Figure 33) and central corneal opacity at the site where the collagen bandage lens is embedded into the corneal structure (Figure 34).

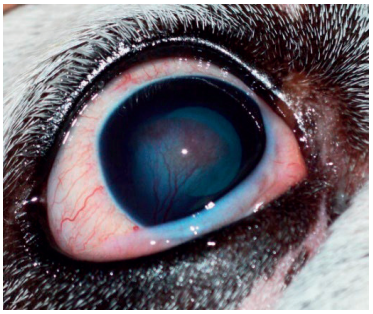


Figure 33. Case from Figure 28, clinical appearance 2 months after the second surgery. Note minimal corneal vascularization and opacity (7 year-old English Bulldog)

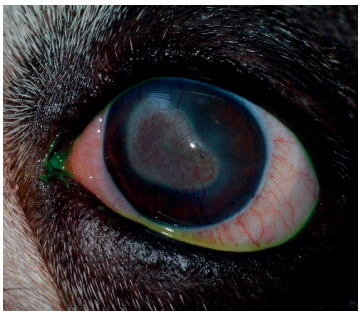


Figure 34. Case from Figure 29, clinical appearance 2 months after the second surgery. Note the central corneal scar (1 year-old Pug)

8/51 dogs in group C (2.34%) developed secondary glaucoma after the surgery likely owing to to pupillary seclusion. These cases subsequently underwent evisceration and intrascleral prosthesis (Figure 35).

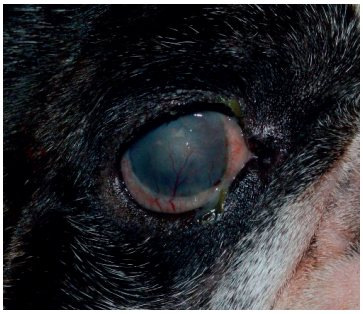


Figure 35. OD Intrascleral prosthesis (6 year-old English Bulldog)

At the six months follow-up 204/342 (59.65%) dogs had a transparent cornea and intact visual reflexes. Topical 1.2% hyaluronic acid and amino acid eye drops gel (an-Hypro®, An-Vision, Germany) was prescribed to all dogs diagnosed with keratoconjunctivitis *sicca* (Gronkiewicz et al., 2017).

113/342 (33.04%) dogs had intact visual reflexes, but with corneal scarring and pigmentation. 17/342 (4.97%) dogs lost their visual reflexes due to corneal scarring and 8/342 (2.34%) dogs underwent intrascleral prosthesis surgery.

In regards to surgical treatment of melting ulcers there are several options to re-establish tectonic support and stabilize the cornea with good visual outcome.

A multicentric retrospective study (2010-2017) on cryopreseved amniotic membrane transplantation for the treatment of complicated corneal ulcers in dogs was published by Costa et al., in 2019. Cryopreserved amniotic membrane transplantation (AMT), unilayer, bilayer and multilayer technique was used in 51/114 melting ulcers in dogs with a mean defect size of 6.2 mm (2-18 mm) with high satisfactory visual and cosmetic outcomes.

Santillo et al., in 2021 used porcine corneal stromal xenograft (BioCorneaVet®) in 40 cases (25 perforations, 8 descemetoceles and 9 deep stromal defect) with a diameter ranging from 3 to 10 mm and reported postoperative complications such as mild to severe corneal vascularization, melting and glaucoma.

Use of BioCorneaVet® is also a surgical option for deep stromal defects providing good tectonic support and preserving anatomical integrity and vision (Sanitillo et al., 2021).

Autologous buccal mucous membrane grafts (Mezzadri et. Al., 2021) and corneoconjunctival transposition (CCT) surgery with and without bioscaffolding matrix (ACell®) can be utilized with good results for corneal ulcer repair in dogs (Keenan et al., 2020).

Four-layer porcine small intestinal submucosa (Vetrix BioSIS plus®) used alone as a scaffold for surgical treatment of deep corneal defects had good results in terms of mechanic support and corneal transparency (Barachetti et al., 2020).

Previously reported surgical treatments for deep melting ulcers include porcine small intestinal submucosa (SIS) and third eyelid flap may be an effective alternative to the traditional conjunctival grafts. The advantage of using a SIS graft include good corneal transparency, preservation of corneal integrity and maintenance of vision (Vanore et al., 2017).

For these techniques special equipment is required (surgical microscope, microsurgical kit), but also good knowledge of the technique of corneal suturing.

The surgical protocol for melting corneal ulcers described in the present study can be performed by any veterinarian as it does not require special techniques. Other benefits of this approach include decreased anaesthesia time and lower procedure costs for the owner.

This study included a large number of cases with large corneal defects treated treated by placement of VetShield® and Softshield®

collagen bandage lenses that act as tectonic support, providing tissue to fill the loss of the corneal structure and promoting healing via vascularization from the limbus (Eshar et al., 2011; Ion et al., 2016; Ionascu, 2017; Willoughby et al., 2002). Collagen shields are manufactured from porcine or bovine collagen and three different collagen shields are currently available on the market with dissolution times of 12, 24, and 72 hours (Guber et al., 2019; Willoughby et al., 2002).

At the same time, the third eyelid behaves like an anatomical bandage (Ionascu, 2017; Vanore et al., 2007; Gellat, 2022) and by applying constant pressure for 21 days it maintains the collagen lens on the corneal surface and helps with the healing of the corneal defect. For the surgical success (regaining vision, mechanical support and corneal transparency) a strict protocol was followed. A complete ophthalmic examination and an ocular ultrasound were performed is mandatory before and after the surgery (and every week until the sutures' removal) to evaluate the diameter of the eyeglobe, the corneal healing process and to detect possible complications that might have developed (Codreanu et al., 2022).

In our study, corneal scarring and pigmentation were reported in 113/342 (33.04%), however these dogs regained their vision 2 months postoperatively. Only 5% of dogs lost their vision due to corneal scarring and 2% dogs underwent intrascleral prosthesis surgery due to secondary glaucoma. The complications reported after this type of surgery are similar to other studies (Osinchuk et al., 2022).

CONCLUSIONS

Surgical treatment of melting corneal ulcers in dogs using VetShield® or Softshield® bandage collagen lenses and third eyelid flap may be an effective alternative with good outcome in terms of corneal transparency and vision improvement. Early diagnosis and surgery increase the chances of success in regain patient's vision.

This novel technique can be performed by any veterinarian as it does not require special equipment or knowledge of microsurgical techniques.

REFERENCES

Barachetti, L., Zanni, M., Stefanello, D., Rampazzo, A., (2020). Use of four-layer porcine small intestinal submucosa alone as a scaffold for the treatment of deep corneal defects in dogs and cats: preliminary results. *Vet Record,* 186(19), 28. https://doi.org/10.1136/vr.105513.

Bowden, A. C., Allbaugh, R. A., Smith, J. S., Mochel, P. J., Sebbag, L. (2022). Kinetics and minimal inhibitory concentrations of ceftiofur in tera film following extended-release parenteral administration (Excede®) in dogs. *Journal of Frontiers in Veterinary Medicine*. 9:975113. https://doi.org/10.3389/fvets.2022.975113.

Chow, D. W. Y., Westermeyer, H. D. (2016). Retrospective evaluation of corneal reconstruction using ACell VetTM alone in dogs and cats: 82 cases. *Veterinary Ophthalmology*, *19*(5), 357–366. https://doi.org/10.1111/vop.12294.

Codreanu, M.D., Dana, C., Bîrțoiu, A. I., Ionașcu, I., Șerdean, C., Diaconescu, A., Constantinescu, R. (2022). *Tratat de ultrasonografie clinică veterinară, Capitolul 2.13 Examenul ecografic al globului ocular, 478-493.* Editura Medicală, București, ISBN 978-973-39-0931-6.

Costa, D., Leiva, M., Sanz, F., Espejo, V., Esteban, J., Vergara, J., Díaz, C., Huguet, E., Cairó, M., Ríos, J., Peña, M. T. (2019). A multicenter retrospective study on cryopreserved amniotic membrane transplatation for the treatment of complicated ulcers in dog. *Veterinary Ophthalmology*, *22*(5), 695-702. https://doi.org/10.1111/vop.12643.

Dulaurent, T., Azoulay, T., Goulle, F., Dulaurent, A., Mentek, M., Peiffer, R. L., Isard, P. F. (2014). Use of bovine pericardium (Tutopatch®) graft for surgical repair of deep melting corneal ulcers in dogs and corneal sequestra in cats. *Veterinary Ophthalmology*,*17*(2),91–99. https://doi.org/10.1111/vop.12047.

Guber, I., Bergin, C., Malde, S., Guber, J., Hamada, S., & Lake, D. (2019, October 1). First experience with Oasis Collagen SOFT SHIELD® for epithelial defect after corneal cross-linking. *International Ophthalmology*, Vol. 39, pp. 2149–2151. https://doi.org/10.1007/s10792-018-01070.

Gelatt, K., Ledbetter, E. C., & Gilger, B. C. (2013). *Veterinary Ophtalmology*.

Gelatt, K., Gelatt, J. P., Plummer, C. E., (2022).

Surgical Procedures for the Conjunctiva and the Nictitating Membrane, *Veterinary Ophthalmic Surgery*, (163-194), https://doi.org/10.1016/B978-0-7020-8163-7.00008-1.

Gronkiewicz, K. M., Giuliano, E. A., Sharma, A., & Mohan, R. R. (2017). Effects of topical hyaluronic acid on corneal wound healing in dogs: a pilot study. *Veterinary Ophthalmology*, *20*(2), 123–130. https://doi.org/10.1111/vop.12379.

Ionascu, I. (2017). *Therapeutic guide of veterinary ophthalmology*. Bucharest, Romania: Curtea Veche, ISBN 978-606-792-016-1.

Ionascu, I., Argăseală, A., Uzun, S., Gârdan, G., Calențaru, V. (2020). A new eye drop formulation

used in the management of corneal ulcers in dogs and cats. *AgroLife Scientific Journal,* 9(1), 164-171, ISSN 2285-5718.

Ionascu, I (2021). *Atlas of Veterinary Ophthalmology, second edition,* Bucharest, Romania, Curtea Veche, ISBN 978-606-792-040-6.

Ion, L, Argaseala, A., Ionascu, I. (2016). The Use Of 72–Hour Dissolvable Collagen Eye Shield (Vetshield™) In Deep Corneal Ulcers In Dogs. ECVO Conference, Budapest, Hungary.

Keenan, A.V., Boveland, S.D., Galarza, R., Moore, P.A. (2021). Corneoconjunctival transposition with or without ACell® deep corneal ulcer repair in 18 dogs. *Veterinary Ophthalmology*, 23(5), 884-891 https://doi.org/10.1111/vop.12815.

Mezzadri, V. R., Crotti, A., Nardi, S., Barsotti, G. (2021). Surgical treatment of canine and feline descemetoceles, deep and perforated corneal ulcer with autologous buccal mucous membrane grafts. *Veterinary Ophthalmology*, 24(6), 599-609, https://doi.org/10.1111/vop.12907.

Osinchuk, S. C., Levitt, S., Sandmeyer, L. S., Parker, S.E. (2022). Evaluation of conjunctival graft procedures and factors that lead to graft complications in canine cases. *Veterinary Ophthalmology, on line acce*s https://doi.org/10.1111/vop.13008.

Santillo, D., Mathieson, I., Corsi, F., Göller R., Guandalini, A. (2021). The use of acellular porcine corneal stroma xenograft (BioCorneaVet™) for the treatment of deep stromal and full thickness corneal defects: A retrospective study of 40 cases (2019–2021). *Veterinary Ophthalmology*, 24(5) , 469-483, https://doi.org/10.1111/vop.12927.

Tsvetanova, A., Powell, R. M., Tsvetanov, K. A., Smith, K. M., Gould, D J., (2021). Melting corneal ulcers (keratomalacia) in dogs: A 5 ‐ year clinical and microbiological study (2014–2018). *Veterinary Ophthalmology,24(3),* (265-278). https://doi.org/10.1111/vop.12885.

Vanore, M., Chahory, S., Payen, G., Clerc, B., (2007) Surgical repair of deep melting ulcers with porcine small intestinal submucosa (SIS) graft in dogs and cats. *Veterinary ophthalmology*, *10*(2), 93–99. https://doi.org/10.1111/j.1463-5224.2007.00515.x

Williams, D. L., Wirostko, B. M., Gum, G., & Mann, B. K. (2017). Topical cross-linked HA-based hydrogel accelerates closure of corneal epithelial defects and repair of stromal ulceration in companion animals. *Investigative Ophthalmology and Visual Science*, *58*(11),4616–4622. https://doi.org/10.1167/iovs.16-20848.

Willoughby, C. E., Batterbury, M., Kaye, S.B. (2002). Collagen corneal shields. *Survey of Ophthalmology*, 47, 174–182. https://doi.org/10.1016/S0039-6257(01)00304-6.

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

THE APPEARANCE OF DIARRHEA IN THE NEONATAL CALF PERIOD - CASE STUDY

Maria Alexandra JALBĂ, Mihai-Alexandru PITRAN, Valentin Alexandru DUȚULESCU Mario CODREANU, Iuliana CODREANU[1]

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăști Blvd, District 1, 011464, Bucharest, Romania, Phone: +4021.318.25.64, Fax: + 4021.318.25.67

Corresponding author email: jalbamariaalexandra@gmail.com

Abstract

Neonatal calf diarrhea syndrome, also known as calf scours or enteritis, is a common condition that affects young calves, particularly those between one and three weeks of age. This syndrome has a multifactorial etiology and has a negative impact on farm economics and welfare. The severity of diarrhea can range from mild to severe, and it can lead to dehydration, electrolyte imbalances, and even death in severe cases. Early recognition and prompt treatment of diarrhea are essential to minimize the negative impact on calf health and productivity. This study was conducted on a private farm at the request of the owner on a 3-week-old female in October 2022. The clinical examination was requested due to changes in the general condition such as apathy, uncontrollable diarrhea, dehydration, colic syndrome, inappetence, high body temperature. This abstract provides an overview of the causes and management of diarrhea in neonates, including prevention strategies and treatment options. Understanding the appearance of diarrhea in the neonatal period is crucial for healthcare providers and caregivers to ensure the optimal health and well-being of newborns.

Key words: *cattle, diarrhea, therapy*

INTRODUCTION

Neonatal calf diarrhea syndrome, also known as calf scours or enteritis, is a common condition that affects young calves, particularly those between one and three weeks of age. (Heinrichs, 2015)

This syndrome has a multifactorial etiology and has a negative impact on farm economics and welfare.The origin of neonatal diarrhea is complicated and the subsequent course and severity of diarrhea are affected by the pathogens present, the condition general and calf susceptibility, quality environment (stress factors), protection against weather conditions, the quality of colostrum management, nutrition (insufficient or inadequate nutrition) and the general level of prevention measures implemented on a farm (Gulliksen et al., 2009).

The severity of diarrhea in newborn calves can vary depending on the underlying cause and the individual calf's immune status and overall health. Mild cases of diarrhea may only involve a few loose or watery stools, while severe cases may result in profuse diarrhea, dehydration, and electrolyte imbalances that can be life-threatening (Torres et al., 2014). Diarrhea can also have negative effects on calf growth and development, including reduced weight gain, delayed weaning, and decreased feed efficiency, which can result in significant economic losses for farmers and producers (Cho & Yoon, 2014).

Table 1. Receptivity of cattle according to age (N. Sattler, 2006)

Age	The pathogen involved
0-4 days	*Escherichia coli, Clostridium perfringens*
4-14 days	Rotavirus
5-15 days	*Clostridium*, type A, B and C
5-10 days	*Coronavirus*
4-30 days	*Cryptosporidium* spp.
5-40 days	*Salmonella* spp.
14-30 days	Parvovirus Rotavirus BVD
+ 18 days	Coccidia

The severity of diarrhea can be influenced by various factors, including the calf's age, management practices, environmental conditions, and infectious agents (various

viruses, bacteria and parasites, including rotaviruses, coronaviruses, *Escherichia coli*, *Salmonella* spp. and *Cryptosporidium*, the most common and economically important being *Salmonella* and *E. coli* species) (Brown & Baker, 2018).

Clinically, diarrhea represents an excretion of fecal matter that contains excessive amounts of water. The consistency varies depending on the severity, so the feces can look pasty to liquid, presenting a whitish, greenish, yellowish color (Lorenz et al., 2011).

This is accompanied by a series of symptoms, such as: loss of appetite, dehydration, hypothermia, prolonged recumbency, prostration, fever (McGuirik et al., 2007).

Cattle diarrhea can be classified into alimentary and microbial (bacterial, viral and parasitic) which require a mandatory differential diagnosis (Thompson et al., 2012).

The differential diagnosis is made against infectious causes which include bacterial infections, viral infections, protozoal infection, non-infection causes (dietary factors, passive transfer failure, intestinal obstruction, metabolic disorders, medication or toxic exposure) and other condition (umbilical infections or hypothermic stress) (Meale et al., 2020).

To establish a definitive diagnosis, a thorough history, clinical examination, and diagnostic testing should be conducted (Gulliksen, et.al., 2009).

The therapeutic approach for neonatal diarrhea in cattle consisted in a combination of supportive care, fluid therapy, nutritional support, and targeted treatment based on the identified cause. Thus, this can include parenteral rehydration, quality nutritional support, antibiotic therapy, antiparasitic medication, improving hygiene and well-being conditions (Pardon et al., 2013).

MATERIALS AND METHODS

This study was conducted on a private farm at the request of the owner on a 3-week-old female in October 2022. The farm was visited for 5 consecutive days, in October 2022.

Personnel walked through the calf pen and visually assessed the health of the calf. This farm is using antimicrobials as treatment for disease in calves and not for prophylactic or metaphylactic use.

Figure 1. Cattle female, 3-week-old

The clinical examination was requested due to changes in the general condition such as apathy, uncontrollable yellow diarrhea, severe dehydration, colic syndrome, inappetence, fever, prolonged lateral decubitus. To establish the diagnosis of diarrhea and determine the underlying cause (etiology) in calves faecal samples were collected from the animal.

The faecal sample was first evaluated macroscopically, and its color and consistency were recorded; than we used VetExpert Rapid Test BoviD-4 Ag to identify the existence of the pathogen that caused the condition.

Test BoviD-4 Ag is a quadruple test for the differentiation of pathogens that cause diarrhea in calves based on the immunochromatographic method, which allows to determine with great precision the presence of *Cryptosporidium*, rotavirus, coronavirus and *E. coli* in the feces of calves.

The therapy was administrated for 5 days thus, in the first day we administered 2 l of Ringer's lactate solution 4 vials, Beformed (vitamin B_1, vitamin B_2, vitamin B_6, vitamin B_{12}, niacin and D-panthenol) in a dose of 15 ml/animal, Buscopan (butyl scopolamine and metamizole sodium) in a dose of 2 ml/animal and

Gabbrovet solution (paromomycin sulfate), in a dose of 2.5 ml/10 kg.

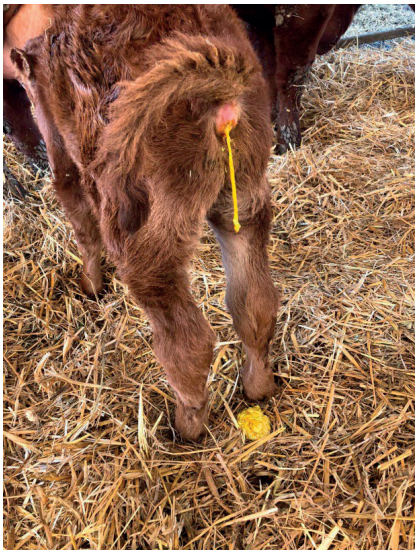


Figure 2. Female calf excreting yellow faecal, liquid consistency

On the second day of treatment, we administered 2 l of Ringer's lactate solution 4 vials, glucose 20 ml, Beformed (vitamin B_1, vitamin B_2, vitamin B_6, vitamin B_{12}, niacin and D-panthenol) in a dose of 10 ml/ animal, Buscopan (butyl scopolamine and metamizole sodium) in a dose of 2 ml/ animal and Gabbrovet solution (paromomycin sulfate), in a dose of 1 ml/10 kg.

On the third day of treatment, we administered 1 l intravenous infusion, composed of Ringer's lactate solution 2 vials, glucose 10 ml, Beformed (vitamin B_1, vitamin B_2, vitamin B_6, vitamin B_{12}, niacin and D-panthenol) in a dose of 5 ml/animal, Buscopan (butyl scopolamine and metamizole sodium) in a dose of 2 ml/animal and Gabbrovet solution (paromomycin sulfate), in a dose of 1 ml /10 kg. In the following 2 days, were administrated Beformed in a dose of 5 ml/ animal and Gabbrovet (paromomycin sulfate) 0.5 ml/10 kg.

RESULTS AND DISCUSSIONS

During the clinical evaluation of all the periods of the disease, it was found that the health of the calf improved and the symptoms have dimmed after the third day of treatment.

In the following two days, we continued the monitoring and administration of the supportive treatment to avoid any recurrence of the disease.

The result of the rapid test was negative for the pathogens included in the kit, thus the etiological agent causing this condition was not elucidated.

In a previous study, 108 cases of diarrhea in calves were analyzed retrospectively, the study classified the diarrhea manifested in cattle into two groups: with an infectious cause and with a non-infectious cause, compared to our case where the animal presented a non-infectious diarrhea (Cho YI et al., 2013).

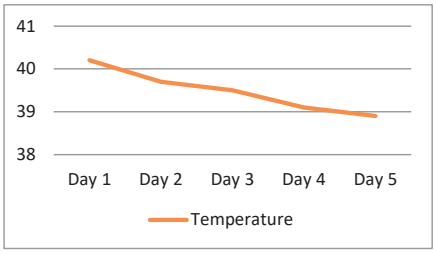


Figure 3. Body temperature monitoring for 5 days consistency

In the specialty literature it was specified that the 2007 National Animal Health Monitoring System (NAHMS) for US dairy reported that approximately 57% of calf mortality at weaning was caused by diarrhea and the majority of cases occurred in calves less than 1 month old (Cho YI et al., 2013). A similar mortality rate (53.4%) for dairy calves due to calf diarrhea was recently reported in Korea. The economic loss associated with calf death in Norway where calf production is 280,000 heads per year was estimated to be approximately 10 million US dollars in 2006 (Cho YI et al., 2013).

Nutrition has a significant role in the occurrence of this pathology, thus it was found that alimentary diarrhea is frequent especially in the case of artificial breastfeeding or is associated with excessive consumption of milk, but characterized by the absence of fever (Heinrichs, 2015).

Previous studies showed that in order to evaluate the infectious etiologies linked to calf diarrhea 165 cow ranches provided a total of 199 and 245 faecal samples from healthy and

diarrheic calves, respectively (Cho et al., 2013; 2014).

Figure 4. Result of faecal sample consistency

Samples were tested by a panel of multiplex PCR assays for 11 enteric pathogens: bovine rotavirus group A (BRV-A), bovine coronavirus (BCoV), bovine viral diarrhea virus (BVDV), bovine enterovirus (BEV), bovine norovirus (BNoV), Nebovirus, bovine torovirus (BToV) *Salmonella* spp., *Escherichia coli*, *Clostridium perfringens* with β-toxin gene and *Cryptosporidium parvum*. Multiple infections were present in more than half of the fecal samples from the diarrheic calves (Cho, 2013; Vandenhole, 2019).

Statistically, calf diarrhea was strongly linked with BRV-A, BCoV, BNoV, nebovirus, *Salmonella* spp., *E. coli*, and *C. parvum* (Browin & Baker, 2018).

C. parvum and BRV-A were identified as the most frequently occurring enteric pathogens for calf diarrhea, with detection rates of 33.7% and 27.1%, respectively, and odds ratios of 173 and 79.9, respectively. Unexpectedly, BNoV and Nebovirus were often found in diarrheic calves, pointing to the possibility that these viruses may play a substantial role in calf diarrhea (Meale, 2020).

CONCLUSIONS

Diarrhea is a common problem in newborn calves, and its appearance is typically characterized by the excretion of fecal matter with excessive water content.

Diarrhea in newborn calves can have multiple causes, including infectious and non-infectious factors.

Early intervention and appropriate treatment can lead to positive outcomes.

Monitoring the calf's progress, including improvements in symptoms and overall health, is important for evaluating the effectiveness of the therapeutic approach.

In the present case, symptomatic treatment was used to improve the symptoms, without a definite diagnosis.

Prevention strategies are the key in managing diarrhea in newborn calves and this includes proper colostrum management to ensure adequate passive transfer of antibodies, maintaining a clean and hygienic environment, implementing good nutrition and feeding practices, and adhering to biosecurity measures.

REFERENCES

Brown, C. C., & Baker, D. C. (2018). *Bovine viral diarrhea*. In Pathology of Wildlife and Zoo Animals (pp. 391-397). Cambridge, Massachusetts: Academic Press.

Cho, Y.I., & Yoon, K.J., (2014). An overview of calf diarrhea - infectious etiology, diagnosis, and intervention. *Journal of Veterinary Science*, 15(1):1-17.

Cho, Y.I., Han, J.I., Wang, C., Cooper, V., Schwartz, K., Engelken, T., & Yoon, K.J., (2013). Case-control study of microbiological etiology associated with calf diarrhea. *Veterinary Microbiology*, 166(3-4):375-85.

de Verdier Klingenberg, K., Svensson, C., & Ekman, T. (2020). Failure of passive transfer of immunity and the fatalism behind its risk factors in newborn calves experience from a Swedish study. *Acta Veterinaria Scandinavica*, 62(1), 1-14.

Garcia, J., Pempek J., Hengy, M., Hinds, A., Campos, D.D., & Habing, G., (2022). Prevalence and predictors of bacteremia in dairy calves with diarrhea, *Journal of Dairy Science*, volume 105, issue 1, P807-817.

Gulliksen, S. M., Jor, E., Lie, K. I., Løken, T., & Åkerstedt, J. (2009). Diarrhoea in the neonatal calf: a meta-analysis of the efficacy of prophylactic and therapeutic interventions. *Acta Veterinaria Scandinavica*, 51(1), 1-15.

Heinrichs, A.J. (2015). Nutrition and calf health. Veterinary Clinics of North America: Food Animal Practice, 31(3):495-510

Krehbiel, C.R., Evans, J.L., Galyean, M.L. (2002) Infectious diseases and management of the newborn calf. Veterinary Clinics of North America: Food Animal Practice, 18(2):273-287.

Lorenz, I., Fagan, J., More, S.J. (2011). Calf health from birth to weaning. Management of diarrhea in pre-weaned calves. *Irish Veterinary Journal*, 64(1):9.

McGuirk, S. M. (2008). Disease management of dairy calves and heifers. *Veterinary Clinics: Food Animal Practice*, 24(1), 139-153.

McGuirk, S. M., & Ruegg, P. L. (2007). Calf diseases and prevention strategies. *Veterinary Clinics of North America: Food Animal Practice*, 23(2), 115-145.

McQuistion, T.E., Copeland, N.K. (1997). Clinical signs and postmortem lesions of rotavirus and coronavirus infections in neonatal calves. Journal of Veterinary Diagnostic Investigation, 9(3):237-239.

Meale, S. J., Chaucheyras-Durand, F., Berends, H., Guan, L. L., & Steele, M. A. (2020). From pre- to postweaning: Transformation of the young calf's gastrointestinal tract. *Journal of Dairy Science*, 103(6), 5815-5828.

Pardon, B., Callens, J., Maris, J., Allais, L., & Deprez, P. (2013). Epidemiological study of neonatal calf diarrhea and antimicrobial resistance of enteropathogenic Escherichia coli in Belgium. *Veterinary Microbiology*, 162(2-4), 773-779.

Quigley, J. D., Drewry, J. J., & Murray, L. D. (1996). Effects of oral colostrum supplementation on calf health and performance. *Journal of Dairy Science*, 79(5), 813-818.

Quigley, J. D., Martin, K. R., & Bemis, D. A. (1991). Effects of feeding colostrum or serum on body temperature and weight gain in neonatal dairy calves. *Journal of Dairy Science*, 74(1), 82-86.

Svensson, C., Linder, A., Olsson, S. O., & Ekman, T. (2003). Failure of passive transfer in newborn dairy calves: the effects of feeding colostrum at different volumes and subsequent health. *Journal of Dairy Science*, 86(4), 1227-1236.

Thompson, P.N., Stone, A., Schultheiss, W.A., et al. (2012). An outbreak of diarrhea associated with neonatal coccidiosis in beef calves. *Veterinary Parasitology*, 186(3-4):450-455.

Torres, A.C., Costa, E.F., de Castro, A.M., et al. (2014). Diarrhea in young calves: Association with enteropathogens, rotavirus genotypes, and host immune response. Veterinary Immunology and Immunopathology, 161(3-4):209-216.

Vandenhole, M., González-Monsalve, B., & Pastell, M. (2019). Invited review: Enteric methane in dairy cattle: Prevalence, challenges, and alternatives. *Journal of Dairy Science*, 102(4), 2826-2840.

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

OUTCOME OF 2 CATS WITH SQUAMOUS CELL CARCINOMA TREATED WITH 1 ½ MANDIBULECTOMY

Daniel LESCAI[1, 2], Bianca BAROIU[2], Anca CRISTEA[2], Anca RUSU[2], Adriana STANCU[2]

[1]Spiru Haret University, Faculty of Veterinary Medicine, 256 Basarabia Blvd, Bucharest, Romania
[2]Oncovet, 2 Dimitrie Salmen Street, Bucharest, Romania

Corresponding author email: daniel.lescai@spiruharet.ro

Abstract

The objective is to describe the outcome and complications of two cats treated with a hemi plus rostral part contralateral mandibulectomy (1 ½ mandibulectomy) technique for the management of oral squamous cell carcinoma (SCC) with bone infiltration. Mandibulectomy can be performed in cats but unlike canine patients, they may require additional supportive care. Two cats were presented for progressive mass growing on the mandible. Both had bone invasion and were diagnosed with SCC by biopsy examination. The procedure involved a left/right mandibulectomy and the rostral part of the right/left hemimandible caudal to the lower canine tooth (1 ½ mandibulectomy), at least 1 cm far from the macroscopically visible lesions. Both cats had feeding tubes placed. The surgical outcome for one of the two cats was excellent, surpassing 302 days of survival, the other cat did not regain the ability to eat and the owners opted for euthanasia 35 days after surgery. Both histopathological reports confirmed SCC and clean margins. The hypothesis of the study reported here was that 1 ½ mandibulectomy would be effective for control of superficial subcentimetrical, caudal to the canine tooth oral SCC with bone invasion in cats but could also lead to permanent loss of feeding function and compromised quality of life.

Key words*: squamous, carcinoma, mandibulectomy, surgery, cats.*

INTRODUCTION

The most frequent oral neoplasia in cats (60-70%) (Stebbins et al., 1989) is feline squamous cell carcinoma (SCC), which is often found in older cats with a median age of 15 years (Bregazzi et al., 2001). The usual tumour sites are the mandible, maxilla and tongue. Bone involvement is common and frequently extensive in both the mandible and maxilla (North & Banks, 2009). While some cats present with visible oral masses, many are seen due to secondary clinical signs, such as ptyalism with bloody, foul-smelling saliva and dysphagia (Snyder, 2012). It is often difficult to determine the true extent of these tumors through physical examinations due to their invasive nature.

The development of oral cavity tumors in dogs and cats has been linked to nutritional and environmental variables, flea collars, and passive smoking (Mikiewicz et al., 2019). Local tumour control is poor, regional lymph node and distant metastases are uncommon and the long-term prognosis is uncertain because the majority of patients are euthanized due to the disease's progression. Regional lymph nodes may be enlarged when a patient first presents, however, they are often hyperplastic because of the production of inflammatory cytokines (North & Banks, 2009).

The overall prognosis for oral SCC is poor. Despite various multimodal therapy approaches, responses are typically only partial and temporary and overall survival continues to be only a few months (Marconato et al., 2020). The treatment of feline oral SCC is difficult, as few therapies (or combinations of therapies) have shown success. The median survival time for feline patients who receive no treatment is only 60 days (North & Banks, 2009). Surgical excision of the visible tumour only rarely results in a prolonged lifespan. The median survival period is extended to only 5 months after a mandibulectomy (Snyder, 2012).

Surgical excision with safety margins is the usual approach to treatment for small tumours of the mandible and maxilla (Murphy, 2016). Early identification of oral SCC in cats is the most significant prognostic indicator whilst they may still be candidates for surgery. Unfortunately, SCC is often advanced when it

is diagnosed, making surgery challenging (Moore & Moore, 2009)

The treatment of choice in oral feline SCC is surgery. A variety of procedures were explored most of which were unsuccessful before the onset of aggressive surgical procedures. Oral tumour survival rates have increased as a result of aggressive surgical resection techniques such as mandibulectomy and maxillectomy (Birchard & Carothers, 1990). More than 1 cm of surgical margins is ideal, however, they are difficult to obtain due to the small feline craniofacial dimensions (Bilgic et al., 2015).

Although mandibulectomies can be performed in cats, they often do not handle the procedure as well as canine patients and may need additional postoperative assistance (Northrup et al., 2006). However, they are frequently unable to feed in the early postoperative period. In order to control this aspect, it is indicated to routinely insert a gastrostomy tube at the time of surgery (Berg, 1998).

This paper aims to describe the procedure, complications and long-term outcome of 2 cats that underwent a 1½ mandibulectomy technique for managing oral SCC with bone infiltration.

MATERIALS AND METHODS

Patients and tumor characteristics

The medical records (May 2021-May 2023) of cats that were presented in our clinic for surgical treatment of oral SCC were reviewed. We considered cases that underwent 1½ mandibulectomies. The cases were selected based on tumour type, size, location of the mass, and degree of bone involvement. Clinical staging for regional and distant metastasis consisted of physical examination, routine complete blood count and serum biochemistry and thoracic and head radiography. The thorough analysis of medical records granted for the collection of additional information, including signalment (breed, age, sex, and weight), concurrent diseases, involved site, tumour size, clinical stage, treatment-related side effects or complications, time and cause of death and date of the most recent follow-up visit.

Case description

Two cats, domestic short hair, were referred for progressive mass growth on the mandible, caudal to mandibular canine teeth. Cat no. 1 is a spayed female, 4 kg body weight, 14 years old, right side previous local biopsy performed. Cat no. 2 is a neutered male, 5.4 kg body weight, 13 years and 10 months old, left side previous biopsy performed. Both biopsy pathology results confirmed SCC. Both cats presented radiological bone involvement. Ptyalism and halitosis were the two most frequent complaints, one of the cats was also showing signs of hyporexia while the other had no change in appetite. At the time of presentation, both cats were in good body condition.

Staging

On diagnostic imaging, both presented bone invasion of the mandible. There was no evidence of distant metastasis. Negative submandibular lymph node involvement with the cytological examination. Three-view thoracic radiographs were also performed for comparison at the time of subsequent follow-up. Both cats were diagnosed with SCC by biopsy examination. The remainder of the clinical examination was unremarkable, with no peripheral lymphadenopathy.

Surgical technique

Examination of the oral cavity under general anesthesia revealed a firm mass that was deforming the right/left ventral aspect of the mandible with the implication of the oral cavity floor and an obvious involvement of the median line, towards the right/left side (Figures 1, 2). Superficial proliferative/ulcerative lesions were caudal to the canine tooth extending towards premolars. Surgery includes right (cat no. 1)/left (cat no. 2) mandibulectomy, at a distance of at least 1 cm caudal to the macroscopic process observed, resulting caudal to the molar, en-bloc resection with skin tissue, and inclusion of the rostral portion of the left (cat no. 1)/right (cat no. 2) hemimandible, behind the canine tooth (Figures 3-8). Osteotomy is achieved with Liston bone cutter forceps, allowing control of hemorrhage by early identification of mandibular canal vessels and bipolar usage. Radiological evidence of complete excision is obtained postoperative and the histopathological examination confirmed it (Figures 9-11). Both cats had feeding tubes placed before surgery termination.

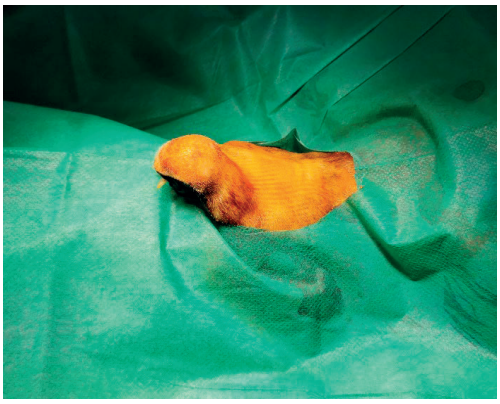


Figure 1. Preoperative image of cat no. 1. The cat is in dorsal recumbency

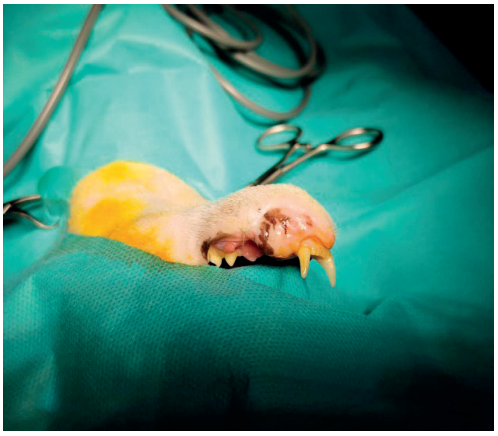


Figure 2. Preoperative image of cat no. 2. There is a large, firm mass of the ventral ramus of the mandible

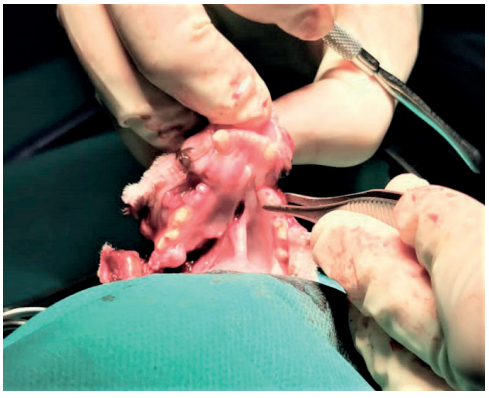


Figure 3. Intraoperative image of cat no. 2. The mass was about to be removed

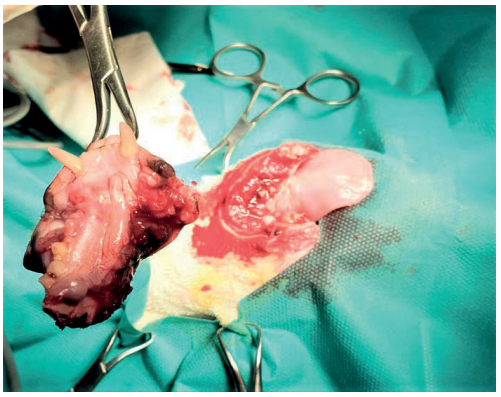


Figure 4. Intraoperative image of cat No.2. The mass was detached

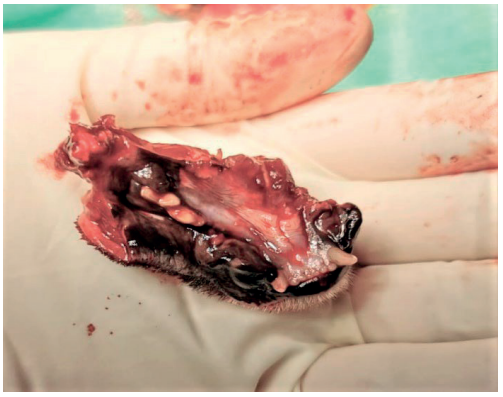


Figure 5. Postoperative image of the removed mass in cat no. 1

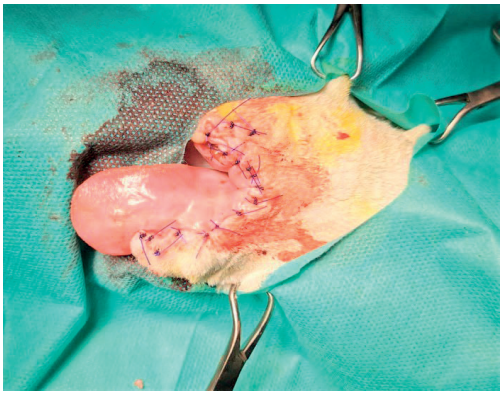


Figure 6. Postoperatively image cat no. 2 after the mass was removed

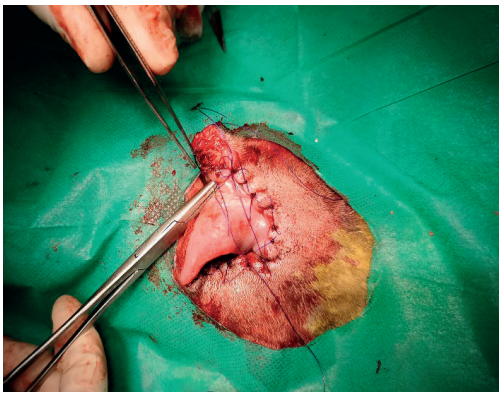


Figure 7. Postoperatively image cat no. 1 after the mass was removed

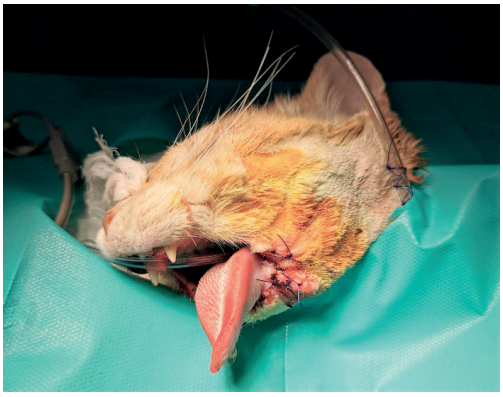


Figure 8. Postoperatively image cat no. 2 after the feeding tube was placed

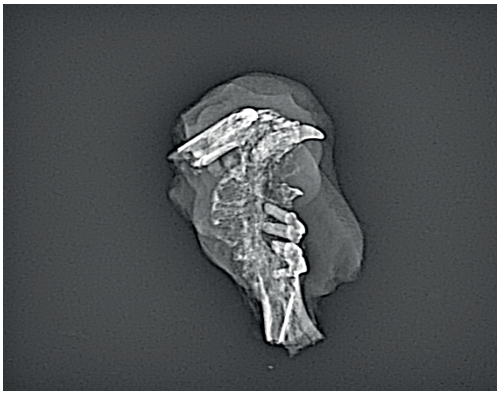


Figure 9. The radiological aspect of the excised mass in cat no. 1

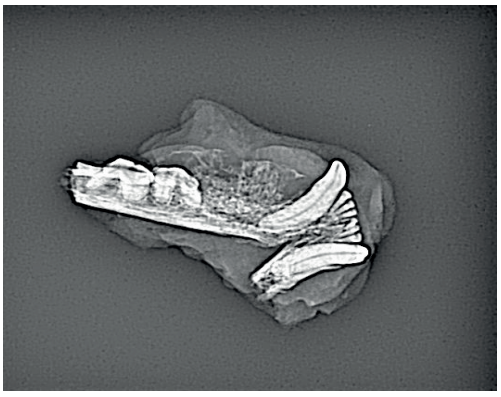


Figure 10. The radiological aspect of the excised mass in cat no. 2

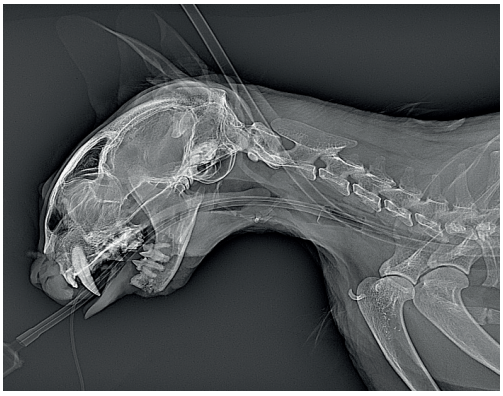


Figure 11. The radiological aspect of cat no. 2 postoperatively

RESULTS AND DISCUSSIONS

Complications

The biology of the SCC implies an increased risk of recurrence and a lower risk of metastasis.

Cats with extended mandibulectomies may develop persistent anorexia as a result, leading to constant feeding assistance.

Considering the extent and nature of the local disease, the need for major intervention, the expected local and general complications and the increased risk of recurrence, the prognosis is poor.

This type of extended intervention involves a difficult-to-predict long-term postoperative evolution, but it is expected that numerous complications and changes in the general condition will occur, with the possibility of other interventions to control local

complications, especially dehiscence over osteotomy sites or high-tension suture points. Another series of complications derive from the mandibular drift and food/debris accumulation on the dental surface along with abundant plaque formation. Also one can observe the hanging tongue.

In the case of persistent local and general complications, without a tendency to reduce and adapt to the new condition, the prognosis is unfavorable.

Possible local complications expected include local inflammation, dehiscence, exposure of bone edges, salivary cysts, hypersalivation, poor cosmesis, permanent anorexia, constant vomiting, ptyalism, dehydration, pain, severe depression, the inability to control and achieve prehension with the tongue and groom voluntarily, behavioral disorders. As for infection, the area is well vascularized and, generally, the risk is low after this surgical procedure.

The complications encountered in both cats were represented by hypersalivation, difficulties in prehension and dehiscence with the exposure of the mandibular edge (Figures 12-14).

Cat no.1 also presented anorexia, exudative inflammation at the level of the esophagostomy site, reduced right paramedian sublingual salivary cyst, 1-2 millimeters dehiscence with a tendency for circumferential granulation and exposure of the mandibular branch, discomfort upon swallowing and elimination of a significant part of the nutritional support orally.

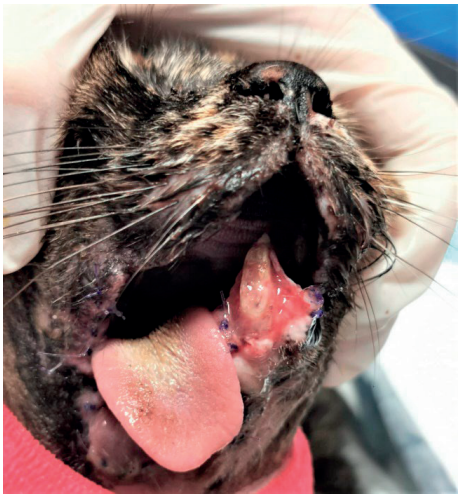


Figure 12. Postoperatively dehiscence with bone exposure in cat no. 1

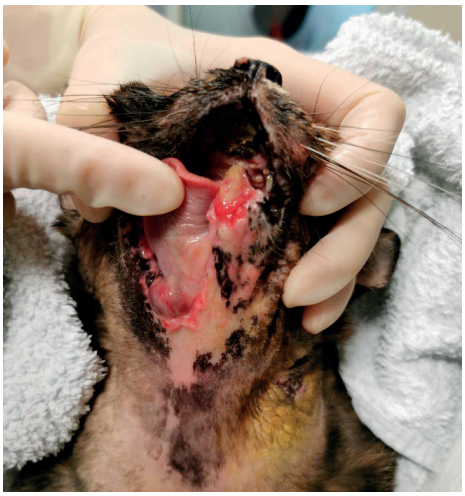


Figure 13. Postoperatively dehiscence with bone exposure in cat no. 1

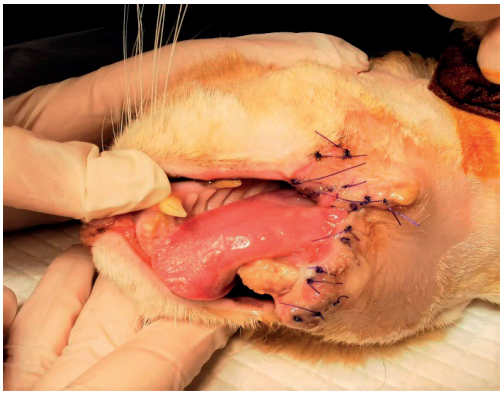


Figure 14. Postoperatively dehiscence with bone exposure in cat no. 2

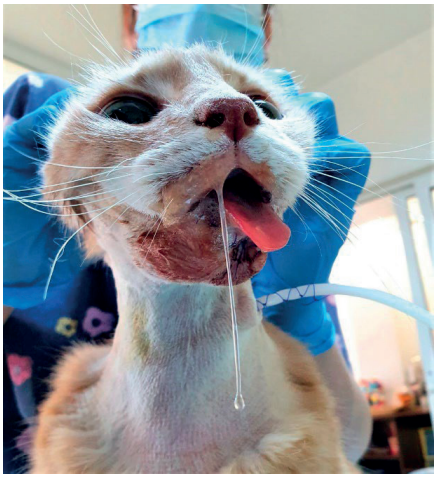


Figure 15. Postoperatively hypersalivation in cat no. 2

Postoperative management

Both cats required systemic antibiotherapy consisting of amoxicillin-clavulanate in a dose of 8.75 mg/kg/day for 14 days (Synulox RTU 100 ml, Zoetis, Belgium).

The analgesia protocol included robenacoxib in a dose of 2 mg/kg, daily for 14 days (Onsior 20 mg/ml, Elanco, France) and buprenorphine in a dose of 0.02 mg/kg, every 8-12 hours for 5 days then every 12 hours at the dose of 0.01 mg/kg for 14 days (Bupaq 0.3 mg/ml, Richter Pharma, Austria).

The esophagogastric tube was used to support caloric intake when there was no tendency for voluntary feeding. The possible complications regarding the feeding tube were inflammation at the place of placement, loss of permeability, migration or rejection due to severe stress, or repeated vomiting. It was estimated that the postoperative critical period is 2-3 weeks.

Cat no. 2 showed interest in food the day following the surgical procedure and 10 days later was consuming food voluntarily. The feeding tube was removed 14 days following the surgical intervention.

A unidimensional descriptive scale was used to accurately assess pain in order to provide targeted postoperative pain treatment (Gruen et al., 2022).

Cat no. 1 required additional analgesia and daily supportive treatment for the first 5 days after surgery, then every 2 days for up to 3 weeks.

The aspects related to the surgical area were manageable locally and did not require additional surgical interventions (Figure 16).

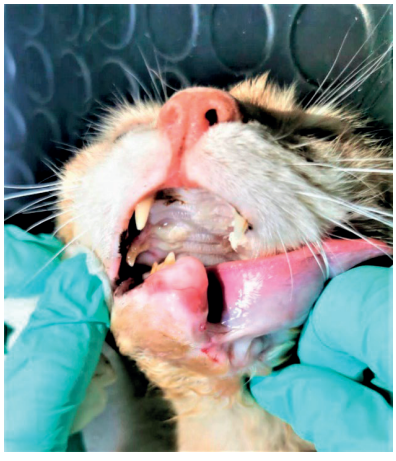


Figure 16. Postoperatively aspect in cat no. 2

The cat appeared interested in food but freely ingested insufficient amounts and refused to be force-fed after the feeding tube was withdrawn three weeks following the procedure.

The ability to eat on his own (cat no. 1) was only partially restored, requiring additional food intake provided by the owners and despite the supportive treatment he was receiving daily in the clinic.

Outcome

The surgical outcome for one of the cats was excellent (cat no. 2), independent food intake was achieved the next day after surgery and the feeding tube was removed two weeks later.

There were no major complications related to the surgery or tumour recurrences during the 302-day follow-up (Figures 17, 18).

The other cat did not regain the ability to eat and the owners opted for euthanasia 35 days later.

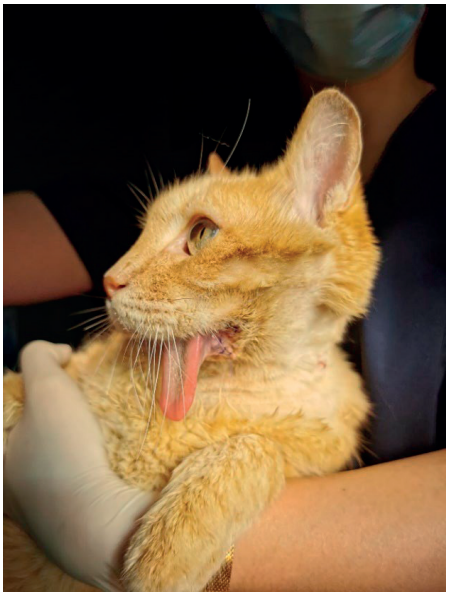


Figure 17. Cat No.2 at 4 weeks follow-up visit

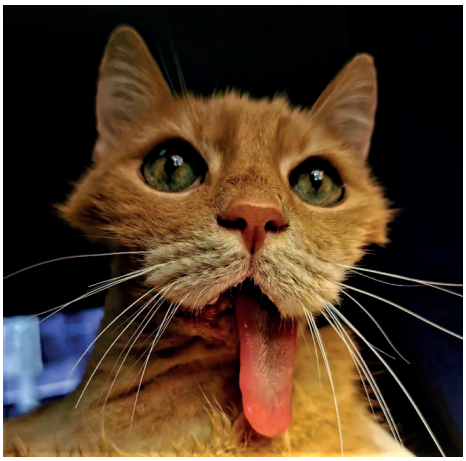


Figure 18. Cat no. 2 at 4 weeks follow-up visit

Discussions

The excision of oral neoplasia is the most common reason for mandibulectomy in cats. Techniques regarding mandibulectomy vary according to the extent of the disease and the surgeon's preferences. In cats, mandibulectomy is relatively rare, partly because the available research suggests against it (Northrup et al., 2006).

The intent of both surgeries was curative and clean margins were histologically confirmed in both cases. A cure can be achieved if clear surgical margins are obtained and the procedure is well tolerated by the cats (Boston et al., 2020).

Feeding tube placement is recommended in all cases of feline mandibulectomy due to the unpredictable intervals between surgical procedures and the willingness of cats to eat voluntarily. In this study, the time frame ranged from two days to two weeks. Owners were informed that postoperative feeding tube assistance was necessary and could be permanent with both cats eventually maintaining their nutritional requirements orally.

Northrup et al. (2006) published a case series involving 42 cats of which 17 had feeding tubes placed at the time of the survey, yet 29 cats showed anorexia in the postoperative period. This underlines the need for vigorous supportive treatment in cats undergoing mandibulectomy and may have contributed to some of the study's poor outcomes (Northrup et al., 2006). It was presumed that mandibulectomy would not achieve local control of most tumors because most feline oral tumors are locally invasive and performing a more aggressive mandibulectomy procedure would result in a permanent loss of basic oral functions and a compromised quality of life.

According to Northrup et al., six cats were treated with radical mandibulectomy, of which three did not fully regain complete oral feeding. In a retrospective study of 8 cats, independent food intake was achieved in 6 cats following radical mandibulectomy and four cats lived longer than one year (Boston et al., 2020).

With a median progression-free interval of nearly 1.5 years, tumor control for the majority of the cats in the Northrup et al. study was remarkably good in contrast to the anticipated result. For cats with SCC, osteosarcoma and fibrosarcoma, the survival rates 1 year after surgery were the same as those 2 years post-intervention, indicating that if a cat lived for 1 year, there was a good probability of long-term survival. The majority of owners (>80%) expressed satisfaction with the outcomes despite the complications implied with mandibulectomy in cats (Northrup et al., 2006).

Wound dehiscence following a mandibulectomy, particularly at the alveolar margin, may occur over the rostral end of the osteotomized mandible, exposing bone. While larger areas might require being surgically debrided and closed, smaller areas of dehiscence may heal by second intention (Verstraete, 2005).

Cats with mandibular SCC who underwent mandibulectomy as sole treatment had a median disease-free interval of 340 days, compared to 911 days for rostral tumours. Unfortunately, just a few patients can benefit from surgical removal with clean margins (North & Banks, 2009).

Partial mandibulectomy or maxillectomy should only be used to treat small tumours as larger tumours or those with insufficient resection margins will require radiotherapy in addition to surgery (Bilgic et al., 2015).

In a retrospective study on 8 cats, the estimated mean survival time was 712 days with three long-term survivors that died of causes unrelated to their main disease. The majority of them underwent radical mandibulectomy to treat extensive oral neoplasia and the procedure

was not associated with poor functional outcomes or significant morbidity during the postoperative period (Boston et al., 2020).

The authors would also like to add that the number of papers regarding the outcome of cats following radical 1 and ½ mandibulectomies is reduced and data from our small study may humbly contribute to the general knowledge regarding the subject. We acknowledge at the same time the small number of cases, only 2 cats, with low statistical power. Although, we learned that client education, owner decision and compliance, early detection of disease and good case selection are paramount for a positive outcome. We may also add that prior surgical visits consisted also of questions about the feeding habits of both cats. Subjectively, based on owners' reports, there was a difference between the two cats. Cat no. 1 reported a more reluctant feeding habit while cat no. 2 had a reported enthusiastic feeding habit, confirmed after surgery. Cosmesis is a subjective issue also and in the case of cat no. 1 is poor while in the case of cat no. 2 is good. We also assessed the quality of life (QOL) as reported by owners and the difference was positively in favour of cat no. 2, doubled by good client satisfaction. Also, on the subjective side, we observed another difference in terms of self-grooming. While cat no. 1 was unable in the first period and only attempted/initiated but did not continue the grooming later after surgery, cat no. 2 was on the opposite side, grooming voluntarily and with relative ease in the postoperative period.

Cat no. 2 survives to date and is disease free, 302 days after surgery. Cat no. 1 survived 35 days, euthanasia by the owner's decision.

Both surgeries resulted in confirmed clean margins. Both cats presented dehiscence over the osteotomy mandibular site in the first 7 days after surgery. Both healed by the second intention. Both cats presented postsurgical submandibular oedema, ptyalism and reduced salivary cysts, which healed spontaneously. Local inflammation is normal after surgery and no sign of local infection was observed in both cats. Cat no. 1 had a low tolerance for the feeding tube, needed frequent position adaptation in the distal oesophagus. On the first day after surgery, the cat vomited and stopped after tube repositioning. Cat no. 1 also presented with exudative inflammation at the esophagostoma site.

It may well be a truism but is worth mentioning that even with small superficial appearances, SCC requires extensive excisions compared to cranium dimensions in cats as well as harbouring bone invasion and extensive profound soft tissue involvement potentially compromising surgical margins. We can say that even with small, in terms of millimetres surface lesions such as SCC, radical surgery is required for the cure, being the case for both cats. As an example, in the case of cat no. 2, the pathology report measured a superficial proliferative-ulcerative lesion of 2/6 mm, between the left canine tooth and the first premolar. It is our opinion, based on our two cases that even with early detection of subcentimetrical superficial lesions the surgical dose could extend to radical 1 and ½ mandibulectomy in cats with the condition of the lesion being located in the rostral part, particularly caudal the canine tooth.

CONCLUSIONS

Mandibulectomy for the management of canine oral tumors has been extensively documented in terms of outcomes and owner satisfaction. In contrast with this, a literature search revealed much less information regarding the outcome of this surgical procedure in cats.

Even though a significant part of bone and soft tissue is usually removed, function and appearance are acceptable. While common, postoperative complications are usually manageable. The early detection of SCC in cats is crucial for establishing a suitable approach in order to achieve long-term survival. Mandibulectomy may be considered in combination with postoperative aggressive pain control and feeding tube management as a treatment option for cats with extensive mandibular neoplasia.

Literature research has been performed on articles concerning mandibulectomies in cats and 30 articles were found. Out of them, 3 articles were assessing the outcomes of the cats that underwent this surgical procedure. The small number of subjects in our study is one of its limitations.

Considering the limited information available on cats treated with 1 ½ mandibulectomy, the locally invasive nature of SCC and the small size of the feline mandible, the hypothesis of the study reported here was that one-and-one-half mandibulectomy would be effective for local control of oral SCC in cats with subcentimetrical superficial lesions with the condition of the lesion being located in the rostral part, particularly caudal the canine tooth but more research is necessary.

We conclude that most of the complications encountered are manageable, of low extent with the exception of feeding ability in the post-surgery period. We could propose as early indicators of prognosis the feeding behaviour before disease/surgery and the self-grooming abilities after surgery, noting that they may be highly subjective. Early disease detection, awareness, avoidance of predisposing factors and, if necessary, prompt aggressive treatment can lead to the best outcomes in cats with oral SCC.

ACKNOWLEDGEMENTS

The authors would like to thank the cat owners for their collaboration.

REFERENCES

Berg, J. (1998). Principles of oncologic orofacial surgery. *Clinical Techniques in Small Animal Practice*, 13(1), 38–41. https://doi.org/10.1016/S1096-2867(98)80025-X

Bilgic, O., Duda, L., Sánchez, M. D., & Lewis, J. R. (2015). Feline Oral Squamous Cell Carcinoma: Clinical Manifestations and Literature Review. *Journal of Veterinary Dentistry*, 32(1), 30–40. https://doi.org/10.1177/089875641503200104

Birchard, S., & Carothers, M. (1990). Aggressive Surgery in the Management of Oral Neoplasia. *Veterinary Clinics of North America: Small Animal Practice*, 20(4), 1117–1140. https://doi.org/10.1016/S0195-5616(90)50088-1

Boston, S. E., Stee, L. L., Bacon, N. J., Szentimrey, D., Kirby, B. M., Nimwegen, S., & Wavreille, V. A. (2020). Outcomes of eight cats with oral neoplasia treated with radical mandibulectomy. *Veterinary Surgery*, 49(1), 222–232. https://doi.org/10.1111/vsu.13341

Bregazzi, V. S., LaRue, S. M., Powers, B. E., Fettman, M. J., Ogilvie, G. K., & Withrow, S. J. (2001). Response of Feline Oral Squamous Cell Carcinoma to Palliative Radioation Therapy. Veterinary Radiology <html_ent Glyph="@amp;" Ascii="&"/> Ultrasound, 42(1), 77–79. https://doi.org/10.1111/j.1740-8261.2001.tb00907.x

Gruen, M. E., Lascelles, B. D. X., Colleran, E., Gottlieb, A., Johnson, J., Lotsikas, P., Marcellin-Little, D., & Wright, B. (2022). Pain Management Guidelines for Dogs and Cats. Journal of the American Animal Hospital Association, 58(2), 55–76. https://doi.org/10.5326/JAAHA-MS-7292

Marconato, L., Weyland, M., Tresch, N., Rossi, F., Leone, V., & Rohrer Bley, C. (2020). Toxicity and outcome in cats with oral squamous cell carcinoma after accelerated hypofractionated radiotherapy and concurrent systemic treatment. *Veterinary and Comparative Oncology*, 18(3), 362–369. https://doi.org/10.1111/vco.12557

Mikiewicz, M., Paździor-Czapula, K., Gesek, M., Lemishevskyi, V., & Otrocka-Domagała, I. (2019). Canine and Feline Oral Cavity Tumours and Tumour-like Lesions: A Retrospective Study of 486 Cases (2015–2017). *Journal of Comparative Pathology*, 172, 80–87. https://doi.org/10.1016/j.jcpa.2019.09.007

Moore, A. S., & Moore, A. S. (2009). Treatment Choices for Oral Cancer in Cats: What is Possible? What is Reasonable? *Journal of Feline Medicine and Surgery*, 11(1), 23–31. https://doi.org/10.1016/j.jfms.2008.11.010

Murphy, S. (2016). Squamous Cell Carcinoma in Cats. In August's Consultations in Feline Internal Medicine, Volume 7 (pp. 526–534). Elsevier. https://doi.org/10.1016/B978-0-323-22652-3.00054-2

North, S. M., & Banks, T. A. (2009). Tumours of head and neck. In *Small Animal Oncology*: An Introduction (pp. 104–105). Saunders/Elsevier.

Northrup, N. C., Selting, K. A., Rassnick, K. M., Kristal, O., O'Brien, M. G., Dank, G., Dhaliwal, R. S., Jagannatha, S., Cornell, K. K., & Gieger, T. L. (2006). Outcomes of Cats With Oral Tumors Treated With Mandibulectomy: 42 Cases. *Journal of the American Animal Hospital Association*, 42(5), 350–360. https://doi.org/10.5326/0420350

Snyder, L. (2012). Oral squamous cell carcinoma: Like owner, like cat. *The Veterinary Journal*, 193(1), 6–7. https://doi.org/10.1016/j.tvjl.2012.02.023

Stebbins, K. E., Morse, C. C., & Goldschmidt, M. H. (1989). Feline Oral Neoplasia: A Ten-Year Survey. *Veterinary Pathology*, 26(2), 121–128. https://doi.org/10.1177/030098588902600204

Verstraete, F. J. M. (2005). Mandibulectomy and Maxillectomy. *Veterinary Clinics of North America: Small Animal Practice*, 35(4), 1009–1039. https://doi.org/10.1016/j.cvsm.2005.03.005

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

PRODUCTION AND EFFICACY EVALUATION OF A *PASTEURELLA* AUTOVACCINE FOR SHEEP

George MOGOȘ[1], Anca BULGARU[1], Elena NEGRU[1], Costin STOICA[2], Mihai DANEȘ[3], Doina DANEȘ[1]

[1]Faculty of Veterinary Medicine/University of Agronomic Sciences and Veterinary Medicine, Splaiul Independentei 105, District 5, Bucharest, Romania
[2]Romvac Company, Centurii Road 7, Voluntari, Ilfov, Romania
[3]Faculty of Veterinary Medicine/Spiru Haret University, 256 Basarabia Avenue, District 2, Bucharest, Romania

Corresponding author email: mogoshge@yahoo.com

Abstract

The aim of the current study was to develop an auto-vaccine based on two Pasteurella strains (P. multocida and P. spp.), previously isolated from an outbreak of respiratory infection in sheep. Preliminary tests were performed to assess the pathogenicity of the isolates on Balb/C mice. For the vaccine production, the Pasteurella strains were cultured on brain-heart infusion (BHI) medium supplemented with horse serum, were inactivated with formaldehyde and adjuvanted with aluminium hydroxide. In vitro and in vivo tests were performed to determine the sterility, safety and efficacy of the finished product. The results showed that the autovaccine had minimal side effects on both laboratory animals, and the target species; also, the biological product was successful in protecting vaccinated mice against the two pathogenic strains in a challenge test.

Key words*: evolution Pasteurella multocida, Pasteurella spp., pneumonia, sheep, vaccine.*

INTRODUCTION

One of the most prevalent bacterial illnesses in small ruminants, pasteurellosis is an important respiratory infection and has a wide geographic distribution, occurring in temperate, subtropical, and tropical regions. The first case of pneumonic pasteurellosis in sheep was documented in 1931, but it wasn't until the 1960s that serotyping and biotyping were important in defining the disease's epidemiology (Aitken, 2007). In sheep, *Mannheimia haemolytica* strains are usually responsible for pneumonic pasteurellosis in all age groups, while *Pasteurella trehalosi* strains induce systemic infection in lambs between the ages of 6 and 10 months. The species *Pasteurella multocida*, considered in rare cases to be the causative agent of respiratory infections in sheep and goats, includes a group of microorganisms characterized by antigenic diversity and multi-host predilection (Weiser et al., 2003). In the upper respiratory tract microbiome of numerous animal species, *P. multocida* is frequently present as a commensal. Environmental factors, stress, viral or mycoplasma infections favour bacterial invasion of lung tissue and the emergence of pneumonia. *P. multocida* can infect and cause illness in people and a variety of animal species (Boumart et al., 2021). As the cause of pneumonia in sheep and goats, the bacterial species has been subjected in a number of studies (Watson and Davies, 2002; Özyildiz et al., 2013; Valadan et al., 2014; Hailu et al., 2017; Boumart et al., 2021). According to Odugbo et al. (2006) and Sadeghian et al. (2011), clinical signs include anorexia, dyspnea with head and neck tightness and open-mouthed breathing, nasal discharge, coughing, listlessness, and death. Diarrhoea has occasionally been reported (Valadan et al., 2014). The best strategy of control, given the sporadic character of the illness, is targeted immunoprophylaxis by routine vaccination (Aitken, 2007). Due to *Pasteurella*'s great antigenic diversity and weak immunogenicity, vaccination against pasteurellosis is challenging (Mânzat, 2001). The purpose of the current study was to produce a vaccine based on two *Pasteurella* strains, previously isolated from an outbreak of respiratory infections in

sheep. The isolates, identified as *P. multocida* and *P.* spp. using Api 20 E and Api 20 NE biochemical tests, were found responsible for the reoccurring respiratory syndrome affecting a flock of sheep, where antimicrobial therapy was unsuccessful long-term (Mogoș et al., 2022). The researchers aimed to determine if the bacterial isolates were suitable for the production of an auto-vaccine, as well as if the finished product was safe to administer to animals and had good immunogenic qualities.

MATERIALS AND METHODS

To determine the pathogenicity of the two *Pasteurella* isolates, 60 Balb/C mice were split into three batches: Batch 1 - control, Batch 2 - *P.* spp. test and Batch 3 - *P. multocida* test. Batches 2 and 3 were subdivided into 4 groups (a, b, c, and d, respectively) of 5 animals each, each group to be inoculated with a different concentration of bacteria (Table 1).

Table 1. Batch organization and dose administered to mice for the pathogenicity test

Batch	Group	Dose
1 - Control	1a	PBS - 1 ml
	1b	PBS - 1 ml
	1c	PBS - 1 ml
	1d	PBS - 1 ml
2 - *Pasteurella* spp.	2a	2,000 CFU*/ml - 1 ml
	2b	200 CFU/ml - 1 ml
	2c	20 CFU/ml – 1 ml
	2d	2 CFU/ml - 1 ml
3 – Pasteurella multocida	3a	2,000 CFU/ml - 1 ml
	3b	200 CFU/ml - 1 ml
	3c	20 CFU/ml - 1 ml
	3d	2 CFU/ml - 1 ml

Legend: CFU/ml - colony forming units per millilitre

The inoculums were prepared by culturing the two bacterial strains in BHI medium for 24 hours at 37°C. The concentration of the cultures was determined using the serial dilutions method. To prepare the inoculums for the pathogenicity test, dilutions were made from each culture in PBS, in order to obtain suspension of 2000 colony forming units per mililiter (CFU/ml), 200 CFU/ml, 20 CFU/ml and 2 CFU/ml. The mice within the test groups were administered 1 ml of the respective dilution intraperitoneally, and the mice belonging to the control group were injected by the same route with 1 ml of sterile PBS each (Table 1). The clinical status of infected animals and their behaviour were monitored for 3 days post-inoculation. The time of appearance of the first clinical signs of disease, their evolution and the outcome were noted. Animals that survived the control infection, as well as animals included in the control group, were euthanized on day 8 post-inoculation.

Post-mortem examinations were performed and samples were collected from the liver, kidney and lungs of each mouse. The samples were cultured on blood agar plates. To determine the bacterial load in the different experimental groups, tissues harvested from lungs, liver and kidney were aseptically homogenized in normal saline to obtain a 10% suspension. Serial dilutions were made and cultured on BHI agar to determine the bacterial titer (CFU / g of tissue).

For the preparation of the *Pasteurella* autovaccine, the bacterial isolates were cultured on BHI medium supplemented with 10% horse serum. The cultures were incubared for 24 hours at 37°C, and then centrifuged 4000 rpm for 20 minutes at a temperature of 4°C. The supernatant was removed and the bacterial mass was washed 3 times in sterile PBS. The bacterial titer was determined for each bacterial suspension and formalin was added to a concentration of 0.2% for inactivation, followed by incubation at 37°C for 72 hours. The inactivated cultured were homogenized and 20% v/v aluminium hydroxide was added as an adjuvant. The pH was adjusted to 7.3 using sodium hydroxide solution.

The safety for laboratory animals was assessed on 25 Guinea pigs, divided into 5 batches of 5 animals/batch. Batches I - IV were administered subcutaneously a different dose of vaccine each, as follows: batch I - 0.5 ml, batch II - 1 ml, batch III - 1.5 ml, and batch IV - 2 ml. The animals in batch 5 represented the control group and were administered 1 ml of PBS/animal. All the animals included in the experiment were vaccinated twice, the booster being administered 3 weeks after the first

vaccination. The animals were monitored daily throughout the experiment and an additional 14 days after the booster, to assess any adverse reactions following vaccination, such as an increase in body temperature, loss of appetite, depression, and other local or systemic reactions attributed to the vaccination.

Safety tests were also performed on the target species. For this experiment, 11 healthy sheep were selected of different ages and physiological status. The test subjects were 2 adult males, two pregnant females, two lactating females and two lambs. The animals were administered two doses of vaccine (2 ml/dose), 21 days apart, subcutaneously. The control group consisted of three sheep (1 male, 1 female and 1 lamb), which were inoculated with sterile PBS following the same protocol. The animals' health status was monitored daily for 35 days following the first vaccination.

The efficacy test for the auto-vaccine was performed using four batches (A, B, C and D) of 10 Balb/C mice each. Batches A and B represented the control animals, and were administered two doses of sterile PBS, 0.5 ml/animal, s.c., 21 days apart. Batches C and D contained the test subjects, which were vaccinated with two doses 0.5 ml auto-vaccine/animal, at 21 days interval. Thirty-five days after the first inoculation, mice belonging to group A and group C were administered intraperitoneally a suspension of *P. multocida* with a concentration of 200 CFU/ml at a dose of 1 ml/animal. Mice belonging to groups B and D were administered intraperitoneally a suspension of *P.* spp. with a concentration of 200 CFU/ml at a dose of 1 ml/animal. Animals were clinically monitored for 7 days after the control infection, noting clinical signs and time of death. At the end of the 7 days of monitoring, all animals in agony, which during the course of the experiment showed clinical signs specific to *Pasteurella* infection, were considered dead and were euthanized. At the end of the experiment, all remaining live animals were euthanized. All of the mice included in the study were subjected to necropsy. Biological samples were collected for bacteriological examination during the necropsy. In the case of mice from the control groups, bacteriological examination was performed to demonstrate the presence of the pathogen. In vaccinated mice, bacteriological examination was carried out to confirm or exclude the presence of the pathogen in the tissues of the animals.

RESULTS AND DISCUSSIONS

The pathogenicity test results for the two strains of *Pasteurella* showed that both isolates caused the death of over 50% of the inoculated mice at a dose of 20 CFU/ml.

In group 2a, 100% mortality was recorded within 24 hours of inoculation; for group 2b, 100% mortality was recorded within 30 hours; for group 2c, 60% mortality was recorded within 36 hours, with surviving mice showing severe clinical signs of respiratory failure. Mice in group 2d survived throughout the experiment without clinical signs of disease (Table 2).

Following the experiment, it was determined that the bacterial isolate of *P.* spp. is pathogenic for mice at a dose of 20 CFU/ml.

In group 3a, all individuals died within 16 hours of inoculation, achieving 100% mortality; all individuals in group 3b died within 22 hours of inoculation, achieving 100% mortality; 4 of the 5 mice belonging to group 3c died within 28 hours, with a mortality rate of 80%, and the fifth mouse survived to the end of the experiment, showing severe respiratory distress; all individuals belonging to group 3d survived, 2 of them showing signs of apathy (Table 3).

Table 2. Pathogenicity test results for *Pasteurella* spp.

Infectious dose (CFU/ml)	Dead subjects	Mortality rate
2000 CFU/mL	5/5	100%
200 CFU/mL	5/5	100%
20 CFU/mL	3/5	60%
2 CFU/mL	0/5	0%

Legend: CFU/ml - colony forming units per millilitre

Table 3. Pathogenicity test results for *Pasteurella multocida*

Infectious dose (CFU/ml)	Dead subjects	Mortality rate
2000 CFU/mL	5/5	100%
200 CFU/mL	5/5	100%
20 CFU/mL	4/5	80%
2 CFU/Ml	0/5	0%

Legend: CFU/ml - colony forming units per millilitre

The experiment demonstrated that the isolated *P. multocida* strain is pathogenic for mice at a dose of 20 CFU/ml. Post mortem examinations revealed predominantly vascular lesions, including congestion, haemorrhages and oedema in most internal organs in the mice that died from the infection. The lung was the main organ affected, with pulmonary oedema and lobar congestion found in most cases. The liver was congested, with haemorrhagic areas and in some cases areas of focal necrosis. The kidneys contained haemorrhages and areas of necrosis. Most of the cadavers showed lesions characteristic of sepsis, such as absence of blood coagulation and splenomegaly. In mice infected with *P. multocida*, the lesions found at necropsy were more severe compared to lesions caused by *P.* spp. infection.

No pasteurellas were isolated from the tissues and organs of the control subjects.

For the subjects in batch 3, infected with *P. multocida*, for group 3a the mean bacterial titre obtained from liver, kidney and lung tissues was 2 x 10^9 CFU/g. For samples collected from cadavers belonging to group 3b, the mean bacterial titre was 7.16 x 10^8 CFU/g, with the highest concntration of bacteria in the kidneys. The mean bacterial titre for for the tissue samples of group 3c was 8.37 x 10^5 CFU/g. For group 3d, from individuals infected with the 2 CFU/ml dose and euthanised after 72 hours, no pasteurellas were recovered.

For the subjects belonging to batch 2, infected with *P.* spp., the mean bacterial titer obtained from tissue samples of mice in group 2a was 4.46 x 10^8 CFU/g. For samples collected from cadavers belonging to group 2b, the mean bacterial titre was 2.67 x 10^7 CFU/g. The mean bacterial titre for the tissue samples belonging to group 2c was 7.1 x 10^4 CFU/g. No pasteurellas were recoverd from the organs of the mice belonging to group 2d, infected with the 2 CFU/ml dose and euthanised after 72 hours.

The cultivation process for the two bacterial isolates resulted in a final concentration of 3 x 10^9 CFU/ml *P. multocida* and 2 x 10^9 CFU/ml *P.* spp. for the bacterial suspensions. After confirming the sterility of the bulk product, the vaccine was dispensed into brown glass vials with rubber stoppers and aluminium caps (Figure 1).

Figure 1. Pasteurella vaccine - finished product

During the safety trials on laboratory animals, throughout the monitoring period, no change in the general condition of the vaccinated Guinea pigs was observed both after the first dose and after the booster. The subjects showed no modification in appetite and defecation and urination were within physiological limits. In 30% of the vaccinated animals, belonging to batches 3 and 4, a slight increase in body temperature was observed within 24-48 hours after the first vaccination, which subsided after 48 hours without any intervention.

The safety trial on the target species revealed no change in the general condition of the vaccinated individuals after the first dose or after the booster. In vaccinated animals, a mild inflammatory reaction was observed at the site of administration within the first 24-72 hours after vaccination, which subsided after 72 hours without treatment. Body temperature remained within physiological limits throughout the clinical monitoring period. No local or systemic reactions were observed in all individuals belonging to the control groups during the 35 days of observation. The results of the *in vivo* tests performed to assess the safety of the *Pasteurella* auto-vaccine have confirmed that the product can be safely administered to both laboratory animals and target species, regardless of the individuals' age and physiological status.

The challenge test performed to determine the efficacy of the auto-vaccine resulted in all mice belonging to batches A and B (control batches) dying within 25 hours of infection. All individuals belonging to batches C and D survived throughout the monitoring period without showing clinical signs of disease.

Necropsy and bacteriological examination of biological samples collected from the dead mice following the control infection established the cause of death as *Pasteurella* septicaemia. Biological samples collected from vaccinated mice subjected to control infection and euthanized were found to be free from *Pasteurella* during bacteriological examination.

The results obtained during the challenge test have demonstrated the fact that vaccination with a high concentration of bacterins has successfully immunized the test subjects, and was able to prevent death, clinical signs of disease and tissue colonization by the two pathogens. Further research will determine the degree of seroconversion that can be obtained by vaccinating the target species with the auto-vaccine.

In the efforts to control pasteurellic pneumonia in sheep, vaccination is the best practical alternative to antibiotic therapy, used both to control and decrease the incidence of the disease and to minimize the use of antimicrobials in economically valuable animals (Verma and Jaiswa, 1998; Kehrenberg et al., 2001). Attempts to produce an effective vaccine against *P. multocida* have not always been successful, with researchers concluding that immunization against sheep pasteurellosis requires either the identification of a bacterial strain that elicits a broad-spectrum immune response or the discovery of an attenuated strain suitable for vaccine production, while establishing optimal cultural conditions to allow expression of a common immunizing antigen. With such strains not having yet been identified, the best alternative remains preparing auto-vaccines from pathogenic strains isolated from the field (Cameron and Bester, 1983). The studies of Berhe et al., (2017) also support this idea, having demonstrated that most animals in a geographical area were infected with at least 4 serotypes of *P. multocida*, which is why they recommend immunising animals with a product containing all the strains or serotypes responsible for the infection. The use of auto-vaccine against pasteurellosis is also supported by a study which shows that following the use of a commercial *Pasteurella* vaccine in a disease outbreak, when tested by indirect haemagglutination using strains from the outbreak, an immune response was obtained in only 16.7% of the vaccinated herd (Qasim et al., 2022).

CONCLUSIONS

The *P. multocida* and *P.* spp. bacterial isolates were pathogenic for mice at the minimum dilution of 20 CFU/ml, and were declared suitable for vaccine production.

For immunoprophylactic purposes, a *Pasteurella* auto-vaccine was prepared from the two bacterial strains isolated from the outbreak.

The auto-vaccine was formulated to a final concentration of 3 x 10^9 CFU/ml *P. multocida* and 2 x 10^9 CFU/ml *P.* spp., inactivated with formalin and adjuvanted with aluminium hydroxide.

To establish the safety of the product administration in animals, tests were performed on both laboratory animals and the target species. The tests showed that the vaccine did not cause systemic adverse reactions and that local reaction at the inoculation site were transient and of low intensity.

To determine the efficacy of the product, a challenge test was performed on mice, whereby the vaccinated individuals remained clinically healthy and necropsy investigation showed no changes specific to the exposure of mice to *Pasteurella*. Necropsy and bacteriological investigation of mice in the control group established the cause of death as septicaemia caused by *P. multocida* and *P.* spp. infection, respectively.

The preliminary trial results described in the current paper demonstrate that the auto-vaccine produced with the field isolates was safe to administer to animals and successful in protecting the vaccinated mice in the challenge test.

REFERENCES

Aitken, I. D. (2007) *Diseases of sheep*, Fourth Edition, Oxford, U. K.: Blackwell Publishing House.

Berhe, K., Weldeselassie, G., Bettridge, J., Christley, R. M., Abdi, R. D. (2017). Small ruminant pasteurellosis in Tigray region, Ethiopia: marked serotype diversity may affect vaccine efficacy. *Epidemiology & Infection, 145*(7): 1326-1338.

Boumart, Z., Bamouh, Z., Semmate, N., Tadlaoui, K. O., El Harrak, M. (2021). Biological Characterisation and Pathogenicity of a *Pasteurella multocida* Isolate from Sheep in Morocco. *Journal of Agricultural Science and Technology A,11*: 53-64.

Cameron, C. M., Bester, F. J. (1983). The inefficacy of polivalent *Pasteurella multocida* vaccines for sheep. *Onderstepoort J. vet. Res., 50*: 101-104.

Hailu, S. M., Kitila, D. B., Gemeda, A. E., Tarekegn, M. (2017). *Pasteurella* organism: Its isolation and identification from pneumonic lungs of goats in Ethiopia. *Journal of Advanced Veterinary and Animal Research, 4*(2): 147–154.

Kehrenberg, C., Schulze-Tanzil, G., Martel, J. L., Chaslus-Dancla, E., Schwarz, S. (2001). Antimicrobial resistance in *Pasteurella* and *Mannheimia*: epidemiology and genetic basis. *Veterinary Research, 32*: 323–339.

Manzat, M. (2001). Boli *Infectioase ale Animalelor – Bacterioze*. Bucharest, RO: Brumar Publishing House.

Mogoş, G., Bulgaru, A., Negru, E., Dinu, H., Daneş, M., Daneş, D. (2022). Isolation and identification of two Pasteurella strains, responsible for an outbreak of pneumonia in sheep. *Scientific Works. Series C. Veterinary Medicine, 68*(2): 78 - 82.

Odugbo, M. O., Odama, L. E., Umoh, J. U., Lamorde, A. G. (2006). *Pasteurella multocida* pneumonic infection in sheep: Prevalence, clinical and pathological studies. *Small Ruminant Research, 66*(1–3): 273-277.

Özyildiz, Z., Tel, O. Y., Yilmaz, R., Özsoy, Ş. Y., Keskin, O. (2013). Pathological and microbiological investigations of pneumonic pasteurellosis in sheep. *Kafkas Univ Vet Fak Derg, 19*(1): 103-108.

Qasim, M. T., Mahdi Mohammed Alakkam, E., Mahdi Mohammed, M., Hachim, S. K., Sabah Jabr, H., Emad Izzat, S., D Al-Dhalemi, M. (2022). Ovine Pasteurellosis Vaccine: Assessment of the Protective Antibody Titer and Recognition of the Prevailing Serotypes. *Archives of Razi Institute, 77*(3): 1207-1210.

Sadeghian, S., Mokhberdezfouli, M. R., Kojouri, G. A., Bazargani, T. T., Tavasoli, A. (2011). *Pasteurella multocida* pneumonic infection in goat: Hematological, biochemical, clinical and pathological studies. *Small Ruminant Research, 100* (2-3): 189-194.

Valadan, M., Jabbari, A. R., Niroumand, M., Tahamtan, Y., Bani Hashemi, S. R. (2014). Isolation and identification of *Pasteurella multocida* from sheep & goat in Iran. *Archives of Razi Institute, 69*(1), 47-55.

Verma, R. S., Jaiswa, T. N. (1998). Haemorrhagic septicaemia vaccines. *Vaccine, 16*: 1184–1192.

Watson, P. J., Davies R. L. (2002). Outbreak of *Pasteurella multocida* septicaemia in neonatal lambs. *Veterinary Record, 151*: 420-422.

Weiser, G. C., Delong, W. J., Paz, J. L., Shafii, B., Price, W. J., Ward, A. C. S. (2003). Characterization of *Pasteurella multocida* associated with pneumonia in Bighorn sheep. *Journal of Wildlife Diseases, 39*(3): 536–544.

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

IS ANTIOXIDANT CAPACITY CONNECTED TO BIOLOGICAL EFFECTS OF *SALVIA GLUTINOSA* L.?

Oana POP (COZMA)[1], Carmen Dana ȘANDRU[1, 2], Diana OLAH[1], Gheorghiță DUCA[2], Mariana RUSU[2], Aurel VASIU[1], Emoke PALL[1, 2], Cristian VĂLEANU[1], Marina SPÎNU[1, 2]

[1]University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Veterinary Medicine, 3-5 Mănăștur Street, 400372, Cluj-Napoca, Romania
[2]Institute of Research and Development for Montanology Cristian, 147 Street XIII, Cristian, Sibiu, Romania

Corresponding author email: marina.spinu@gmail.com

Abstract

The active principles extracted from plants prove to be useful in preventing or treating various diseases through their influence at molecular level. This potential of plants could reduce the negative effects of existing therapies due to lesser side effects and the results of bioavailability studies are encouraging. Hydro-alcoholic Salvia glutinosa L. extracts were examined for their antioxidant potential, anti-bacterial activity and in vitro immune stimulating effects. The dry aerial part of the plant (herba) was used for these experiments, after minced and solubilized in ethanol and also in aqueous solution. The antioxidant capacity was investigated by free radical scavenging effect over 1,1-diphenyl l-2-picrylhydrazyl (DPPH) radical, the antimicrobial effect by the Kirby Bauer radial diffusion test and the immune stimulating effect by tests on whole blood cultures. The results indicated a stronger antioxidant capacity (RSA% 88.89), antibacterial effect (G - E. coli, P. aeruginosa, x = 12 mm < G+, S. aureus, ATCC/clinical strains, x = 23 mm) and immune stimulation of the ethanol extract compared to the aqueous one. These results indicated the plant as a potential complex source to be implemented in alternative therapy.

Key words*: Salvia glutinosa* L.*, extract, antioxidant, antimicrobial, immune stimulating, therapy.*

INTRODUCTION

In many parts of the world medicinal plants have been used as components of the alternative therapy for numerous diseases (Palombo, 2011, Petrovska, 2012). Phytochemicals present in medicinal plants have discovered to be beneficial for the prevention and treatment of various diseases due to its capacity to act in multiple biological mechanisms (Zhang et al., 2011; Pereira et al., 2019). Other findings also suggested that some of the plants used in traditional medicine could provide antioxidant components useful for both therapy and preventive purposes.

One of the largest genera of *Lamiaceae* comprising approximately 980 species is genus *Salvia* (Hu et al., 2018). Originally from Europe and West Asia, *Salvia glutinosa* L. is a perennial herbaceous plant with wide distribution especially in the mountainous regions, being naturalized in several areas due to the appearance, smell and medicinal qualities it offers. The height (50-100 cm) at which it can reach is different depending on the pedo-climatic conditions. The morphological characteristics of this plant are: high and sticky stem, lanceolate leaves and provided with sticky glandular brushes, corolla consisting of yellow petals, with brown punctures, also provided with glandular brushes (Yin et al., 2023).

In Europe, *Salvia glutinosa* is widespread in mountain areas, mainly in deciduous forests, but other preferential areas are also reported, such as mixed coniferous and deciduous forests, wetlands at the edge of forests or on alpine pastures. There are numerous studies that describe the composition in active principles of *Salvia glutinosa*, which differs depending on the development conditions of the plant (microclimate, soil, geographical area), but also depending on the solvent used for extraction (Grdiša et al., 2015). Among the biologically active substances in the composition of *Salvia* are flavonoids, which are secondary metabolites of plants (Kennedy and Wightman, 2011).

The pharmacological effects of *Salvia* essential oils are based on the presence of more than 100 active compounds, which can be categorized

into monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, diterpenes, nonisoprenoid compounds and oxygenated sesquiterpenes (Fu et al., 2013; Szentmihályi et al., 2004). The most abundant components are 1,8-cineole, camphor, and a wide variety of thujenes (Russo et al., 2013).

The purpose of this study was to determine the effect of *Salvia glutinosa* extracts on bacteria and leukocytes, in connection to the antioxidant activity of the plant, to clarify the link between the *in vivo* effects of sage decoction used in traditional medicine on skin wounds and the true mechanisms underlying the repair processes in these cases.

MATERIALS AND METHODS

The aqueous and ethanolic extracts of *Salvia glutinosa* were evaluated by a three step procedure, envisaging the antioxidant activity (a), the antibacterial effect (b) and the *in vitro* immune activity on whole blood cultures (c), as follows:

a. The total polyphenol content in aqueous solutions and in ethanolic extracts was estimated using the technique described by Blainski et al. (2013). The absorbance was read at 750 nm using a Shimadzu UV-VIS 1700 spectrophotometer. The standard curve was maintained by using different concentrations of gallic acid: 0, 0.25, 0.50, 0.75 and 1 mg/ml. The content in total polyphenols was expressed in gallic acid equivalents, mg GAE/100 ml sample.

To express the antioxidant activity of the plant extracts, the free radical scavenging effect was assessed over 1,1-diphenyl l-2-picrylhydrazyl (DPPH) radical, method proposed by Odriozola-Serrano et al. (2013) 100 µl of each sample was mixed with 3.9 ml of DPPH (0.025 g/l) maintained properly in the dark for 30 min. The absorbance of the samples was measured at 515 nm (Shimadzu 1700 UV-VIS) against a methanol/water blank (percent over standard DPPH absorbance).

b. The *in vitro* antimicrobial potential of watery and ethanolic extracts of *Salvia glutinosa* was evaluated by well-diffusion method against the following reference strains: *Staphylococcus aureus* ATCC 6538P, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 10145. For each bacterium, an inoculum was prepared by suspending 24 h pure culture in Mueller Hinton (MH) broth in order to dilute approximately to 10^{-6} colony forming units (CFU)/mL according to McFarland scale. The bacterial suspensions were "flood-inoculated" onto the surface of MH agar and dried. The extracts (60 µL) were placed into wells (three wells of six-millimeter diameter for each extract) made into the MH agar using a sterile matrix. The assay included 70% ethanol as the negative control and gentamicin as standard antibiotic. All tests were performed in triplicate. The diameters of the growth inhibition zones were measured after 24 h of incubation at 37°C. The data were statistically analysed for significance of the treatment effect by using Microsoft Excel software.

c. For monitoring the in vitro effect of the *Salvia glutinosa* extracts on leukocytes, blood samples were collected from adult bovine, by puncturing the jugular vein, during the official testing for transmissible disease surveillance. The samples were diluted with RPMI 1640 (Sigma-Aldrich, USA) medium (1:4) with 5% FCS (Gibco) and penicillin and streptomycin (Sigma-Aldrich). The diluted samples were added 96-well plate, 100 µl/well, 6 variants: (1) untreated control culture, (2) phytohaemagglutinin-M (PHA-M) (1 µl/well) (3) Alcohol (1.5 µl/ well), (4) concanavalin A (Con A) (1 µl/ well), (5) alcoholic extracts of *Salvia officinalis* (1.5 µl /well), (6) alcoholic extracts of *Vaccinium myrtillus* (1.5 µl/well). The cultures were incubated for 48 h at 37°C and 5% CO_2. Glucose concentrations were measured in the initial medium and in all variants, using orto-toluidine colorimetric test. 12.5 µl of the cultural medium were transferred to 0.5 ml of orto-toluidine reagent, heated for 8 minutes, added in cold water and evaluate using a spectrophotometer at 610 nm wavelength (Sumal PE2, Karl Zeiss, Germany), using the reagent as a blank. For transformation index (TI) the following formula was used: TI %=[(MG–SG)/MG] ´100, where TI, blast transformation index, MG, glucose concentration in the initial culture medium and SG, glucose concentration in the sample after incubation.

The data were statistically analysed for significance of the treatment effect by using Microsoft Excel software.

RESULTS AND DISCUSSIONS

Investigated by the lipide peroxidation system, the antioxidant activity of aqueous methanolic extracts of various sage species (*S. candelabrum*, *S. ringens*, *S. tomentosa*, *S. nemorosa*, and *S. glutinosa*) were dependant on concentration and similar to those observed for *S. officinalis* (Miura et al., 2002; Zupkó et al., 2001). The results of the Folin-Ciocâlteu method are presented in Figure 1 and reveal an increased amount of polyphenols in the ethanolic extracts, compared to the aqueous extracts, respectively the decoction and infusion of *Salvia glutinosa*. The differences between the two types of preparations lie, on the one hand, in the greater solubilizing capacity of alcohol compared to polyphenols and, on the other hand, in the temperature of obtaining the decoction and the infusion. The water used to prepare the teas was pre-heated to 100°C, which led to the denaturation of an important fraction of polyphenols, considering the fact that the optimal temperature for their recovery is 20-50°C (Brglez Mojzer et al., 2016).

The antioxidant activity measured by the DPPH test was similar to that described in literature for Romanian *Salvia glutinosa* (Mocan et al., 2020) that is X = 79.57±0.71 mg TE/g of extract for the tea and X = 88.21±0.63 mg TE/g of alcoholic extract. The radical scavanging activity varied in intensity based on plant sampling season (Figure 2).

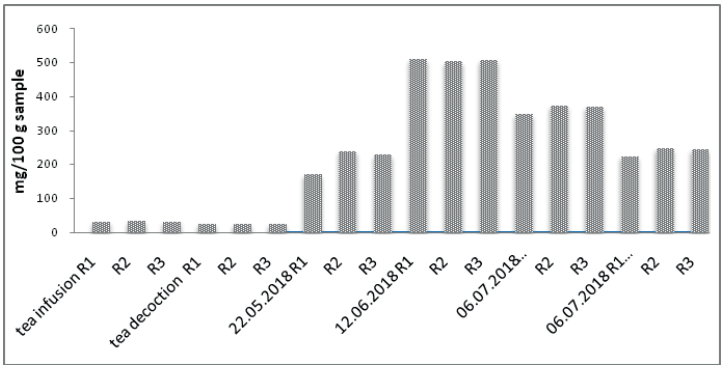


Figure 1. Total polyphenol content of different extracts of *Salvia glutinosa*. The R1, 2, 3 indicate the number of the replica for each sampling date ($p<0.05$ for the month of June versus May, and alcoholic extracts versus tea or decoction)

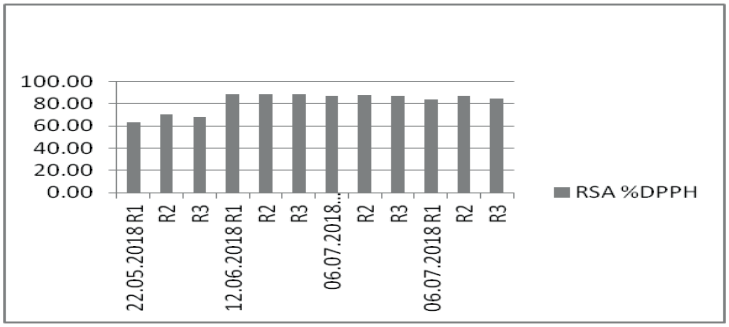


Figure 2. The radical scavenging activity (RSA%) depended on the sampling period of the plants, with higher values in June-July

The antimicrobial efficacy of the ethanolic extract and aqueous preparations of *Salvia glutinosa* showed different activity against different ATCC and field isolates of bacterial strains (Figure 3). As it is indicated by the inhibition diameters, the alcoholic extract was more active than the tea against all bacteria except *E. coli*, where surprisingly, the tea was

more active. These data are similar to those in literature, citing the efficacy of *Salvia glutinosa* alcoholic extract against numerous bacteria, including *B. cereus* (Mocanu et al., 2020). Nevertheless, the efficacy of the tea against *E. coli* was not mentioned, although the same effect is recognised for *S. officinalis* tea (Vogl et al., 2013). The statistically significant differences ($p<0.05$ - $p<0.001$) were recorded only when comparing the activity of antibiotics with that of the plant extracts.

In the blast transformation test of the leukocytes, the alcoholic extract induced stimulation of the mononuclear cells, when compared to the tea or decoction (Figure 4).

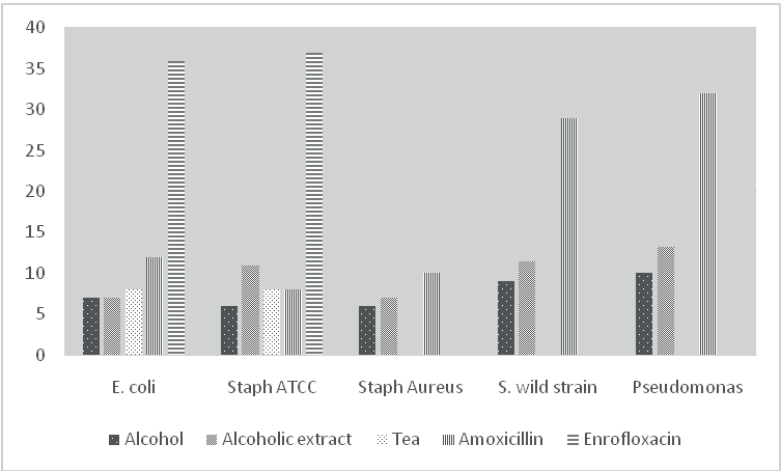


Figure 3. Antimicrobial efficacy of different *Salvia glutinosa* extracts when compared to ethanol and antibiotics

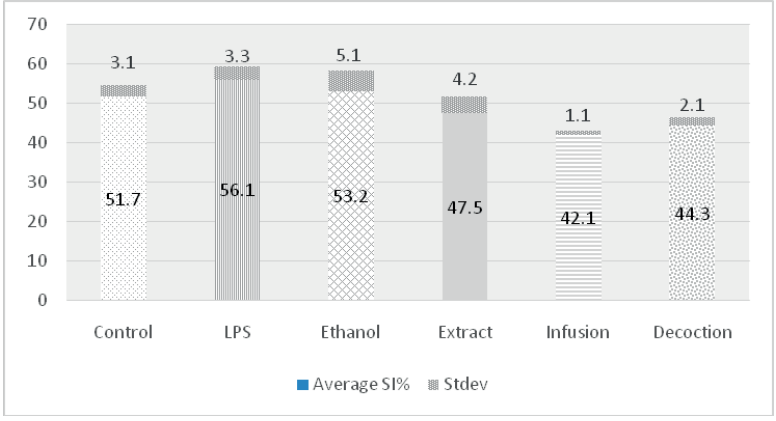


Figure 4. The blast transformation indices calculated for various *in vitro* treatments: the alcoholic extract induced an increased stimulation of the leukocytes when compared to aqueous preparations

The need to discover new methods in diminishing the antibiotic resistance phenomenon directed research towards the investigation of the bacteriostatic or bactericidal capacity of some plant extracts. Current studies in the field, which verify the bioavailability of *Salvia officinalis* and other *Salvia* species extracts' biological activity at cellular level are becoming more and more numerous, thus emphasizing the bactericidal and antioxidant properties of this genus.

The data in the literature supports the antimicrobial properties of *Salvia glutinosa*. Testing the ethanolic extract of *Salvia* on some clinical isolates and some reference strains of *Streptococcus pyogenes* reveals that a

concentration of 125 µg of Salvia extract is necessary to alter the structure of the bacterial wall and exert a bactericidal effect (MBC) (Wijesundara et al., 2019). The results of the present study followed the same dose-effect pattern, based on the type of bacterial isolate investigated. This locally resourced plant provides encouraging solutions to implement in antibacterial therapy, further enhanced by the radical scavanging activity and its immune stimulating effect.

CONCLUSIONS

The bioavailability of the active principles of *Salvia glutinosa* L. in different cell culture systems (bacteria and cells of the circulating immune system) depends on several factors, such as: the extraction method, the type of cells used, the harvesting period and the amount of polyphenols.

The antioxidant effect due to the increased amount of polyphenols can be exploited for protection against oxidative damage and for anti-inflammatory properties in veterinary medicine as well. The ethanolic extracts active against bacteria could represent an alternative to antibiotic therapy especially against infections involving *Staphylococcus* spp., but the range of dosages needs to be expanded. The immune stimulating efficacy of the extracts represents an asset for their combined positive biological effects.

The observations made on all three different cell types justify the use of the obtained extracts as adjuvant therapy in the therapy of skin wounds.

AUTHORS' CONTRIBUTION

All authors had equal contribution in the study design, sampling, sample processing, data processing and writing the paper.

REFERENCES

Blainski, A., Lopes, G., de Mello, J. (2013). Application and Analysis of the Folin-Ciocalteu Method for the Determination of the Total Phenolic Content from Limonium Brasiliense L. *Molecules*, *18*(6), 6852–6865.

Brglez Mojzer, E., Knez Hrnčič, M., Škerget, M., Knez, Ž., Bren, U. (2016). Polyphenols: Extraction Methods, Antioxidative Action, Bioavailability and Anticarcinogenic Effects. *Molecules (Basel, Switzerland)*, *21*(7), 901.

Fu Z. H., Wang H., Hu X., Sun Z., Han C. (2013). The pharmacological properties of *Salvia* essential oils; 3(7): 122–127.

Grdiša M, Jug-Dujaković M, Lončarić M, Carović-Stanko K, Ninčević T, Liber Z, Šatović Z. (2015). Dalmatian sage (*Salvia officinalis* L.): A review of biochemical contents, medical properties and genetic diversity. *Agric Conspec Sci*.; 80(2): 69–78.

Hu G.-X., Takano A., Drew B.T, Liu E.-D., Soltis D.E, Soltis P.S., Peng H., Xiang C.-L. (2018). Phylogeny and staminal evolution of *Salvia* (Lamiaceae, Nepetoideae) in East Asia, *Annals of Botany*, 122: 4, 649–668,

Kennedy D. O., Wightman E.L. (2011). Herbal Extracts and Phytochemicals: Plant Secondary Metabolites and the Enhancement of Human Brain Function, *Adv Nutr.*; 2(1): 32–50.

Miura, K., Kikuzaki, H., & Nakatani, N. (2002). Antioxidant activity of chemical components from sage (*Salvia officinalis* L.) and thyme (*Thymus vulgaris* L.) measured by the oil stability index method. *Journal of agricultural and food chemistry*, *50*(7), 1845–1851.

Odriozola-Serrano, I.; Aguilo-Aguayo, I.; Soliva-Fortuny, R.; Martin-Belloso, O. (2013). Pulsed electric fields processing effects on quality and health-related constituents of plant-based foods. *Trends Food Sci. Technol*., 29, 98–107.

Palombo EA, Traditional Medicinal Plant Extracts and Natural Products with Activity against Oral Bacteria: Potential Application in the Prevention and Treatment of Oral Diseases, Evidence-Based Complementary and Alternative Medicine Volume 2011, Article ID 680354

Pereira, A. S. P., den Haan, H., Peña-García, J., Moreno, M. M., Pérez-Sánchez, H. ve Apostolides, Z. (2019). Exploring African Medicinal Plants for Potential Anti-Diabetic Compounds with the DIA-DB Inverse Virtual Screening Web Server. *Molecules*, *24*(10), 2002.

Petrovska B. B. (2012). Historical review of medicinal plants' usage. *Pharmacognosy reviews*, 6(11), 1–5.

Russo, A., Formisano, C., Rigano, D., Senatore, F., Delfine, S., Cardile, V., Rosselli, S., & Bruno, M. (2013). Chemical composition and anticancer activity of essential oils of Mediterranean sage (*Salvia officinalis* L.) grown in different environmental conditions. Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association, 55, 42–47.

Szentmihályi K., Csedo C., Then M. (2004). Comparative study on tannins, flavonoids, terpenes and mineral elements of some *Salvia* species. 629:463–470.

Yin D., Luo W., Yang Z., Shuai Qi, Chen K., Gou A. (2023). Morphological and biochemical studies of *Salvia guaranitica* St. Hil. under simulated deposition with different amounts of dust, Ecotoxicology and Environmental Safety, 249:114404.

Zhang, L., Ravipati, A. S., Koyyalamudi, S. R., Jeong, S. C., Reddy, N., Smith, P. T., Bartlett, J., Shanmugam, K., Münch, G., & Wu, M. J. (2011). Antioxidant and anti-inflammatory activities of selected medicinal plants containing phenolic and flavonoid compounds. *Journal of agricultural and food chemistry*, *59*(23), 12361–12367.

Zupkó I, Hohmann J, Rédei D, Falkay Gy, Janicsák G, Máthé I. (2001). Antioxidant activity of leaves of *Salvia* species in enzyme-dependent and enzyme-independent systems of lipid peroxidation and their phenolic constituents. *Planta Medica*,67, 366-368.

Mocan, A., Babota, M., Pop, A., Fizeșan, I., Diuzheva, A., Locatelli, M., Carradori, S., Campestre, C., Menghini, L., Sisea, C. R., Soković, M., Zengin, G., Păltinean, R., Badarau, S., C Vodnar, D. C., & Crișan, G. (2020). Chemical Constituents and Biologic Activities of Sage Species: A Comparison between *Salvia officinalis* L., *S. glutinosa* L. and *S. transsylvanica (Schur ex Griseb. & Schenk) Schur*. *Antioxidants (Basel, Switzerland)*, *9*(6), 480.

Vogl, S., Picker, P., Mihaly-Bison, J., Fakhrudin, N., Atanasov, A. G., Heiss, E. H., Wawrosch, C., Reznicek, G., Dirsch, V. M., Saukel, J., & Kopp, B. (2013). Ethnopharmacological *in vitro* studies on Austria's folk medicine--an unexplored lore in vitro anti-inflammatory activities of 71 Austrian traditional herbal drugs. *Journal of ethnopharmacology*, *149*(3), 750–771.

Wijesundara Niluni M., Rupasinghe H. P. Vasantha (2019). Bactericidal and Anti-Biofilm Activity of Ethanol Extracts Derived from Selected Medicinal Plants against Streptococcus pyogenes, *Molecules,* vol. 24, 1165

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

THE EFFECT OF GENERAL ANESTHESIA ON UREA AND CREATININE VALUES IN A GROUP OF DOGS

Maria Roxana TURCU, Cătălin MICȘA, Lucian IONIȚĂ, Ruxandra COSTEA

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: turcu.mariaroxana@yahoo.com

Abstract

All anesthetic drugs act on renal function, the kidney being the primary organ involved in the excretion of the anesthetic drugs. Also, anesthesia influences kidney function, especially through hemodynamic and neuroendocrine changes. Anesthesia and renal function are highly interconnected and can potentially influence each other. Understanding the anesthetic effects on renal function can help to develop safe perioperative care and to optimize the after-surgery outcome. The study will focus on how general anesthesia influences renal parameters, blood urea nitrogen (BUN) and creatinine (CREA). The results of our study show the impact of general anesthesia for both BUN and CREA, in correlation with the medication used for premedication, and the patient`s condition.

Key words*: general anesthesia, kidneys, urea, creatinine.*

INTRODUCTION

In a healthy organism, the kidney plays an important role in fluid, electrolyte, and acid-base regulation and also in filtration, reabsorption and secretion. Under normal physiological conditions, all these roles can be performed properly and the kidneys will need to receive about 20- 25% of the cardiac output via renal arteries, being among the most highly perfused organs in the body (Evans et al., 2020) and highly metabolically active. Even a short period of renal ischemia can lead to acute renal injury (Tranquili et al., 2013).

Blood urea nitrogen (BUN) is synthetized in the liver from the amino acids that arise from the catabolism of both exogenous and endogenous proteins. The excretion of BUN is achieved through glomerular filtration. Creatinine is a molecule that is synthesized mainly in the liver, kidneys, and pancreas and found in tissues with high-energy demands, like muscles (Bonilla et al., 2021). Crea is metabolized and excreted almost entirely through kidneys, via glomerular filtration. For healthy canine patients, the normal BUN concentration is 8 to 25 mg/dL, while in CREA is 0.3 to 1.3 mg/dL (Nelson & Couto, 2019).

Unfortunately, the renal disease frequently goes undiagnosed because the elevation in BUN and CREA occurs when the renal function is 50-70% compromised (Rezende & Mama, 2015).

MATERIALS AND METHODS

The study was conducted on 25 canine patients, aged between 1 and 14 years old. The study took place at the Faculty of Veterinary Medicine Bucharest and describes how general anesthesia affects the BUN and Crea values.

For biochemical blood determinations, we used the veterinary biochemical analyzer SMT-120V (Figure 1.).

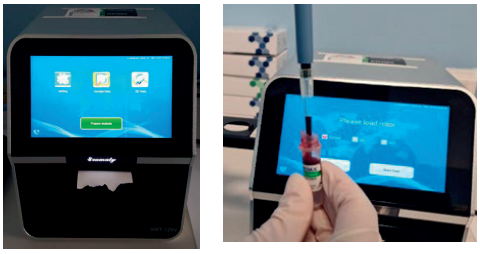


Figure 1. Biochemical lab analyzer SMT-120V. Preparing the blood for the analysis

The operating system is based on the spectrometry technique. The sample used for this machine can be serum, plasma, or blood and it needs to be collected in lithium heparin tubs.

All general identification data were recorded for the entire group: breed, age, gender, and body weight.
Before surgery, every patient had a pre-anesthetic examination that included: heart rate, pulse, respiratory rate, non-invasive blood pressure, assessing the colour of the mucosa membranes, and rectal temperature. Also, every patient had a complete blood count and biochemistry evaluation that included BUN and CREA. All patients were assigned an anesthesia risk score according to the American Society of Anesthesiologists (ASA) physical status classification system modified for veterinary medicine (Costea, 2016).

Table 1. ASA score of every patient

	ASA1	ASA2	ASA3	ASA4	ASA5
Number of dogs	8	9	8	0	0

Patients underwent different types of surgeries, ophthalmological surgeries, and urogenital surgeries, including emergency surgery for pyometra. Depending on the type of the procedure the animal underwent, different time duration existed. For example, the ophthalmological surgeries were shorter compared with the urogenital surgeries.
Multiple anesthetic protocols were taken into consideration based on the ASA score (Table 1). The premedication was administered intramuscularly to all the patients. Apart from the premedication, all patients benefit of induction with Propofol (2-5 mg/kg, intravenous), intubation and maintenance with Isoflurane and Oxygen 100%. Lactated Ringer was administrated throughout the surgery and in the recovery period, at a rate of 5 ml/kg/h.
The group was divided into 4 categories based on the premedication used, as follows:

- First group: Butorphanol (0.3 mg/kg), Midazolam (0.2 mg/kg) and Ketamine (2 mg/kg);
- Second group: Butorphanol (0.3 mg/kg) and Ketamine (2 mg/kg);
- Third group: Dexmedetomidine (2 mcg/kg), Butoraphanol (0.3 mg/kg) and Ketamine (2 mg/kg);
- Fourth group: Acepromazine (0.02 mg/kg), Butorfanol (0.3 mg/kg) and Ketamine (2 mg/kg).

For the purpose of this study, the parameters taken into consideration were BUN and CREA, and the tests were taken on the same device for a better comparison of the results.

RESULTS AND DISCUSSIONS

The patients selected for this study were included in the I-II-III ASA score (Table 1) and the anesthetic protocols were chosen based on the classification of patients in these anesthetic risk groups (Table 2).
The type of the procedure influenced the duration of the surgery and also the postoperative analgesia. The type of the procedure also influenced the duration of the general anesthesia. For example, the ophthalmological surgeries were shorter and, in some situations, did not require such depth anesthesia compared to the urogenital surgeries.
From the total of 25 cases, the premedication was distributed as follows (Figure 2):

- The first group (MBK) consisted of 8 cases, which represented 32% of the total number of cases, and was premedicated with Butorphanol (0.3 mg/kg), Midazolam (0.2 mg/kg) and Ketamine (2 mg/kg)
- The second group (BK) consisted of 9 cases, which represented 36% of the total number of cases was premedicated with Butorphanol (0.2 mg/kg) and Ketamine (2 mg/kg);
- The third group (DBK) consisted of 7 cases, which represented 28% of the total number of cases was premedicated with Dexmedetomidine (2 mcg/kg), Butorphanol (0.3 mg/kg) and Ketamine (2 mg/kg);
- The fourth group (ABK) consisted of 1 case, which represented 4% of the total number of cases was premedicated with Acepromazine (0.02 mg/kg), Butorphanol (0.3 mg/kg) and Ketamine (2 mg/kg).

The first part of the study aimed to quantify the effect of anesthesia in relation to the age groups of the patients studied. In Figure 3, are presented the data obtained for the total number of patients divided into age groups: the first group is represented by dogs aged 1 to 6 years old, and the second group dogs aged 7 to 14 years old. Globally, for the whole study group, an increase in both BUN and CREA was registered, but different results emerged in different age groups.

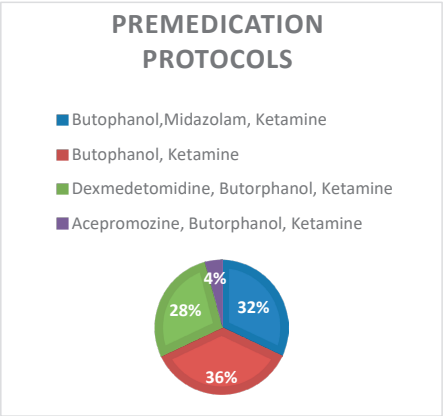


Figure 2. The premedication distribution in the study group

Table 2. Classification of patients in anesthetic risk groups

ASA group	Number of patients	Protocol
I	7	DBK
I	1	ABK
II	9	BK
III	8	MBK

BUN increased by 5% and CREA increased by 13% in the group of dogs aged between 1 and 6 years old. As a comparison, the BUN increased by 3% and CREA decreased by 1% in the group were the dogs where between 7 and 14 years old (Figure 3).

Date from the hole group		
Row label	Average of BUN (mg/dl)	Average of Crea (mg/dl)
Before	21.972	1.2136
After	21.168	1.1624
Difference	4%	4%

Dogs with age between 1 and 6 years		
Row label	Average of BUN (mg/dl)	Average of Crea (mg/dl)
Before	17.770	0.897
After	16.957	0.794
Difference	5%	13%

Dogs with age between 7 and 14 years		
Row label	Average of BUN (mg/dl)	Average of Crea (mg/dl)
Before	27.318	1.616
After	26.527	1.630
Difference	3%	-1%

Figure 3. Date obtained in the study group divided by age

For geriatric patients, the renal system shows important structural changes. A 50% decrease in functional nephrons is not unusual in the aging animal, along with decreased renal blood flow and a decreased glomerular filtration rate (Baetge & Matthews, 2012). Ketamine metabolization and excretion can be longer, especially for this category of patients. The CREA level varied in the sense of increase or decrease values, correlated with the pathologies and protocols chosen, data confirmed by the literature (Patel, 2009).

The second part of the study aimed to quantify the effect of anesthesia in relation to the different protocols used. Figure 4 illustrates the difference between BUN and CREA before and after premedication based on the drugs that were used. For the protocol Dexmedetomidine-Butorphanol-Ketamine (DBK) the BUN increased by 7% and CREA decreased by 7%. On the Butorphanol-Midazolam-Ketamine group (MBK) BUN increased by 1% and CREA increased spectacularly by 59%. Midazolam decreases the glomerular filtration rate and the renal plasma flow while Ketamine increases levels of urea and creatinine (Alizadeh & Fard, 2019). Finally, in the group Butorphanol-Ketamine (BK), BUN decreased by 2% and CREA increased by 24%. Possibly the differences compared to the MBK group, are correlated with the lack of Midazolam in the protocol.

Dexmedetomidine-Butorphanol-Ketamine		
Row label	Average of BUN (mg/dl)	Average of Crea (mg/dl)
Before	15.985	0.825
After	14.885	0.888
Difference	7%	-7%

Butorphanol-Midazolam-Ketamine		
Row label	Average of BUN (mg/dl)	Average of Crea (mg/dl)
Before	16.337	0.931
After	16.212	0.587
Difference	1%	59%

Butorphanol-Ketamine		
Row label	Average of BUN (mg/dl)	Average of Crea (mg/dl)
Before	18.522	0.79
After	18.811	0.637
Difference	-2%	24%

Figure 4. Data obtained in the study group divided based on premedication

The important variations for the MBK and BK protocols can be correlated with the ASA status of the patients, respectively with the important pre-anesthetic status of the patients. The values obtained for the DBK protocol can be correlated with the synergistic effects of the alpha-2 agonist dexmedetomidine and respectively ketamine, on the cardiovascular system, respectively on metabolism and renal excretion.

In addition to the premedication protocols used, the potential effect on renal function of the medication used for induction and maintenance should also be considered Propofol, used for the induction of anesthesia, is metabolized by the process of glucuronidation in hepatic and extrahepatic sites including the kidney (Hiraoka et al., 2005). Halogenated agents can have a significant impact, more important for sevoflurane versus isoflurane, since the production of fluoride ions by sevoflurane is the main difference (Tsukamoto et al., 1996).

CONCLUSIONS

In conclusion, there are differences in the renal parameters that were taken into account for this study (BUN and CREA). The parameters were measured before premedication and in the early phase of recovery. Changes can be associated with the age of the patient, the type of surgery and different anesthetic protocols used.

ASA II-III patients, as well as geriatric patients, are more exposed to BUN and CREA variations during anesthesia

The DBK protocol used for ASA I and II patients had a medium impact on BUN and CREA variations

The important differences between the protocols are very likely based on the doses used for premedication, in relation to the patient's condition.

Further studies, implying a detailed correlation between the patient's clinical condition, the surgical procedure, its duration and the chosen protocol are necessary.

REFERENCES

Alizadeh, R., & Fard, Z. A. (2019). Renal effects of general anesthesia from old to recent studies. *Journal of cellular physiology*, 234(10), 16944-16952.

Baetge, C. L., & Matthews, N. S. (2012). Anesthesia and analgesia for geriatric veterinary patients. *Veterinary Clinics: Small Animal Practice*, 42(4), 643-653.

Bonilla, D. A., Kreider, R. B., Stout, J. R., Forero, D. A., Kerksick, C. M., Roberts, M. D., & Rawson, E. S. (2021). Metabolic basis of creatine in health and disease: a bioinformatics-assisted review. *Nutrients*, 13(4), 1238.

Costea, R. (2016). Anaesthesia considerations for critically ill patients. *European Journal of Companion Animal Practice*, 26(3), 27-35.

Evans, R. G., Smith, D. W., Lee, C. J., Ngo, J. P., & Gardiner, B. S. (2020). What makes the kidney susceptible to hypoxia? *The Anatomical Record,* 303(10), 2544-2552.

Gil, A. G., Illera, J. C., Silván, G., Lorenzo, P. L., & Illera, M. (2002). Changes in hepatic and renal enzyme concentrations and heart and respiratory rates in new zealand white rabbits after anesthetic treatments. *Journal of the American Association for Laboratory Animal Science*, 41(6), 30-32.

Grimm, K. A., Lamont, L. A., Tranquilli, W. J., Greene, S. A., & Robertson, S. A. (Eds.). (2015). *Veterinary anesthesia and analgesia*. John Wiley & Sons

Hiraoka, H., Yamamoto, K., Miyoshi, S., Morita, T., Nakamura, K., Kadoi, Y., Horiuchi, R. (2005). Kidneys contribute to the extrahepatic clearance of propofol in humans, but not lungs and brain. *British Journal of Clinical Pharmacology*, 60(2), 176–182.

Kim, J. W., Kim, J. D., Yu, S. B., & Ryu, S. J. (2013). Comparison of hepatic and renal function between inhalation anesthesia with sevoflurane and remifentanil and total intravenous anesthesia with propofol and remifentanil for thyroidectomy. *Korean journal of anesthesiology*, 64(2), 112-116.

Nelson, R. and Couto, C., 2019. Small animal internal medicine

Patel, P. B. (2009). Evaluation of atropine-diazepam-ketamine induced anaesthesia during neutering programme in adult and geriatric dogs. Indian *Journal of Veterinary Surgery*, 30(2):109.

Rezende, M., & Mama, K. (2015). Anesthesia for patients with renal disease. *Clinician's Brief*, 3, 41-44.

Sahin, S. H., Cinar, S. O., Paksoy, I., Sut, N., & Oba, S. (2011). Comparison between low flow sevoflurane anesthesia and total intravenous anesthesia during intermediate-duration surgery: effects on renal and hepatic toxicity. *Hippokratia*, 15(1), 69.

Setiawan, P. G. M., Tunjung, W. A. S., & Nurhidayat, L. (2016). Effect of diazepam on kidney function and histological structure of white rat's kidney. *Berkala Penelitian Hayati*, 22(1), 1-6.

Tranquilli, W. J., Thurmon, J. C., & Grimm, K. A. (Eds.). (2013). *Lumb and Jones' veterinary anesthesia and analgesia*. John Wiley & Sons.

Tsukamoto, N., Hirabayashi, Y., Shimizu, R., & Mitsuhata, H. (1996). The effects of sevoflurane and isoflurane anesthesia on renal tubular function in patients with moderately impaired renal function. *Anesthesia and Analgesia*, 82(5), 909–913

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

THORACIC TRAUMA UPDATES IN FELINE HIGH-RISE SYNDROME. WHAT CHANGED IN 30 YEARS? 50 CASES IN ONE YEAR

Seralp UZUN[1], Iuliana IONASCU[1], Florin DUMITRESCU[1], Dragos-Marian DUMITRASCU[1], Tiberiu Sebastian IANCU[1], Radu JERCAU[2], Catalina GEORGESCU[2], Dumitru MAGALEAS[2], Georgeta FILIP[2], Ada IONESCU[2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independenței, District 5, Bucharest, Romania
[2]University of Agronomic Sciences and Veterinary Medicine of Bucharest, University Veterinary Emergency Hospital "Prof. univ. dr. Alin Bîrţoiu", 105 Splaiul Independenței, District 5, Bucharest, Romania

Corresponding author email: seralp.uzun@gmail.com

Abstract

High-rise syndrome is a general definition of multiple traumatic injuries that cats experience after falling from a height of 2 or more floors of high-rise buildings in urban areas. This falling generally results with multiple injuries including thoracic, abdominal, orthopedic and craniomandibular or craniomaxillofacial trauma. The combination of multiple traumatic injuries can be life threatening.50 cats diagnosed with high-rise syndrome between period December 2021-December 2022 in Veterinary Emergency Hospital "Prof. univ. dr. Alin Bîrţoiu", University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine. Thoracic trauma was diagnosed in 82% of cats. Pneumothorax was diagnosed in 54% of cats and pulmonary contusions diagnosed in 50% of cats. Some cats diagnosed both pneumothorax and pulmonary contusion. Past 30 years, some authors pointed and rated the injuries they diagnosed in their articles and case reports. The aim of this study is to point increased thoracic trauma after high-rise syndrome related with hitting more hard grounds than past years in urban areas.

Key words*: high-rise syndrome, pneumothorax, pulmonary contusion, thoracic trauma.*

INTRODUCTION

Feline high-rise syndrome (FHRS) refers to combination of some traumatic injuries that cats experience after falling from a certain height (8 meters or 24 feet or 2 floors) generally in urban areas. High-rise syndrome, mostly reported and described in cats by several authors in the past (Robinson 1976; Dupre et al.,1995; Flagstad et al., 1998; Papazoglou et al., 2001). This syndrome is also been reported in dogs (Gordon et al., 1993), but it is rare than in cats. In humans the terms “high-flyer syndrome” or “jumpers syndrome” were also used by some authors (Reynolds et al., 1971; Smith et al., 1975).

Some authors reported the relationship between the height of the fall and the severity of the injuries feline high-rise syndrome the severity of the injuries (Flagstad et al., 1998; Papazoglou et al., 2001) while some others focused severity of the traumatic injuries linearly with the height of the fall (Dupre et al.,1995).

According to popular belief, falls from a height usually happen while chasing a bird or an insect on the balcony or losing the balance and slipping on the edge of the balcony railing as well as window.

The aim of this study is statistically evaluating 50 cats which were diagnosed with FHRS between December 2021 and December 2022 in the Veterinary Emergency Hospital "Prof. univ. dr. Alin Bîrţoiu" of USAMV of Bucharest and focus on increased thoracic trauma percentages than previous reports and articles.

All kind of injuries were documented, likewise the timing of the fall (night or day time), the ground they hit (soft soil garden or concrete) to understand the severity of the injuries with thoracic trauma and also to understand if there is a relationship with height.

MATERIALS AND METHODS

In the period between December 1, 2021 and December 1, 2022 50 cats were treated after high-rise syndrome in the Veterinary Emergency Hospital "Prof. univ. dr. Alin Bîrţoiu" of University of Agronomic Sciences and Veterinary Medicine of Bucharest (USAMVB). Only the cats which felt from second and higher floors included in this study. Trauma triage was performed to all the cases. Depending on the triage further diagnostic tools were performed such as biochemical and hematological analysis. Fast thoracic (T-FAST) and fast abdominal (A-FAST) ultrasounds were performed to all patients as a general trauma approach protocol. X-rays or computed tomography (CT) performed only after patients were stabilized.

To evaluate the traumatic injuries, we used following criteria:

- Thoracic trauma, pulmonary contusions, pneumothorax, hemothorax and traumatic diaphragmatic hernia (TDH) - score 3;
- Epistaxis, craniomaxillofacial fractures, colon vertebrae fractures and dislocations, hard palate fractures and abdominal trauma with bladder rupture or urethral rupture - score 2;
- Orthopedic fractures and luxations - score 1

Based on this scoring system, each kind of injuries summed up. As an example, if a cat diagnosed with pneumothorax and pulmonary contusions with a fractured femur scored as; 3+3+1 = 7 whereas if a cat had two metatarsal fractures with fractured radius and ulna scored as; 1+1+1+1 = 4. Each fracture scored with 1 point. Thus, if a cat had more than one meta-carpal or metatarsal fractures each counted as 1.

All cats were evaluated with impact force they exposed after hitting the ground to understand the relationship between the weight of the cats and the height they felt from. At the same time, to find out the minimum impact force that can cause thoracic trauma, pneumothorax and/or pulmonary contusion. To calculate the impact force "Newton's second law of motion" is needed. This law basically describes of the changes that a force can produce on the motion of a body.

Second law of motion states that the time rate of change of the momentum of a body is equal in both magnitude and direction to the force imposed on it. The momentum of a body is equal to the product of its mass and its velocity and the equation is $F = ma$, where F (force), m (mass) and a (acceleration) are both vector quantities. Secondly the "conservation of energy" must be considered. Conservation of energy manifests that energy isn't created or destroyed, just transformed from one form into another.

The conservation of energy is needed to calculate how much kinetic energy an object has just before the point of impact. This is the energy which the object has all come from the gravitational potential it has before falling and the equation is $E = mgh$, where E is the energy, m is the mass of the object, g is the acceleration due to gravity constant *(9.81 m s $^{-2}$ or 9.81 meters per second squared)*, and h is the height the object falls from.

The other information that we know from the impact force calculation is the penetration. As an example, if an object penetrates into the ground after the impact, the impact force that it will be exposed is smaller. In another words, greater penetration implies smaller impact force while higher impact force will be exposed by the object if it hits to harder ground because hard ground means less penetration. The other information is that if the object bounces back, the impact force will be even greater because of greater chance in momentum and many cats bounce back little or more after falling and hitting the ground.

With all those information the formula will be;

$$\text{average impact force} = \frac{\text{kg} \times 9.81\ \text{m}/s^2 \times \text{m}}{\text{d}}$$

d is the average distance that the object bounces after the impact. For our study we assumed d as 0.1m = 10 cm for all the cats hit the ground.

In modern building constructions the average height of the second floors are between 4.7 and 5.8 meters depending on the construction materials used.

In this study the average of the second floor considered as 5.25 meters and each floor after second floor considered as 3 meters.

According to the average impact force formula and all the data, if a cat with 3.5 kg body

weight falls from 5th floor (14.25 meters height), the average impact force that the cat exposed will be 4887.7 Newton(s) N.

RESULTS AND DISCUSSIONS

During the defined period age ranges of the cats with HRS were between 3 months and 11 years old. It was determined that the majority of cats felt from a height were one year old or below, 44% (22/50). The oldest cat was 11 years old (Figure 1).

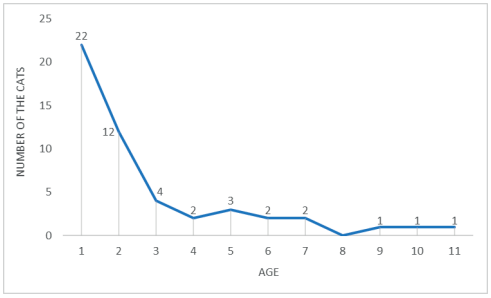


Figure 1. Age dispersion of the cats with HRS

Maximum number of the falls happened in July (13 cats), while the minimum was in January (1 cat). In March and April, we didn't receive any cats felt from balcony or window.
Remarkably 13 cats were brought to the hospital between October and January which mostly owners said that cats jumped from the window or felt from the edge of the balconies right after they arrived home and opened the windows to have fresh air in the house (Figure 2).

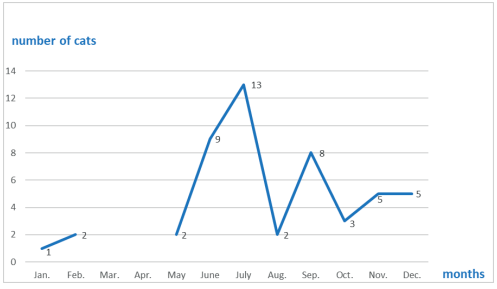


Figure 2. Number of cats and months they felt from balconies or window

46% of the cats were female (23/50), 24% were male (12/50), 10% of the cats were spayed females (5/50) and 20% of the cats were neutered males (10/50) (Figure 3).

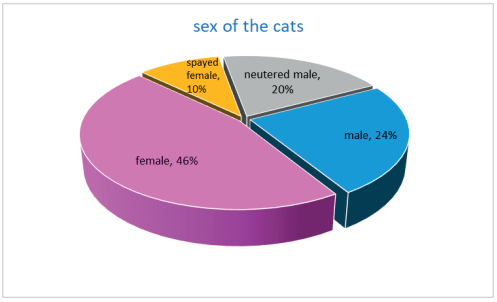


Figure 3. Sex dispersion of the cats with HRS

31 cats fell at night time while 19 cats felt during the day time. 5 neutered males felt at the night time and 5 neutered males felt in the day time. 2 male cats felt in the day time, while 10 non-neutered males felt at the night time. Similar results are also found in female cats. 3 spayed females felt in day time, while 2 spayed felt at night. 9 females felt in the day time and remarkably 14 non-spayed females felt at night (Figure 4). This valuable information helped us to understand cats' behavior. We all know that cats are night hunters by nature. Therefore, high-rise syndrome were observed mostly at night period. The other finding was that spayed and neutered cats probably were less active at night time than the non-spayed and non-neutered cats.
This also might be evidence of behavioral changes after spaying or neutralizing. Besides we learned 2 female and 1 male cats knowledge high-rise syndrome 1 year before approximately the same period.
Thoracic trauma was diagnosed in 82% (41/50) of cats. All those cats received supplemental O_2 via flow-by, mask or O_2 cabin technique due to respiratory distress, dyspnea, or cyanosis. Pneumothorax was present in 65.8% (27/41) of these cats with thoracic trauma. Thoracentesis carried out bilaterally to all those 27 cats without rushing the radiography to avoid extra stress and as well as not to worsen the respiratory situation.
Thoracentesis is our hospital's protocol for dyspneic, thoracic trauma patients due to its diagnostic and therapeutic purposes.
12h to 24h hours later, when the cats stabilized, radiographic images confirmed also the existence of pulmonary contusions in 55.5 % (15/27) of the cats which were also had pneumothorax. 24.3% (10/41) of the cats with

thoracic trauma presented only pulmonary contusions.

All the cats with pneumothorax treated with thoracentesis or chest tube placement.

Only one 9 years old 4.2 kg one female cat which felt from 8th floor was diagnosed with spontaneous pneumothorax. For this cat thoracentesis performed in each 8 h for the first 36 h and in each 12 h for the following 24 h. Atelectasis, collapsed lung lubes on the right side diagnosed via computed tomography (CT) and the lung lobectomy surgery performed via sternotomy. Unfortunately, this patient passed away one hour later the surgery in ICU. The other 1 year old, 3 kg body weight, cat which felt from 6th floor died in 30 minutes after entering to emergency room due to hemothorax. Nevertheless, the survival rate was 96% (48/50).

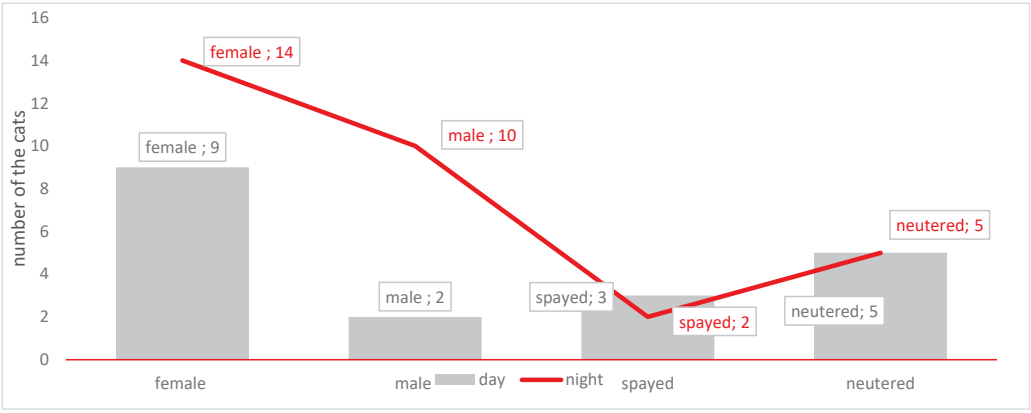


Figure 4. Cats sex dispersion and falling period relations

4% (2/50) cats were diagnosed with traumatic diaphragmatic hernia and both cats were taken to surgery 24 h to 36 h later after they came to hospital.

4% (2/50) cats had rib fractures and 1 cat clinically diagnosed with flail chest.

Thoracic trauma was diagnosed regardless of floor difference. While pneumothorax and pulmonary contusion were diagnosed together in 33.3% of the cats which felt from the 5th floor. Pulmonary contusion and pneumothorax were diagnosed together in 44.4% of the same number of the cats which felt from the 6th floor. One cat which felt from the highest floor (10th floor), diagnosed with only pneumothorax and the oldest cat (11 years old) which felt from 5th floor also diagnosed with only pneumothorax (Table 1).

Table 1. Thoracic trauma relation with the height

	Pneumothorax	Pulmonary contusions	Pneumothorax & Pulmonary contusions	Hemothorax	Flail chest	Traumatic diaphragmatic hernia	Without thoracic trauma
2nd floor	3		2				2
3rd floor		2	1				1
4th floor	2	1	2				
5th floor	2	1	3	1			2
6th floor	3		4		1		1
7th floor	1	3					3
8th floor		3	3			2	
10th floor	1						

Incidence of thoracic trauma in female cats were 86.2% (25/29) while the incidence of thoracic trauma in male cats were 76.1% (16/21).

Incidence of pneumothorax diagnosed together with pulmonary contusions in the majority of cats at 1 year old and below was 31.8% (7/22). This value was even greater than the pneumothorax and pulmonary contusion component diagnosed together in all cats in other age groups 25% (7/28).

Impact force calculated for each cat to understand the relationship between impact force and thoracic trauma.

Cats diagnosed with thoracic trauma had an average body weight of 5.7 kg and the average impact force they experienced was 2919.75 N, felt from the 2nd floor.

The average body weight of the cats which felt from 3rd floor was 4.5 kg and the average impact force they experienced was 3638.25 N. The average body weight of the cats which felt from 4th floor was 3.2 kg and the average impact force they experienced was 3555.55 N. The average body weight of the cats which felt from 5th floor was 4 kg and the average impact force they experienced was 5638.3 N. The average body weight of the cats which felt from 6th floor was 3.3 kg and the average impact force they experienced was 5530.32 N. The average body weight of the cats which felt from 7th floor was 3.8 kg and the average impact force they experienced was 7441.85 N. The average body weight of the cats which felt from 8th floor was 3.9 kg and the average impact force they experienced was 8848.15 N.

All cats evaluated with a special traumatic injuries score system. Each injury added to main thoracic trauma scores. Only 4 cats diagnosed with thoracic trauma did not present any other injuries. Rest of the cats with thoracic trauma also presented with multiple other injuries. 12% (6/50) of the cats diagnosed with hematuria. Other than hematuria, abdominal injuries not observed in 50 cats.

11 cats were hypothermic, body temperatures were between 35.1°C-36.3°C. Those cats were kept in oxygen incubators with heating pads until their body temperature turn back to normal range.

Tibia was the most frequently fractured bone (11 cats). Tibia followed by radius bone fractures (9 cats). 8 of the cats also presented with ulna fractures.

One cat had radius and ulna fractures in both legs. 8 cats diagnosed with metacarpal fractures. Those cats had more than one metacarpal bone fracture. In total 21 metacarpal fractures were diagnosed. Fractured femur diagnosed in 6 cats. 42% (21/50) of the cats diagnosed with single one bone fracture. 58% (29/50) of the cats diagnosed with multiple fractures (Table 2).

3 cats clinically diagnosed with hard palate fracture. 2 cats were diagnosed with craniomaxillofacial fractures and 1 cat with mandibular fracture. Esophagostomy feeding tubes placed 3 of those 6 cats.

Coxofemoral luxations diagnosed in 5 cats. femoral head ostectomy (FHO) performed to those cats.

Titanium implants used in all radius, tibia, femur and pelvic fractures. Only in one cat intramedullary pin used due to economic reasons of the owner.

Cats with the rib fractures treated with cage rest.

Spinal fusion surgery performed to a cat with colon vertebrae fracture. This cat hospitalized 21 days and discharged home after.

Table 2. Fractures and luxations dispersions in cats after HRS

Fractured bones and injuries	Number of cats
Metacarpal fractures	8
Radius	10
Ulna	9
Scapula	1
Craniomaxillofacial	2
Mandibula	1
Hard palate fractures	3
Rib fractures	2
Vertebral fractures	2
Pelvic fractures	4
Sacroiliac fractures /luxations	3
Coxofemoral luxations	5
Femur	6
Tibia	11
Metatarsal fractures	1

According to special traumatic injuries score system (TIS), Female cats were diagnosed with more severe injuries than male cats. Average traumatic injuries score of female cats were higher than male cats. Even though, interestingly the average TIS were greater in 1 year old and younger males than the same age range female cats (Average TIS = 6.3 male cats, age 1 and below/ Average TIS = 5.13 female cats age 1 and below). No remarkable

difference found between young and old cats with TIS (Average TIS = 5.7 in cats, 1 year old and below/ Average TIS = 5.4 in 2 years and older cats).

High-rise syndrome is seen mostly younger cats. Younger cats fall from balconies and windows whilst playing, chasing a bird or butterfly (Vnuk et al., 2004). The mean age of the cats in our study was 2.76 years. 44% (22/50) of the cats were 1 year old and below. Whitney and Mehlhaff (1987) reported that 65% of the cats were under age 3. The mean age of the cats in our study were higher then that reported by Dupre et al. (1995) - 2.5 years, Flagstad et al. (1998) - 2.3 years, Vnuk et al. (2004) - 1.8 years and even older than the mean age of the cats reported by Papazoglou et al. (2001) - 1.2 years.

Papazoglou et al. (2001) reported 51% males, 46% females, 1% neutered males, 1% spayed females and 1% unrecorded gender status. Whitney and Mehlhaff (1987) reported 48% females and 48 % males and 4% unrecorded. In that study also mentioned that 23% males were neutered and 27% females were spayed. Vnuk et al. (2004) reported 53.8% were females, 42% males, neutered males 3.4% and undetermined 0.8% in their study. In our study 46% of the cats were females, 24% males, 10% spayed females and 20% neutered males. The study of Papazoglou et al, were covering the years between 1988-1998 in Greece with the greatest percentage of males experienced high-rise were in their study (51%). The study of Vnuk et al, were covering the years between 1998-2001 in Croatia which male percentage were 24%. Our study covers one year between December 2021 and December 2022 in Bucharest, Romania with a percentage of the males 24%. In our study neutered males were 20% which were higher than the studies of Papazoglou et al. and Vnuk et al.

The decreasing percentage of males experienced high-rise syndrome may be due to increasing numbers of neutralized males in years.

Yet, we do not know the percentage of cats fell from balconies or windows in day time and/or at night from many other authors' studies. Papazoglou et al. (2001) reported the majority of the cats fell during day time in their study, while Whitney and Mehlhaff (1987) reported 40% fell at night. In our study 38% of the cats fell from high buildings in the day time and 62%, were fell at night. The nighttime fall rate of non-spayed female cats 28% (14/50) was significantly higher than the daytime fall rate of non-spayed female cats in our study 18% (9/50). The result we found was even more interesting when we made the same comparison between non-neutered male cats (4% (2/50) day time fall - 20% (10/50) night time). This differences and the higher value of night time falls, inevitably brings to mind the question of whether cats are jumping from a balcony or a window with the instinct of hunting rather than a game or sexual urge.

Thoracic trauma was diagnosed in 82% of the cats. Only pneumothorax was diagnosed in 12 cats. Only pulmonary contusion was diagnosed in 10 cats. However, 15 other cats diagnosed with pulmonary contusion and pneumothorax together. This was clear finding that the incidence of pneumothorax were 54% and 50% pulmonary contusion. Whitney and Mehlhaff (1987) diagnosed 90% of cats with thoracic trauma, pneumothorax in 63% of cats and pulmonary contusions in 68% of cats in their study. Papazoglou et al. (2001) reported thoracic trauma in only 13% of the cats, pneumothorax 4% and pulmonary contusions as 6.8% in 207 cats between 1988 and 1998 in Greece. Flagstad et al. (1998) diagnosed pneumothorax only in 7.1% of the cats in Denmark. Vnuk et al. (2004) reported 33.6% pneumothorax and 20% pulmonary contusions in 119 cases between 1998 and 2001 in Croatia. Merbl et al. (2013) reported 21.5% pneumothorax and 18.7% pulmonary contusions in 107 between 1999 and 2009 in Israel.

The percentage of pulmonary contusions were extremely higher than other authors' reports except study of Whitney and Mehlhaff (1987). Whitney and Mehlhaff recommended thoracic radiographies in all cats and thoracic radiography carried out in 69% of the cats in their study. Papazoglou et al. (2001) reported thoracic radiographies were carried out in all cats. Vnuk et al. (2004) reported thoracic radiographies carried out only in cats which were showing the abnormal respiration. In our study thoracic radiographies carried out on all cats but only after they were clinically stabilized. Besides all the pneumothorax cases

diagnosed with thoracentesis in the emergency room before radiographs. Thoracentesis is our protocol before thoracic radiographs or CT scans not to worsen the situation in dyspneic patients. Besides timing of radiographs are seriously important for diagnosis in pulmonary contusions. Because pulmonary contusions may not appear up to 6-12 h post trauma. The low appearance of pulmonary contusions in other reports may due to timing of the radiographs or due to some animals with thoracic trauma may have minimal clinical signs or even none (Aron and Roberts, 1993). In those animals, possible thoracic trauma was not diagnosed.

If we look closely to the cat numbers and the years of the reports, we can think more cats will be observed with thoracic trauma in the future, due to the change in living style, moving to higher buildings. We think that concretization in cities and the risk of cats falling on concrete grounds more frequently increased.

In addition, 27 cats diagnosed with pneumothorax in our study and 15 of those cats were also diagnosed with pulmonary contusion which the percentage were 55.5%. This strong finding reinforcing the fact that it would not be strange to suspect the presence of pulmonary contusion in cats which are clinically diagnosed with pneumothorax in HRS.

Pneumothorax and pulmonary contusions and other thoracic traumas were diagnosed in all floors that cats fell from. This clinical finding disproves the thesis that cats would have less thoracic trauma if they fall from lower floors.

Vnuk at al. (2004) reported 38,5% cats with forelimb fractures. Papazoglou et al. reported 68% of the limb fractures were forelimb fractures. Merbl et al. (2013) reported 16,8% forelimb fractures. Zaghloul and Samy (2018) reported 20% out of 45 cases between 2015 and 2018 in Egypt but not reported any thoracic trauma. Catalkaya et al. (2022) reported 22.2% forelimb fractures in 72 cases in 2019 in Turkey but also not reported any thoracic trauma, pneumothorax or pulmonary contusions. This difference may be due to their reports mostly focused on orthopedic injuries. In our study forelimb fractures percentage was 68% in total bone fractures. Unlikely the cats with hindlimb fractures, all the cats diagnosed with forelimb fractures also diagnosed with thoracic trauma, pneumothorax and/or pulmonary contusions. This interesting clinical finding makes us believe that there can be high incidence of thoracic trauma existence in the cats with forelimb fractures after high-rise syndrome.

Unlike the studies of other authors, in our study the impact force calculations were made to understand if there is a relation between thoracic trauma and the impact force that cats are experiencing while hitting the ground.

All average impact force and the average body weight, which play a role in the formation of pneumothorax and pulmonary contusion, are listed floor by floor in our study.

One of the interesting findings regarding the effect of impact force on thoracic trauma was that 2 cats with the same body weight (3.3 kg) also both felt from 8th floor in different times. Both cats clinically diagnosed with pneumothorax and pulmonary contusions. The impact force they faced was 7519 N. Another interesting finding was with the cat 4.4 kg body weight. This cat felt from 10th floor and experienced 12612,6 N impact force while hitting the ground. However, the cat diagnosed with only pneumothorax and without any orthopedic injuries. Next surprising clinical finding was the cat with 6,5kg body weight fell from 6th floor. This cat also experienced extremely high impact force, 10988.2 N and diagnosed with only fractured tibia.

Instead of these two exception cases, we do believe there might be strong effect of the impact force on thoracic trauma. But yet to prove this we need to study on many more cases.

REFERENCES

Aron, D.N., Roberts, R.E. (1993). Pneumothorax. In: Bojrab, A. (Ed.), Disease Mechanism in Small Animal Surgery. *Lea & Febiger, Philadelphia*, pp. 396e403.

Catalkaya, E., Altan, S., Ersoz-Kanay, B., Yayla, S., Saylak, N. (2022). Clinical and etiologically evaluation of cats with high-rise syndrome: assessment of 72 cases (A retrospective study). *MAE Vet Fak Derg, 7*(1):20-25,2022DOI: 0.24880/maeuvfd.957535

Dupre, G., Allenou, A., Bouvy, B. (1995). High- rise syndrome: retrospective study on 413 cats. *Veterinary Surgery 24*, 294.

Flagstad, A., Arnbjerg, J., Jensen, S.E. (1998). Feline high-rise syndrome in the greater metropolitan area of

Copenhagen. A four-year retrospective study. *The European Journal of Companion Animal Practice, 9*, 165-171.

Gordon, L.E., Thacher, C., Kapatkin, A. (1993). Highrise syndrome in dogs: 81 cases (1985-1991). *Journal of the American Veterinary Medical Association, 202*, 118-125.

Merbl, Y., Milgram, J., Moed, Y., Bibring, U., Peery, D. and Aroch, I. (2013). Epidemiological, Clinical and Hematological Findings in Feline High-Rise Syndrome in Israel: A Retrospective Case-Controlled Study of 107 Cats. *Israel Journal of Veterinary Medicine, Vol. 68*(1).

Papazoglou, L.G., Galatos, A.D., Patsikas, M.N., Savas, I., Leontides, L., Trifonidou, M., Karayianopoulou, M. (2001). High-rise syndrome in cats: 207 cases (1988e1998). *Australian Veterinary Practitioner, 31* (3), 98-102.

Reynolds, B.M., Balsano, N.A., Reynolds, F.X. (1971). Falls from heights: a surgical experience of 200 consecutive cases. *Ann. Surg., 174,* 304-310.

Robinson, G.W. (1976). The high-rise trauma syndrome in cats. *Feline Practice, 6,* 40e43.

Smith, M.D., Burrington, J.D., Woolf, A.D. (1975). Injuries in children sustained in free falls: an analysis of 66 cases. *Journal of Trauma, 15,* 987-991.

Zaghloul A. E. and Samy A. (2018). High Rise Syndrome: A Correlation Between Height and Affections In 45 Cats from Urban Areas. *Alexandria Journal of Veterinary Sciences AJVS, Vol. 59*(1): 43-48.

Whitney, W.O., Mehlhaff, C.J. (1987). High-rise syndrome in cats. *Journal of the American Veterinary Medical Association, 191*, 1399-1403.

ANIMAL PRODUCTION, PUBLIC HEALTH AND FOOD QUALITY CONTROL

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

SURVEY ON FACTORS AFFECTING HONEY BEES COLONIES IN ROMANIA: PRELIMINARY DATA

Daniela CERBU (BOANFA)[1], Gheorghe DOBRE[2], Emanuel MITREA[1], Andreea Cristina PALTIN[1], Ioan Liviu MITREA[1], Mariana IONITA[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania
[2]Romapis - Romanian Beekeeping Association, 21 N. Balcescu Blvd, District 1, 010044, Bucharest, Romania

Corresponding author email: danacerbu@yahoo.com

Abstract

The longevity of honey bees, although genetically conditioned, is impacted by numerous factors including diseases, parasites, pesticides, predators, but also by environmental and socio-economic factors. Additionally, the decrease in pollen resources reduces the queen's brood and finally the longevity of the bee colony. Therefore, a survey, based on clinical examinations in apiaries and a questionnaire completed by beekeepers was conducted in 2023 to analyze the major factors that adversely impact the honey bees colonies. For this a total of 50 beekeepers from five counties in Central and South-eastern Romania were enrolled in the study. Among the factors causing honey bee losses, diseases (including varroosis, wax moth, nosemosis, others) and unfavourable climatic conditions, such as longer dryness and cold periods, rains, or strong winds were reported. These findings emphasize on the importance of continuous monitoring, investigations, and specific control measures to be taken in order to preserve the health and activity of honey bee colonies.

Key words*: honey bee, colony survival, colony losses, Romania.*

INTRODUCTION

Honey bee - *Apis mellifera* Linnaeus 1758 (the European honey bee) - is well known as the most important pollinator of agricultural crops and natural vegetation, but as well as important producer of honney and bee-by products (Pirk et al., 2014; Bekić et al., 2014). Subsequently, the role of honey bees is vital in agriculture. For instance, bees maintain 78% of the native flora and bring revenues to the European Union of over 1.4 bilion euros, while in the USA, the California almond industry alone is worth $2 billion annually and relies on over 1 million honey bee hives for cross-pollination (Ratnieks and Norman, 2010). There are studies reporting that 52 of the 115 leading global food commodities depend on honey bee pollination for either fruit or seed set (Klein et al., 2007).

Managed honey bees are considered ideally suited for the pollination of large monocrop plantings. Subsequently, honey bees are recognized as the most important pollinator for most crop monocultures worldwide (Delaplane and Mayer, 2000; van Engelsdorp & Meixner, 2010).

However, managed honey bee populations are impacted by various and multiple factors including diseases (viral, bacterial, fungal, microsporidial (i.e. *Nosema* spp.), parasites (i.e. *Varroa destructor*), pesticides, predators, pests (i.e. moths), colony collapse disorder (CCD), but also by environment, and socio-economic factors (Morse and Flottum, 1997; Genersch, 2010; van Engelsdorp & Meixner, 2010). Each of these can act alone or in combination, and can adversely affect the productivity and survival of honey bee colonies (Oldroyd, 2007).

Bee colony loss it is a problem that it is reported worldwide (Higes et al., 2010; Lee et al., 2015; Pirk et al. 2009; Smith et al., 2013). Beekeepers knowledge of the common bees diseases and clinical and laboratory bees surveillance in order to prevent infection of new colonies are extremely useful to highlight the factors that lead to honey bee loses (Dumitru et al., 2020). Biosecurity measures in beekeeping and beekeepers knowledge regarding the risk factors are important to prevent possible sources of contamination of honeybees or honey (Borum et al., 2022).

In Romania, beekeeping is common occupation, being considered "national wealth" (Law 383/2013). It is well known the importance of continous monitoring and surveillance to identify risk factors affecting the survival and productivity of managed honey bee colonies in a particular geographical area.
Therefore, a questionnaire-based survey among beekeepers from several counties in Romania was undertaken aiming to identify major causes that lead to honey bees colonies losses and implicitly to the decrease of bee by-products, especially honey production.

MATERIALS AND METHODS

A survey based on a set of 3 questionnaires (A, B, C) elaborated by Romapis (romapis.org) (Federation of Beekeeping Associations from Romania), from which there were selected questions that were relevant to the purpose of this study, was performed. The questionnaires were distributed during of March-May 2023 period, among Romapis members and beekeepers that voluntarily answered to the questionnaires. The tree sets of questionnaires included:

(i) Questionnaire A - with questions for highlighting the management of the apiary and health status of bees:

- How often do you inspect the apiary?
- Extreme weather conditions in 2022?
- Do you keep bees stationary or do you migrate with your bees?
- Do you buy/sell biological materials (queens, bees swarm, bees colonies)?
- Which pathological conditions (diseases, parasites, pests, others) noticed in your colonies in recent year?
- Have you requested consultancy from a veterinarian?
- Have you treated against any disease your apiary or used antibiotics treatments?
- Did you feed your bees (sugar, old honey or proteic food)?
- Do you participate in apicultural fairs, conferences or meetings?
- How many bee colony you had at July 31, 2022 and how many did you have at the beginnig of winter?

(ii) Questionnaire B - on bee products:

- type of flora for harvesting;
- bee-products obtained.

(iii) Questionnaire C:

- movement investigations of bee colonies during 2013-2022.

The questionnaires were collected and answers were introduced into a database using Excel Microsoft spreadsheet software for analysis.

RESULTS AND DISCUSSION

Results

In order to identify risk factors that lead to losses in Romanian bee colonies, a questionnaire based survey was undertaken. For this, a total of 50 beekeepers from 32 localities and five counties (Brasov, Prahova, Giurgiu, Valcea, Ialomita) in Center and Southestern Romania were enrroled in the study.
The main results are presented by each questionnaire, as following.

(i) For the Questionnaire A

- Regarding the apiary's management: of the 50 beekeepers enrolled in the study, the majority (52%; n = 26), answered that they inspect the apiary weekly, while 36% (n = 18) daily, and 12% (n = 6) at more than 2 weeks (Figure 1). Also, 26% (13/50) of the surveyed beekeepers are taking notes in the apiary's notebook.

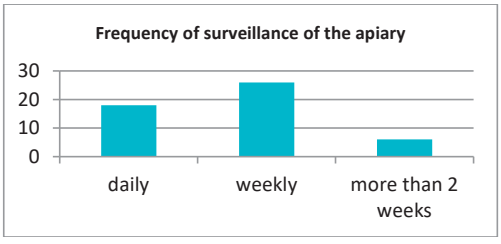


Figure 1. Frequency of surveillance of the apiary (answers from 50 beekepers in five counties from Center and Southern Romania)

- With regards to registering extreme weather conditions in 2022, the following were reported: draught (74%; n = 34), long cold period or rain (50, and strong wind (26%) (Figure 2).

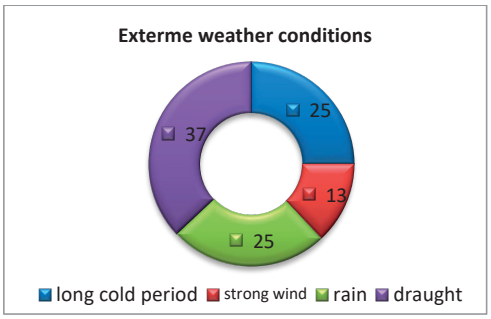


Figure 2. Extreme weather conditions registered in 2022 (answers from 50 beekepers in five counties from Center and Southern Romania)

○ With regards to the beekeeping operational type, 18 beekeepers (36%) were migrating with their bees to other types of harvesting than from the originating area.

○ Another important factor followed was the exchange of biological material (bees, queens, swarms) between apiaries: 32% (n = 16) of the beekepers reported the practice of these exchanges, 62% (n = 31) no practiced, and 3 did not answer.

○ Amongst the pathological conditions notticed in the surveyed apiaries, the most reported was varoosis (80% of the beekeepers noticed it), followed by the wax moth and Chalkbrood/Stonebrood, reported by 29 (58%) and 27 (54%), respectively beekeepers, while other conditions were reported by lower frequency, such as nosemosis (22.0%) or American/European foulbrood (4%). Details are presented in Table 1.

Other pathological conditions (i.e. paralysis, malformations) were observed by 14.0% (n = 7) of the beekeepers.

Table 1. Pathological conditions noticed by beekeepers (number of apiaries)

Pathological condition	American/ European foulbrood	Nosemosis	Varoosis	Galleriosis	Chalkbrood/ Stonebrood
Positive apiaries	2	11	40	29	27
Negative apiaries	41	37	10	18	17
No answer	7	2	0	3	6

○ Collaboration with a veterinarian was mentioned by 31 (62.0%) of the questioned beekeepers, while 12 (34.0%) answered no, and 7 did not answer this question.

○ Honey bee treatments: all of the surveyed beekeepers stated that they used antivarroa treatment, 32% (n = 16) treated for nosemosis. The antibiotic treatment was mentioned by 8% (n = 4) of the beekeepers.

○ Regarding feeding techniques, 41 (82%) of the beekeepers specified that they had to feed the bee families, 31 (62%) of them also using old recovered honey.

○ Assessing the beekeepers level of information concerning good practices in beekeeping, 35 (70.0%) answered that they do participate to bee conferences or beekeeping fairs, while 15 of them had never participated of any instruction regarding apiculture or beekeeping.

○ Regarding the number of colonies monitored on July 31 in 2022, 24 of the 50 surveyed answered, summarizing a total of 2043 colonies, of which 1919 colonies were introduced at winter.

(ii) For the Questionnaire B - on the harvesting flora type and bee-products obtained, answers were collected from 40 apiaries located in two counties (Prahova, n = 16 and Brasov, n = 24).

○ The survey showed that the main type of flora for harvesting was acacia (87.5%), followed by sunflower and rapeseed (about 50%), linden, meadow, others (from 30% to 22%) (Table 2).

Table 2. Harvesting flora type in surveyed apiaries from Central and Southeastern of Romania

Flora type	Number of apiary, by originating county		Total (n = 40)	
	BV*	PH*	No.	%
Acacia	22	13	35	*87.5*
Rapessed	8	10	18	*45.0*
Sunflower	14	*9*	23	*57.5*
Linden	10	*2*	12	*30.0*
meadow	7	*2*	*9*	*22.5*
Others	9	*1*	*10*	*25.0*
Fruit trees	2	1	*3*	*7.5*
Mint	0	*1*	*1*	*2.5*

*BV: Brasov county; PH: Prahova county

○ In terms of bee-by products obtained, other than honey, the most reported were swarms, fertilized queens and wax (Table 3).

Table 3. The bee-by products obtained in the surveyed apiaries, in the 2022 year (stratified by county)

Bee-by product		Honney	Swarms	Fertilized queens	Wax	Propolis	Pollen
Total	**n**	**40**	**27**	**26**	**26**	**19**	**5**
	%	***100***	***67.5***	***65.0***	***65.0***	***47.5***	***12.5***
BV	**n**	24	21	21	17	13	2
	%	*100*	*87.50*	*87.50*	*70.8*	*54.14*	*8.33*
PH	**n**	*16*	*6*	*5*	*9*	*6*	*3*
	%	*100*	*37.5*	*31.3*	*56.3*	*37.5*	*18.7*

(ii) For the Questionnaire C

We investigated the movement of bees colonies during 2013-2022 year and if the beekeepers sold or bought bees families. However, since not all of them recorded those movements in the apiary book, not all 50 beekeepers investigated were able to complete that the questionnaire C; some of them were new beekeepers.

Therefore, the answers were collected from 36 beekeepers, one of them having apiary only since 2022. The details are presented in Table 4.

Table 4. Movement of the bee colonies over the last 10 years (survey of 36 beekeepers) from two counties, Romania

Year		2013	2014	2015	2016	2017	2018	2019	2020	2021	2022
No. colonies	BV	792	1024	1087	1249	1570	1706	1660	1811	1974	2014
	PH	610	605	315	701	789	792	810	891	815	1058
No. beekepers	BV	14	14	15	16	18	20	21	21	23	23
	PH	9	9	9	9	9	10	10	11	10	13
	total	23	23	24	25	27	30	31	32	33	36

Discussion

Through this questionnaire based survey aiming for investigating potential causes that lead to honeybees colonies losses and subsequently decrease of bee by-products it was revealed that the apiaries enrolled in the survey were affected by various, commonly reported factors, including management practices, diseases, but also enviromental and climatic conditions. These are in agreement with recent studies reporting on influence of the season and bee technologies on the epidemiology of bee diseases, in Romania (Dumitru et al., 2020).

It is well known that regular apiary surveillance is vital for early detection of any signs of illness. Additionally, implementing a biosecurity plan and keeping records are imperative in preventing colonies loss.

As mentioned, in Romania, beekeeping is a an occupation practiced by most beekeepers in a stationary form, apiaries with 26-76 colonies being the most common, according to data provided in 2016 (romapis.org). This fact was confirmed also in our survey, where 64% of the 50 beekeepers were not migrate with their bees. Beekeeping migration, if the sanitary-veterinary norms in force and good beekeping practices are observed, is very beneficial both for bees and economically, in terms of honey production; also, the mortality is slightly lower than in stationary apiaries (Lee et al., 2015). However, there are some studies that include beekeeping migration in terms of losses in bee colonies (Pirk et al., 2014).

By means of QA we noticed that not all beekeepers implement the rules of biosecurity in beekeeping. This could be also influenced also by the operation type. It is acknowledged that recent data about the operation type in a particular geographical area is of high relevance to better document and identify potential causes of colony losses. In a study, Lee et al (2015) surveying the annual colony losses in the USA, showed that beekepers tend to have different management practicess, according to the by operation type. Therefore, backyard beekeepers tend to be stationary, have fewer colonies, and manage less rigorously (Lee et al., 2015).

Also, the apiary's notebook should also include notes on climatic conditions, as climate changes affect flora and implicitly the bees by decreasing pollen resources and longevity of bee colonies (Jones et al., 2021), as showed also in the present survey.

Extreme weather conditions in the 2022 year in many counties of Romania led to a decrease in honey production by up to 45% acording to ACA, Romanian Beekeepers Association.

Biological material trades are one among the main factors for the emergence and

dissemination of diseases (Mutinelli, 2011), especially if the biological material, mainly queen bees, are not accompanied by health certificates (Borum, 2022).

The main bee pathological conditions, such as nosemosis, varroosis, galleriosis, American/ European foulbrood, Chalkbrood/Stonebrood were reported also in the present survey. Therefore, introduced, monitoring and proper measure for their control, must be implemented, as they can can cause high mortality and subsequently clonoy losses (Mitrea, 2011).

Collaborating with a veterinarian and the request for veterinary services is necessary for the prevention and early detection of any possible bee disease (Kyle et al., 2021).

Sampling and analyzing bees or bee-by products must be part of the biosecurity plan in each beekeeping farm (Mitrea, 2002).

Medicinal residues in honey and hive by-products would be significantly reduced if the treatments instituted were diminished, especially to avoid parasites drug resistance (Mitrea, 2002) a desideratum that can be achieved through beekeepers education (Jacques et al., 2017). In this regards, some reports on essential oils efficacy or other compunds against honey bees nosemosis, due to their antiseptic properties, in order to obtain residue-free bee products (Chioveanu et al., 2004; Dumitru et al., 2017, 2018).

In 2022, long periods of drought were reported, followed by periods of cooler temperatures than the average of 2022. Thus, beekeepers specified that they had to feed the bee families out of necessity, due to lack of harvesting, using old recovered honey this signals another potential factor incriminated in the loss of colonies, by perpetuating certain diseases, such as nosemosis (Dumitru et al., 2018; Salkova et al., 2022).

Beekeeping health status monitoring of the apiaries by implementing good beekeeping practices, declaring and registering with the County animal laboratory and official veterinarians is a moral and legal obligation of apiary owners, due to possible thefts between compliant and unregistered apiaries. All this, along more rigorous information of beekeepers, can greatly reduce losses among bee colonies.

Regarding the number of colonies monitored on July 31, 2022, only 24 of the 50 surveyed answeredș these 24 apiaries belong to the Fagaras Country area; the result showed a loss over 6%. It is well known that documenting colony losses is critical to characterise the losses into broad frame and to identify potential causes of mortality, especially in different areas.

Also from the 40 beekeepers originating form Brasov and Prahova counties we managed to centralize the bee-by products obtained during the studied year (in Questionnaire B). However, Apilarnil and royal jelly, although extremely beneficial to health, were not among the products targeted by beekeepers the surveyed areas.

Refusal to answer regarding the diseases reported in apiaries, along with the confirmation of 30% of beekeepers regarding the fact that they do not participate in beekeeping fairs, conferences or counsels, highlights the fact that the beekeeping sector in Romania still has many gaps in term of biosecurity and good practices in beekeeping, those affecting honey bees colonies.

An increased number of bee colonies in the last decade along with the decrease in honey production shows that the increased density of apiaries for commercial purposes may contributes to the spread of diseases. Recent surveys show an increasing trend in beekeepers number, although bees colonies number worldwide is far from being sufficient to ensure entomophilous cultivated plants areas pollination. Industrial agriculture involves large areas requireing plants pollination, bees providing 80% of their pollination (Borum et al., 2022) in addition to entomophilous plants specific to each area, being ranked as one of the main causes leading to bee colonies loss (Shanahan, 2022).

CONCLUSIONS

The findings of the present study emphasize the importance of continuous monitoring, investigations, and specific control measures to be taken in order to preserve the health and longevity of honey bee colonies. Additionally, it is showed the need for implementing up-to-date information programs regarding beekeeping for beekeepers in Romania.

ACKNOWLEDGEMENTS

We thanks to all beekeepers that participated and answered to the questionnaire.

REFERENCES

Bekić, B., Jeločnik, M., Subić, J. (2014). Honey bee colony collapse disorder (*Apis mellifera* L.) possible causes. *Scientific Papers. Series "Management, Economic Engineering in Agriculture and Rural Development",* 14 (2), PRINT ISSN 2284-7995, 13-18.

Borum, A.E. (2022). Biosecurity and good beekeeping practices in beekeeping. *Uludag Bee Journal*, 22(2): 246-76.https://doi.org/10.31467/uluaricilik.1175874.

Chioveanu, G., Ionescu, D., Mardare, A. (2004). Control of nosemosis-Treatment with "Protofil"; *APIACTA,* 39: 31-38.

Delaplane, K S; Mayer, D F (2000). *Crop pollination by bees*. CABI Publishing; New York, USA.

Dumitru, A., Chioveanu, G., Ionita, M., Dobre, G., & Mitrea, I. L. (2017). "In vitro" studies on using natural essential oils in treatment of nosemosis in honeybees: Determination of the therapeutic Dose. *Sci. Work. Vet. Medic. Ser. C*, *63*, 165-170.

Dumitru, A., Chioveanu, G., Ionita, M., Dobre, G., & Mitrea, I. L. (2018). *In vitro* trial on using amprolium clorhidrat to control Nosema infection in honeybees. *Sci. Works Ser. C Vet. Med*, *64*, 111-116.

Dumitru, A.S., Chioveanu, G., Dobre, G., Ioniță, M., Mitrea, I.L. (2020). Evolution of nosemosis in the apiary: influence of the season and bee technologies. *AgroLife Scientific Journal*, *9*(1).

El Agrebi, N., Steinhauer, N., Tosi, S., Leinartz, L., de Graaf, D.C., Saegerman, C. (2021). Risk and protective indicators of beekeeping management practices. *Science of the Total Environment* 799:149381. doi: 10.1016/j.scitotenv.2021.149381.

Genersch E., von der Ohe W., Kaatz H., Schroeder A., Otten C., Büchler R, Berg S, Ritter W., Mühlen W., Gisder S., Meixner M, Liebig G., Rosenkranz P. (2010). The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. *Apidologie.* doi:10.1051/apido/2010014

Higes, M., Martín-Hernández, R., Martínez-Salvador, A., Garrido-Bailón, E., González-Porto, A.V., Meana, A., Bernal, J.L., Del Nozal, M.J., Bernal, J. (2010). A preliminary study of the epidemiological factors related to honey bee colony loss in Spain. *Environmental Microbiology Reports* 2(2):243-250.

Jacques, A., Laurent, M.; EPILOBEE Consortium; Ribière-Chabert, M., Saussac, M., Bougeard, S., Budge, G.E., Hendrikx, P., Chauzat, M.P. (2017). A pan-European epidemiological study reveals honey bee colony survival depends on beekeeper education and disease control. *PLoS One* 9;12(3):e0172591. doi: 10.1371/journal.pone.0172591.

Jones, L., Brennan, G.L., Lowe, A., Creer, S., Ford, C.R., de Vere, N. (2021). Shifts in honeybee foraging reveal historical changes in floral resources. *Communication Biology* 14; 4(1): 37. doi: 10.1038/s42003-020-01562-4.

Klein A-M, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T (2007). Importance of pollinators in changing landscapes for world crops. *Proc R Soc B* 274: 303–313.

Kyle, B., Lee, K., Pernal, S.F. (2021). Epidemiology and Biosecurity for Veterinarians Working with Honey bees (*Apis mellifera*). *Veterinary Clinics North America Food Animimal Practice* 37(3): 479-490. doi: 10.1016/j.cvfa.2021.06.004.

Lazar, S., Dolis, M. (2004). *Apicultura practica.* Editura ALFA, Iasi, 220.

Lee, K.V., Steinhauer, N., Rennich, K. et al. (2015). A national survey of managed honey bee 2013–2014 annual colony losses in the USA. *Apidologie* 46, 292–305 doi.org/10.1007/s13592-015-0356-z.

Mitrea, I.L. (2002). Controlul parazitologic – concept biologic, medical si economic. *Scientia Parasitologica*, 1, 79-89.

Mitrea, I.L., (2011). *Parazitologie si boli parazitare*. Editura Ceres Bucuresti 590-594.

Morse, R.A, Calderone, N.W. (2000). The value of honey bees as pollinators of U.S. crops in 2000. *Gleanings Bee Culture* Suppl., pp. 1–15.

Mutinelli, F. (2011). The spread of pathogens through trade in honey bees and their products (including queen bees and semen): overview and recent developments. *Revue Scientifique et Technique,* 30(1): 257-71. doi: 10.20506/rst.30.1.2033.

Oldroyd, B.P. (1999). Coevolution while you wait: *Varroa jacobsoni*, a new parasite of western honeybees. *Trends Ecol Evol* 14: 312–315.

Pietropaoli, M., Ribarits, A., Moosbeckhofer, R., Köglberger, H., Alber, O., Gregorc, A., Smodiš Škerl, M.I., Presern, J., Bubnič, J., Necati Muz, M., Higes, M., Tiozzo, B., Jannoni-Sebastianini, F., Lubroth, J., Cazier, J., Raizman, E., Zilli, R., Della Marta, U., Formato, G. (2021). Biosecurity measures in European beekeeping. *Revue scientifique et technique (International Office of Epizootics).* 39(3).

Pirk, C.W.W., Human, H., Crewe, R.M., vanEngelsdrop, D. (2014). A survey of managed honey bee colony losses in the Republic of South Africa - 2009 to 2011. *Journal of Apicultural Research* 53(1): 35-42 .

Ratnieks Francis L. W., Carreck Norman L. (2010). Clarity on Honey Bee Collapse? The worldwide losses of honey bee colonies continue to puzzle researchers and the beekeeping industry. *Science,* 327(5962): 152-153. DOI: 10.1126/science.1185563

Salkova, D., Shumkova, R., Balkanska, R., Palova, N., Neov, B., Radoslavov, G., Hristov, P. Molecular (2021). Detection of *Nosema* spp. in Honey in Bulgaria. *Veterinary Sciences* 28; 9(1): 10. doi: 10.3390/vetsci9010010.

Shanahan, M. (2022). Bees and Industrial Agriculture: What Researchers are Missing, and Why it's a Problem. *Journal of Insect Science* 1; 22(1): 14. doi: 10.1093/jisesa/ieab090.

Smith, K.M., Loh, E.H., Rostal, M.K., Zambrana-Torrelio, C.M., Mendiola, L., Daszak, P. (2013). Pathogens, pests, and economics: drivers of honey bee colony declines and losses. *Ecohealth* 10(4): 434-45. doi: 10.1007/s10393-013-0870-2.

van Engelsdorp, M.D. Meixner (2010). A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *Journal of Invertebrate Pathology* 103, S80–S95.

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

COMPARATIVE STUDY OF SOME TRACE ELEMENTS AND MACROMINERALS IN PIG LEG DEPENDING ON THE COOKING METHOD

Gheorghe Valentin GORAN, Emanuela BADEA, Carmen Daniela PETCU, Oana Diana MIHAI, Liliana TUDOREANU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independenței, District 5, 050097, Bucharest, Romania

Corresponding author email: gheorghe.goran@fmvb.usamv.ro

Abstract

A comparative study of trace elements and macrominerals in pork can provide valuable information about how the safety and nutritional level of the meat is affected by the cooking method. This study aimed to assess the effects of three cooking methods (roasting, boiling, and microwaving) on the mineral composition of pig leg. In this study, minerals' concentration in raw and cooked pork samples were determined by ICP-OES. Roasting, boiling, and microwaving can lead to different effects on the minerals and the toxins present in the meat. The studied cooking methods influenced the mineral composition and nutritional value in cooked pig leg samples compared to raw ones, with impact on the minerals' intake. Generally, macromineral levels increased in cooked pork samples and trace elements decreased, with roasting improving the mineral nutritional value of pig leg.

Key words: *pig leg, trace elements, macrominerals, cooking method.*

INTRODUCTION

Meat plays a crucial role in providing the organism with essential minerals and nutrients (De Smet & Vossen, 2016). It is a significant dietary source that provides the body with vital minerals, such as iron, zinc, and selenium, essential in maintaining overall health and preventing various diseases. Meat can be a valuable part of a healthy diet when consumed in moderation and alongside a variety of other nutrient-rich foods. However, there are risks associated with excessive meat consumption (Giromini & Givens, 2022; Pereira & Vicente, 2013).

The quality of food has been negatively impacted by pollution, which has become a major source of exposure for people to harmful minerals (Chen et al., 2022; Wang et al., 2018). As a result, it is important to consider the accumulation of metallic pollutants in animal tissues, as well as the potential impact of toxic metals on essential metal levels in animals raised in industrialized areas (Chałabis-Mazurek et al., 2021; Shahjahan et al., 2022).

Some cooking methods can lead to the loss of nutrients, while others can increase the bioavailability of these nutrients. Overall, proper thermal preparation of meat is important for both food safety and nutritional quality (Rao et al., 2022; Suleman et al., 2020). The cooking method can affect the level of trace elements and macro-minerals in meat. The degree of mineral loss in cooked food is influenced by various factors such as cooking method and food type. Roasting is observed to result in less mineral loss compared to boiling, especially for foods of the same type. (Biel et al., 2019; Goran et al., 2016; Oz et al., 2016).

The pink meat of pork is a popular and widely consumed meat globally, providing essential nutrients such as protein, vitamins, and minerals (Biesalski, 2005).

The objective of this study is to investigate how the mineral levels of pork leg are affected by three distinct cooking methods, namely roasting, boiling, and microwaving. This study can provide valuable insights into how to prepare pork in a way that preserves its nutritional value and contributes to overall health and wellbeing.

MATERIALS AND METHODS

Samples of pork leg were obtained from the gluteus muscle of crossbred barrows farmed in

Romania. The barrows were between 6-8 months old and weighed approximately 100 kg. The meat samples were cut into similar square dimensions (approximately 2*2*2 cm pieces), weighed, labelled, and packed in temperature-resistant food plastic bags. Each bag contained four samples of 20 g ± 5% each. A total of 48 samples were divided into four groups: boiled, roasted, microwave irradiated, and raw. For each cooking method, the time for cooking was estimated after several tests to achieve edible samples. The bags were positioned in the centre of the electric/microwave oven tray/plate for roasting and microwave cooking, with no contact between the meat samples and oven tray/plate. The samples were then cooled for 5 hours at an ambient temperature of 20°C, after which the liquid was discarded, and the samples were stored at 6°C for 24 hours. Before grinding using the knife mill, raw and cooked samples at 6°C were drained off.

0.5 g (wet weight - ww) was taken from each sample and digested using a Berghof Spedwave MWS-2 microwave oven as follows: Step 1: 120°C, 50% power; Step 2: 180°C, 75% power; Step 3: 100°C, 40% power.

The digested samples were diluted with ultrapure water to a volume of 25 mL and analyzed using a Thermo iCAP ICP-OES spectrometer (RF1100 W). The spectrometer had a reading time of 30 seconds and a washing time of 30 seconds, with a nebulizer gas flow of 0.5 L/min, an auxiliary gas flow of 0.5 L/min, and a sample injection pump flow of 50 rpm. Calibration curves were created using standard solutions of 0.001 ppm, 0.01 ppm, 0.1 ppm, 1 ppm, 5 ppm, 10 ppm, and 50 ppm, prepared by diluting a multi-element ICP MERCK standard that contained 1000 mL/L of Al, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, Pb, Se, Sr, and Zn. The concentrations of minerals that were below the method detection limit are not reported in this study.

Non parametric statistical analysis was performed for the obtained data using SPSS software. The Kruskal-Wallis test by ranks was used to determine whether there were significant differences between the levels of all analyzed mineral concentrations based on the cooking method. The difference between groups was considered to be statistically significant when P value was below 0.05, and it was highly significant when P value was below 0.001.

RESULTS AND DISCUSSIONS

Table 1 presents the average mineral levels of both raw and cooked pork leg. Among the 20 elements tested (Al, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, Pb, Se, Sr, and Zn), only Al, Ba, Ca, Cu, Fe, K, Li, Mg, Na, Sr, and Zn were detected at levels above the limit of detection, and significant differences were observed between the various cooking methods for K, Fe, and Zn. The primary macro-minerals present in both raw and cooked meat were K (with average levels ranging from 267.115-370.431 ppm), followed by Na (with average levels ranging from 27.035-44.848 ppm), and Mg (with average levels ranging from 22.064-27.187 ppm).

Table 1. Total mineral levels* (ppm ww) in pork leg samples after different cooking method preparation

Element	Cooking method	Mean	Std Dev	Std Err Mean	*p*-value
Al	Raw	0.425	0.1313	0.0758	0.668
	Boiled	0.424	0.2574	0.1486	
	Roasted	0.559	0.0866	0.0500	
	Microwaved	0.467	0.0689	0.0398	
Ba	Raw	0.194	0.1033	0.0596	0.117
	Boiled	0.346	-	-	
	Roasted	0.049	0.0354	0.0251	
	Microwaved	-	-	-	
Ca	Raw	5.026	0.7488	0.4323	0.121
	Boiled	5.911	0.4473	0.2582	
	Roasted	6.573	0.7554	0.4361	
	Microwaved	6.530	0.7621	0.4400	

Cu	Raw	0.191	0.1068	0.0617	0.536
	Boiled	0.240	0.3393	0.2399	
	Roasted	0.144	0.0842	0.0486	
	Microwaved	0.064	0.0410	0.0236	
Fe	Raw	4.126 [a]	3.0095	1.7375	**0.022**
	Boiled	1.136 [b]	0.3985	0.2301	
	Roasted	1.751	0.2568	0.1483	
	Microwaved	1.446 [b]	0.0573	0.0331	
K	Raw	267.115 [a]	32.7415	18.9033	**0.049**
	Boiled	305.780	11.5961	6.6950	
	Roasted	370.431 [b]	55.2186	31.8805	
	Microwaved	293.281	14.7102	8.4929	
Li	Raw	0.518	0.0876	0.0506	0.183
	Boiled	0.459	0.2819	0.1627	
	Roasted	0.341	0.0718	0.0415	
	Microwaved	0.272	0.0562	0.0325	
Mg	Raw	22.064	3.1389	1.8122	0.369
	Boiled	23.507	1.1136	0.6429	
	Roasted	27.187	3.8062	2.1975	
	Microwaved	23.420	1.0497	0.6060	
Na	Raw	27.035	2.4004	1.3858	0.094
	Boiled	36.130	2.8528	1.6470	
	Roasted	44.848	10.6204	6.1317	
	Microwaved	33.666	4.2989	2.4820	
Sr	Raw	0.245	0.0560	0.0323	0.101
	Boiled	0.470	-	-	
	Roasted	0.221	0.0867	0.0500	
	Microwaved	0.095	0.0758	0.0437	
Zn	Raw	1.068 [a]	0.0387	0.0224	**0.050**
	Boiled	1.679	0.2964	0.1711	
	Roasted	1.864	0.1277	0.0737	
	Microwaved	1.994 [b]	0.2305	0.1331	

*Levels connected by different letters are significantly different. Comparison can't be made between different elements levels.

The mineral composition is influenced by various factors, including but not limited to species, breed, gender, age, muscle type, diet, genetics, and cooking method (Goran et al., 2016; Lebret & Čandek-Potokar, 2022; Tornberg, 2005; Wereńska et al., 2022).

Cooking of meat typically leads to higher macro-mineral levels compared to raw samples due to water loss during cooking. The extent of mineral loss depends on the cooking method and the solubility of the minerals. On the other hand, levels of Zn and Al were generally lower in raw samples than in cooked meat, independent of the cooking method.

The statistical analysis of the minerals' concentrations of the pork cooked by different methods showed no significant influence on the overall pork leg nutritional value (Table 1). However, the level of Zn was found to be significantly higher in microwaved meat compared to the raw samples.

The mean level of Fe showed significant differences after cooking ($p < 0.05$). Pork is known as one of the main sources of heme Fe, which is the form of Fe with the highest bioavailability (Menezes et al., 2018). Previous studies have indicated that the levels of soluble heme Fe generally decrease from raw meat to cooked meat, depending on the increasing cooking temperature (Goran et al., 2016; Purchas et al., 2004). Similarly, in the present study, the Fe levels in pork showed statistically significant decreases after cooking, regardless of the cooking type. This may be due to the difference between the heme Fe levels of haemoglobin and myoglobin origin, which are soluble and insoluble heme Fe, respectively (Cabrera et al., 2010).

The mean Zn values significantly increased ($p < 0.05$) in cooked pork samples compared to raw meat. Moreover, the different cooking methods used for pork showed significant differences ($p < 0.05$), with microwaved pork samples having the highest average level of Zn. These significant differences in Zn mean level in cooked pork samples were likely due to an

increase in the insoluble Zn fraction in denatured proteins in pork leg (Goran et al., 2016; Menezes et al., 2018).

The K level increased significantly ($p < 0.05$) after roasting, which may be attributed to the reduction of K leaching into the cooking water, potentially induced by the use of temperature-resistant plastic bag cooking. Previous research has indicated that dry cooking methods can result in increased mineral level due to water evaporation (Borela et al., 2022; Goran et al., 2016; Omojola et al., 2015). The average levels of the other studied macro-minerals Ca, Mg, and Na were higher in roasted pork, but not significantly different compared to raw pork samples, results which are consistent to their lower leach into lost cooking liquids.

Al, Ba, Cu, Li, and Sr levels in pork cooked samples, did not register statistically significant differences between raw and cooked samples. The average levels of Al in roasted pork were higher than in raw pork samples, but the difference was not statistically significant. Ba level in microwaved samples could not be observed due to average levels below analysis method detection limits.

The mean levels of Cu and Sr in cooked pork samples showed an increase in boiled samples, but the differences were not significant. The mean level of both trace minerals in the other cooked pork samples decreased, but not significantly when compared to the average levels found in raw meat samples. A higher level of Sr was observed in boiled pork samples, while it decreased in the other cooked samples. This trend was inversely related to the lower levels of Ca in boiled samples, which increased in the other cooked samples. This is likely because Sr shares similar properties with Ca (Goran et al., 2016; Zhao et al., 2016). There were no significant ($p > 0.05$) differences observed in the average Li levels in cooked pork samples, with the highest levels being found in raw pork samples and the lowest levels in the microwaved pork samples.

CONCLUSIONS

The chemical composition and nutritional value of pork can be influenced by different cooking methods, which in turn can impact the intake of essential mineral nutrients. Thermal preparation has been shown to affect the levels of Fe, K, and Zn in cooked pork samples compared to raw ones, with significant differences observed in boiled and microwaved samples for Fe levels, and in microwaved samples for Zn levels. Regarding macro-elements, only roasted samples showed significant differences, specifically for K. Roasting appears to be the most effective pork cooking method for improving the mineral nutritional value of the meat. This information can serve as a recommendation for consumers to choose the most effective cooking method for maintaining or improving the nutritional quality of pork.

REFERENCES

Biel, W., E, C.-P., & A, K. (2019). Offal Chemical Composition from Veal, Beef, and Lamb Maintained in Organic Production Systems. *Animals (Basel), 9*(8), 489. doi:10.3390/ani9080489

Biesalski, H. (2005). Meat as a component of a healthy diet – are there any risks or benefits if meat is avoided in the diet? *Meat Science, 70*(3), 509–524. doi:10.1016/j.meatsci.2004.07.017

Borela, V., de Alencar, E., Mendonça, M., Han, H., Raposo, A., Ariza-Montes, A., . . . Zandonadi, R. (2022). Influence of Different Cooking Methods on Fillet Steak Physicochemical Characteristics. *Int. J. Environ. Res. Public Health, 19*, 606. doi:10.3390/ijerph19010606

Cabrera, M., Ramos, A., Saadoun, A., & Brito, G. (2010). Selenium, copper, zinc, iron and manganese level of seven meat cuts from Hereford and Braford steers fed pasture in Uruguay. *Meat Science, 84*(3), 518–528. doi:10.1016/j.meatsci.2009.10.007

Chałabis-Mazurek, A., Valverde Piedra, J., Muszyński, S., Tomaszewska, E., Szymańczyk, S., Kowalik, S., . . . Schwarz, T. (2021). The Concentration of Selected Heavy Metals in Muscles, Liver and Kidneys of Pigs Fed Standard Diets and Diets Containing 60% of New Rye Varieties. *Animals (Basel), 11*(5), 1377. doi:10.3390/ani11051377

Chen, L., Zhou, M., Wang, J., Zhang, Z., Duan, C., Wang, X., . . . Fang, L. (2022). A global meta-analysis of heavy metal(loid)s pollution in soils near copper mines: Evaluation of pollution level and probabilistic health risks. *Sci Total Environ, 835*, 155441. doi:10.1016/j.scitotenv.2022.155441

De Smet, S., & Vossen, E. (2016). Meat: The balance between nutrition and health. A review,. *Meat Science, 120*, 145-156. doi:10.1016/j.meatsci.2016.04.008.

Giromini, C., & Givens, D. (2022). Benefits and Risks Associated with Meat Consumption during Key Life Processes and in Relation to the Risk of Chronic Diseases. *Foods, 11*(14), 2063. doi:10.3390/foods11142063

Goran, G., Tudoreanu, L., Rotaru, E., & Crivineanu, V. (2016). Comparative study of mineral composition of beef steak and pork chops depending on the thermal preparation method. *Meat Science, 118*, 117-121. doi:10.1016/j.meatsci.2016.03.031

Lebret, B., & Čandek-Potokar, M. (2022). Review: Pork quality attributes from farm to fork. Part I. Carcass and fresh meat. *Animal, 16*(Supplement 1), 100402. doi:10.1016/j.animal.2021.100402

Menezes, E., Oliveira, A., França, C., Souza, G., & Nogueira, A. (2018). Bioaccessibility of Ca, Cu, Fe, Mg, Zn, and crude protein in beef, pork and chicken after thermal processing. *Food Chem, 240*, 75-83. doi:10.1016/j.foodchem.2017.07.090

Omojola, A., Ahmed, S., Attoh-Kotoku, V., & Wogar, G. (2015). Effect of cooking methods on cholesterol, mineral composition and formation of total heterocyclic aromatic amines in Muscovy drake meat. *J Sci Food Agric., 95*(1), 98-102. doi:10.1002/jsfa.6687

Oz, F., Aksu, M., & Turan, M. (2016). The Effects of Different Cooking Methods on Some Quality Criteria and Mineral Composition of Beef Steaks. *Journal of Food Processing and Preservation, 41*(4), e13008. doi:10.1111/jfpp.13008

Pereira, P., & Vicente, A. (2013). Meat nutritional composition and nutritive role in the human diet. *Meat Science, 93*(3), 586-592. doi:10.1016/j.meatsci.2012.09.018

Purchas, R., Rutherfurd, S., Pearce, P., Vather, R., & Wilkinson, B. (2004). Cooking temperature effects on the forms of iron and levels of several compounds in beef semitendinosus muscle. *Meat Science, 68*, 201–207.

Rao, J., Meng, F., Li, Y., Chen, W., Liu, D., & Zhang, J. (2022). Effect of cooking methods on the edible, nutritive qualities and volatile flavor compounds of rabbit meat. *J Sci Food Agric., 102*(10), 4218-4228. doi:10.1002/jsfa.11773

Shahjahan, M., Taslima, K., Rahman, M., Al-Emran, M., Alam, S., & Faggio, C. (2022). Effects of heavy metals on fish physiology - A review. *Chemosphere, 300*, 134519. doi:10.1016/j.chemosphere.2022.134519

Suleman, R., Wang, Z., Aadil, R., Hui, T., Hopkins, D., & Zhang, D. (2020). Effect of cooking on the nutritive quality, sensory properties and safety of lamb meat: Current challenges and future prospects. *Meat Science, 167*, 108172. doi:10.1016/j.meatsci.2020.108172

Tornberg, E. (2005). Effects of heat on meat proteins – Implications on structure and quality of meat products. *Meat Science, 70*(3), 493–508. doi:10.1016/j.meatsci.2004.11.021

Wang, M., Zhang, R., He, K., & Chen, L. (2018). Concentrations and health risks of heavy metals in vegetables from typical farmland in the Pearl River Delta, South China. *Ecotoxicology and Environmental Safety, 147*, 548-556. doi:10.1016/j.ecoenv.2017.09.029

Wereńska, M., Haraf, G., Okruszek, A., Marcinkowska, W., & Wołoszyn, J. (2022). The Effects of Sous Vide, Microwave Cooking, and Stewing on Some Quality Criteria of Goose Meat. *Foods, 12*(1), 129. doi:10.3390/foods12010129

Zhao, Y., Wang, D., & Yang, S. (2016). Effect of organic and conventional rearing system on the mineral level of pork. *Meat Science, 118*, 103-107. doi:10.1016/j.meatsci.2016.03.030

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

COMPARATIVE STUDY ON THE VARIATION OF THE SERUM CORTISOL LEVEL DEPENDING ON THE CATTLE SLAUGHTERING METHOD

Oana Diana MIHAI, Carmen Daniela PETCU, Dana TĂPĂLOAGĂ, Corina PREDESCU, Gheorghe Valentin GORAN, Elena MITRĂNESCU, Emanuela BADEA, Oana Mărgărita GHIMPEȚEANU, Emilia CIOBOTARU-PÎRVU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independenţei, District 5, 050097, Bucharest, Romania

Corresponding author email: carmenpetcufmvb@gmail.com

Abstract

Over time, meat has played an essential role in human evolution and is an important component of a healthy and balanced diet, a fact due to the nutritional richness that varies depending on a number of factors. Pre-slaughter stress is a crucial factor in meat quality and safety. Animals intended for slaughter are stressed by a variety of endogenous and exogenous factors. Slaughter is a complex process, and there is clear evidence in the literature, that pre-slaughter stress is harmful to the meat quality. Therefore, the purpose of stunning is to render animals unconscious during bleeding, without causing pain or stress. In some countries, cattle are slaughtered by a religious method, without stunning, namely the halal slaughter practiced by Muslims and the kosher slaughter practiced by Jews. The study was carried out during 2020-2022, on two batches of conventionally slaughtered cattle (with stunning), on one batch of halal slaughtered cattle (without stunning and on one batch of traditionally slaughtered cattle (without stunning). Within the slaughterhouses, the technological flow of slaughtering cattle was followed and blood samples were collected in order to extract serum and dose cortisol. Cortisol was dosed in a specialized laboratory using the immunoenzymatic method with chemiluminescence detection. Comparing the analyzed batches, it can be seen that higher average values of the cortisol level were recorded in the batches slaughtered in the traditional halal system, compared to the conventionally slaughtered batches. The values recorded for all four batches, exceed the reference range of 0.47-0.75 µg/dL. Excessive handling of cattle induces their stress, therefore special attention must be paid to the rest period before slaughter, to physiologically rebalance the body, but also to the slaughtering process, to minimize stress levels and ultimately improve meat quality obtained, because it has been shown that there is a direct correlation between the quality of the meat and the way the animals are slaughtered, more precisely with stunning or without stunning.

Key words*: cattle, cortisol, halal, slaughter, stress.*

INTRODUCTION

Meat has played an essential role in human evolution and represent an important component of a balanced and healthy diet, a fact due to its nutritional richness (Williamson et al, 2005; McNeill & Van Elswyk, 2012; Pereira & Vicente, 2012; Petcu, 2013; Predescu et al., 2018; Mihai et al., 2021).

The nutritional composition of meat varies depending on a number of factors, such as: breed, sex, age, diet, fattening status, body weight, animal health, rational feeding, animal movement, but also on the slaughtering method, here thinking to slaughter with stunning (conventional) or slaughter without stunning (traditional, halal, kosher) (Williams, 2007; Banu et al., 2009).

In the living organism, a series of energetic and biochemical transformations occur, which are in close interdependence and which stop with the suppression of the animal's life (Ionescu & Diaconescu, 2010; Mihai et al., 2021).

Stress is defined as a complex cascade of events, consisting of a stimulus - stressor, which causes a subsequent reaction in the brain - stress perception and activates physiological reactions - stress response (Dhabhar & McEwen, 1997; Ciliberti et al., 2017).

Acute stress lasts from a few minutes to several hours, and chronic stress persists several hours a day, for weeks or months (Dhabhar, 2002; Ciliberti et al., 2017).

The increasing global commercialization of beef has led to an increase in the number of animals slaughtered (Blokhuis et al., 2008),

resulting in animal welfare issues, related to transport to the slaughterhouse and handling before slaughter have worsened (Miranda de la Lama et al., 2014; Pérez-Linares et al., 2015).
Due to the increasingly frequent change in the mentality of consumers and their desire to consume only safe food that does not have a negative impact on their own health, on the environment, nor on the way animals are reared, cattle welfare has become a social issue and broadened the concept of food quality (Savu et al., 2002; María, 2006; Sepúlveda et al., 2008; Pérez-Linares et al., 2015; Petcu, 2015).
Pre-slaughter stress is a crucial factor in meat production. Animals destined for slaughter are stressed by a variety of endogenous and exogenous factors, which adversely affect complex post-mortem biochemical reactions (Franco et al., 2015). The most common factors are: weather changes, transport conditions, how the animals are handled, the waiting period before slaughter, the stunning procedure and the method of slaughter (Apple, 2005).
The magnitude of any negative effect depends on the type, duration and intensity of the stressors, before the animal is slaughtered, and how susceptible it is to them (Ferguson et al., 2001; Ferguson & Warner, 2009; Pérez-Linares et al., 2015).
These pre-slaughter adverse effects not only have an impact on animal welfare, but also have negative effects on meat quality. Particularly in cattle, there is evidence that pre-slaughter stress is detrimental to meat quality (Ferguson, 2008). These adverse effects on beef quality can lead to Dark Firm Dry (DFD) meat (Franco et al., 2015; Pérez-Linares et al., 2015).
In the period before slaughter, stress and physical activity contribute to the depletion of muscle glycogen and, as a result of this increased pH, the beef changes its appearance, and can be classified as DFD (dark, firm and dry) (Pérez-Linares et al., 2015).
DFD meat is highly susceptible to bacterial problems due to the increased pH ($\geq$ 5.8) (Van de Water et al., 2003). It is difficult to market DFD meat, because the consumer associates its dark colour with improper storage conditions or assumes that the beef is outdated (Mounier et al., 2006).
The slaughter process is quite complex, as it is characterized by several stressful stages, caused by numerous factors. Consequently, the purpose of stunning is to render animals unconscious during bleeding without causing pain or stress. In many countries, however, it is a common practice to slaughter cattle by a religious method, practiced without stunning, and here we refer to the halal slaughter practiced by Muslims and the kosher slaughter practiced by Jews (Önenç & Kaya, 2004; Barrasso, 2021).
Based on available data, it has been shown that almost 26 million Muslims and 1.1 million Jews live in the European Union. Thus, animals slaughtered according with religious rituals are reared in the EU (Europe's Growing Muslim Population, 2017).
During slaughter, animal stress can be physical, psychological, or both physical and psychological (Lawrie, 1966; Barrasso, 2021).
The physiological response to stress involves the secretion of stress hormones such as cortisol and catecholamines (adrenaline and noradrenaline) (Linares et al., 2008; Terlouw et al., 2021).
Cortisol determination is one of the most widely used methods for assessing stress in animals. It can be dosed from blood (serum or plasma), saliva, urine, faeces, milk and hair (Casal et al., 2017).

MATERIALS AND METHODS

The study was conducted in the period 2020-2022 on two batches of conventionally slaughtered cattle (with stunning), on one batch of halal slaughtered cattle (without stunning) and on one batch of traditionally slaughtered cattle (without stunning). Blood samples were collected as follows:
- Batch 1: 15 blood samples collected in November 2020 (cold season), from a batch of 100 cattle, different breeds, slaughtered after stunning, in a slaughterhouse.
- Batch 2: 15 blood samples collected in October 2021, from a batch of 60 cattle, different breeds, slaughtered without stunning in a slaughterhouse, by the halal method, specific to Muslims.
- Batch 3: 15 blood samples collected in August 2022 (warm season), from a batch of 98

cattle, different breeds, slaughtered after stunning, in a slaughterhouse.
- Batch 4: 6 blood samples collected in December 2022 (cold season), from traditionnally slaughtered cattle.
In the slaughterhouses, the technological flow of slaughtering cattle was followed and blood samples were collected.
In the case of **conventional slaughter**, the cattle enter the adduction corridor and are mechanically stunned, using a stun gun with a captive bolt, positioned at the level of the head. Dividing the skull to find the ideal location for penetrating the brain is done imaginary, by drawing two lines starting from the eye to the opposite horn. Immediately after the stunning, hanging on the conveyor line takes place, and the bleeding stage will take place in the shortest possible time.
In the case of **halal slaughter**, the cattle are slaughtered without stunning. Each cattle is placed in the individual rotating containment box and the designated person in the slaughterhouse performs the sectioning of the blood vessels in one movement, using a sharp knife, not before saying *Bismillah*.
In the case of **traditional slaughter**, cattle are slaughtered without stunning.
The present study aims to carry out laboratory analyzes aimed at measuring the level of cortisol in blood samples (Figure 1), collected at the time of bleeding (approximately 9 ml of blood collected in a BD Vacutainer - Clot Activator Tube). The blood samples were identified by labelling and transported immediately to a specialized laboratory, cortisol being dosed by the Immunoenzymatic method with chemiluminescence detection.

Figure 1. Blood samples collected from cattle slaughtered in the slaughterhouse

In the present study, cortisol was assayed from serum (Figure 2) obtained from blood samples collected from the bleeding wound.
To determine these parameters, specialized training and laboratory equipment, as well as specific materials and reagents, are necessary.

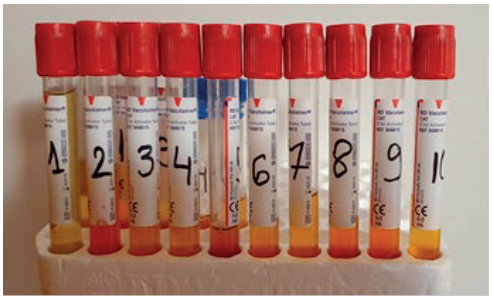


Figure 2. Serum samples used for cortisol dosing

RESULTS AND DISCUSSIONS

It has been shown that there is a direct correlation between the way animals are slaughtered (with or without stunning) and the stress during slaughter, which directly influences the meat quality.
Also, the period of slaughter, in direct correlation with the temperature of the environment, can represent different types of stress for the animal.
How animals react to these stressors, depends on their individual emotional reactivity (Deiss et al., 2009).

Results and discussions regarding blood cortisol levels

It has been shown that stress before slaughter has a negative impact on the hormonal system of animals and implicitly on the meat quality (D'Eath et al., 2010; Mihai et al., 2021).
One of the most used methods for assessing stress in animals is the determination of cortisol, because it provides information about the activity of the hypothalamic-pituitary-adrenal axis (Casal et al., 2017; Mihai et al., 2021).
The determination of the cortisol level in the blood samples collected after the slaughter of the cattle, highlighted different values, which exceed the reference interval, established by Jackson and collaborators in 2002, namely 0.47-0.75 μg/dL.

Study 1 - determination of cortisol in blood samples collected from conventionally slaughtered cattle in November 2020 (Conventional 1)

Following the analysis of the cortisol level of the 15 blood samples collected from conventionally slaughtered cattle in November 2020, it was observed that all samples had higher values compared to the reference interval (0.47-0.75 µg/dL).

The results obtained after the cortisol dosage from the samples of batch 1 are presented in Table 1.

Table 1. Results obtained after cortisol dosing in cattle slaughtered in the conventional system from batch 1 (Conventional 1)

No.	Slaughtering date	Sex	Age	Cortisol level µg/dL
1.	19.11.2020	F	14 years	**1.64**
2.	19.11.2020	F	3 years	**1.61**
3.	19.11.2020	F	4 years	**1.02**
4.	19.11.2020	F	10 years	**3.87**
5.	19.11.2020	F	5 years	**1.10**
6.	19.11.2020	F	4 years	**2.53**
7.	19.11.2020	F	6 years	**4.15**
8.	19.11.2020	F	14 years	**4.98**
9.	19.11.2020	F	3 years	**7.73**
10.	19.11.2020	F	13 years	**3.82**
11.	19.11.2020	F	5 years	**2.92**
12.	19.11.2020	F	13 years	**4.13**
13.	19.11.2020	F	3 years	**4.34**
14.	19.11.2020	F	4 years	**2.15**
15.	19.11.2020	F	10 years	**2.35**

Study 2 - determination of cortisol from blood samples collected from halal slaughtered cattle in October 2021 (Halal)

In October 2021, 15 blood samples collected from cattle slaughtered in a halal system in a slaughterhouse were analyzed in a specialized laboratory. All immunoassay cortisol values were above the reference interval. The lowest value recorded was 1.46 µg/dL, and the highest value 7.57 µg/dL, the accepted reference interval being 0.47-0.75 µg/dL.

Sample number 1 recorded a value 10 times higher compared to the maximum reference value. The results are presented in Table 2.

Table 2. Results obtained after cortisol dosing in cattle slaughtered in the halal system from batch 2 (Halal)

No.	Slaughtering date	Sex	Age	Cortisol level µg/dL
1.	11.10.2021	M	4 years	**7.57**
2.	11.10.2021	F	3 years	**7.34**
3.	11.10.2021	F	4 years	**5.26**
4.	11.10.2021	M	5 years	**2.82**
5.	11.10.2021	M	5 years	**1.46**
6.	11.10.2021	F	9 years	**3.27**
7.	11.10.2021	F	8 years	**6.12**
8.	11.10.2021	F	3 years	**5.20**
9.	11.10.2021	M	9 years	**6.98**
10.	11.10.2021	F	5 years	**7.10**
11.	11.10.2021	F	9 years	**2.27**
12.	11.10.2021	F	8 years	**5.02**
13.	11.10.2021	F	3 years	**5.20**
14.	11.10.2021	M	9 years	**4.68**
15.	11.10.2021	F	5 years	**6.10**

Study 3 - determination of cortisol in blood samples collected from conventionally slaughtered cattle in August 2022 (Conventional 2)

Following the analysis of the cortisol level of the 15 blood samples collected from conventionally slaughtered cattle in August 2022, it was observed that all samples had higher values compared to the reference interval (0.47-0.75 µg/dL). Sample number 9 recorded a value 7 times higher compared to the reference interval, namely 5.55 µg/dL.

It is found that animals slaughtered in the warm season recorded lower cortisol values, which are slightly closer to the reference interval, compared to animals slaughtered in the cold season, which recorded higher values.

The results obtained after the cortisol dosage from the samples of batch 3 are presented in Table 3.

Table 3. Results obtained after cortisol dosing in cattle slaughtered in the conventional system from batch 3 (Conventional 2)

No.	Slaughtering date	Sex	Age	Cortisol level µg/dL
1.	30.08.2022	F	10 years	**1.20**
2.	30.08.2022	F	4 years	**2.22**
3.	30.08.2022	F	5 years	**1.66**
4.	30.08.2022	F	5 years	**1.98**
5.	30.08.2022	F	5 years	**1.07**
6.	30.08.2022	F	12 years	**1.91**
7.	30.08.2022	F	9 years	**3.49**
8.	30.08.2022	F	14 years	**1.17**
9.	30.08.2022	F	8 years	**5.55**
10.	30.08.2022	F	13 years	**2.44**
11.	30.08.2022	F	3 years	**1.15**

No.	Slaughtering date	Sex	Age	Cortisol level µg/dL
12.	30.08.2022	M	3 years	**1.19**
13.	30.08.2022	F	3 years	**1.35**
14.	30.08.2022	F	10 years	**3.23**
15.	30.08.2022	F	5 years	**1.51**

Study 4 - determination of cortisol in blood samples collected from traditionally slaughtered cattle in December 2022 (Traditional)

Following the analysis of the cortisol level of the 6 blood samples collected from traditionally slaughtered cattle in December 2022, it was observed that all samples had higher values compared to the reference interval (0.47-0.75 µg/dL). Sample number 1 recorded 9.10 µg/dL, the value exceeding 12 times the accepted reference maximum value, established by Jackson & Cockcroft. The results obtained after the cortisol dosage from the samples of batch 4 are presented in Table 4.

Table 4. Results obtained following cortisol dosing in cattle slaughtered in the traditional system from batch 4 (Traditional)

No.	Slaughtering date	Sex	Age	Cortisol level µg/dL
1.	14.12.2022	M	8 months	9.10
2.	14.12.2022	M	10 months	6.14
3.	14.12.2022	M	8 months	7.00
4.	14.12.2022	M	8 months	4.30
5.	14.12.2022	F	12 months	4.14
6.	14.12.2022	M	8 months	5.22

Results and discussions regarding the statistical analysis of the data

The results obtained from the summary statistics (mean values, standard deviation, standard error of the mean, median, maximum and minimum values) of serum cortisol samples collected from slaughtered animals are presented in Figure 3.

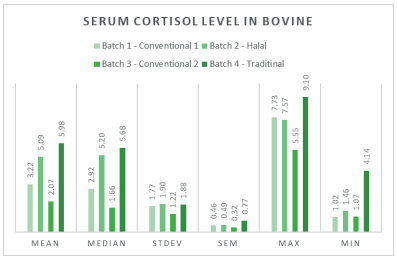


Figure 3. Summary statistics of serum cortisol level in bovine (mean values, median, standard deviation, standard error of the mean, max. and min. values) (µg/dL)

Analysing the four batches statistically, following an ANOVA one-way analysis of variance test, using the GraphPad Prism Statistical Software, it resulted that there were significant differences within the cortisol levels recorded for all the batches ($P<0.05$) (Figure 4).

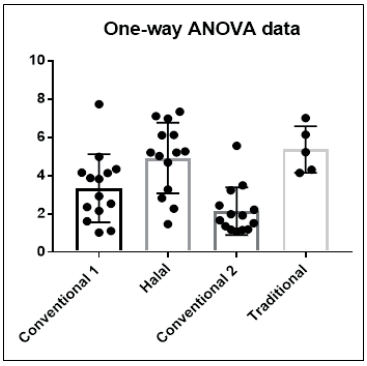


Figure 4. Summary statistics of ANOVA one-way analysis

Comparing the results obtained, it can be observed that higher values of the cortisol level were recorded in the batches slaughtered in the traditional and halal system, compared to the batches slaughtered conventionally.

The first batch was slaughtered in November, the second batch in October, the third batch in August and the fourth batch in December.

The highest cortisol value was recorded in one of the traditionally slaughtered calves.

According to numerous studies, an important factor that influences the stress level of animals is the environment temperature (Guerrini & Bertchinger, 1982; Linares et al., 2008; Deiss et al., 2009; Salaberger, 2016; Mihai et al., 2021).

The values recorded for all four batches exceed the reference interval of 0.47-0.75 µg/dL.

Handling operations before animal slaughter, certainly induce a stress response, affecting the condition of the animal (Śmiecińska et al., 2011). Optimal preparation of animals before slaughter, by ensuring rest immediately after transport, alleviates stress and physiologically balances the organism (Gispert et al., 2000; Fischer, 2001; Śmiecińska et al., 2011; Petcu, 2015).

Recent studies in northwestern Mexico, reported an incidence of DFD meat during the warm season (summer), and concluded that both pre-slaughter and post-slaughter factors contribute

to the occurrence of DFD meat. The resting period before slaughter, the relative humidity of the environment and the time elapsed between slaughters were relevant factors during the pre-slaughter period, while the chilling temperature and the time spent by the carcass in storage areas were found to be important post-slauthering factors (Pérez-Linares et al., 2008; Sotelo-Flores, 2008; Leyva-García et al., 2012; Pérez-Linares et al., 2015).

In order to determine the significance of the differences between the experimental groups, the t-test (student test) was applied.

The results obtained from the summary statistics of t-test (Student test) for all pairwise comparisons are presented in Table 5.

Table 5. Summary statistics of serum cortisol level in bovine - t-test (Student test)

Batches analyzed	P - value	Significantly different (P < 0.05)	Mean ± SEM (Standard error of the mean)
Conventional 1 - Halal	0.0095	Yes	3.223 ± 0.458, n=15 5.093 ± 0.4915, n=15
Conventional 1 - Conventional 2	0.0483	Yes	3.223 ± 0.458, n=15 2.075 ± 0.3151, n=15
Conventional 1 - Traditional	0.005	Yes	3.223 ± 0.458, n=15 5.983 ± 0.7656, n=6
Halal - Conventional 2	< 0.0001	Yes	5.093 ± 0.4915, n=15 2.075 ± 0.3151, n=15
Halal - Traditional	0.3431	No	5.093 ± 0.4915, n=15 5.983 ± 0.7656, n=6
Conventional 2 - Traditional	< 0.0001	Yes	2.075 ± 0.3151, n=15 5.983 ± 0.7656, n=6

Batch 1 slaughtered conventionally in November and batch 2 slaughtered halal obtained statistically significant differences (P < 0.05) (Figure 5).

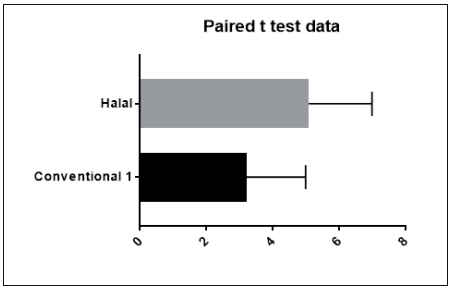


Figure 5. Mean value between batch 1 and batch 2

Batch 1 slaughtered conventionally in November and batch 3 slaughtered conventionally in August obtained statistically significant differences (P < 0.05) (Figure 6). The samples of batch 3 were collected in the warm season (August), compared to the samples of batch 1, which were collected in the cold season (November).

Batch 1 recorded a mean cortisol value of 3.22 µg/dL, a value that is higher compared to the mean cortisol value recorded for batch 3, namely 2.07 µg/dL. Both batches of animals were conventionally slaughtered in the same slaughterhouse, the difference in results correlating, most likely with the season in which these animals were slaughtered.

The time of harvesting samples may be an explanation for the fact that samples collected from animals slaughtered in the summer recorded lower cortisol levels, compared to samples collected from animals slaughtered in the late autumn, a fact that is also explained by Guerrini and Bertchinger in their studies, showing that the lowest values of plasma cortisol were recorded during the exposure of the animals to a warm environment, and the highest values were recorded when the animals were exposed to a cool and humid environment.

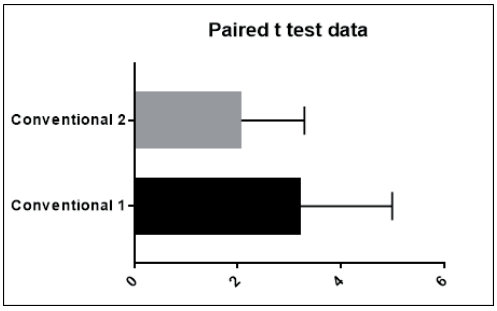


Figure 6. Mean value between batch 1 and batch 3

Batch 1 slaughtered conventionally in November and batch 4 slaughtered traditionally obtained statistically significant differences ($P < 0.05$) (Figure 7).

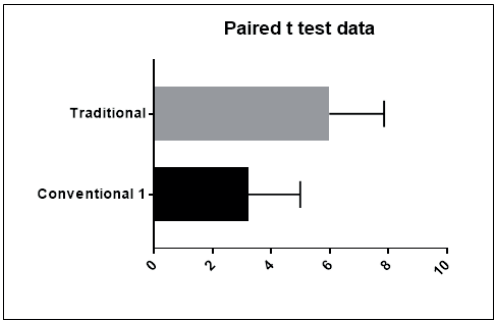


Figure 7. Mean value between batch 1 and batch 4

Batch 2 slaughtered halal and batch 3 slaughtered conventionally in August obtained statistically significant differences ($P < 0.05$) (Figure 8).

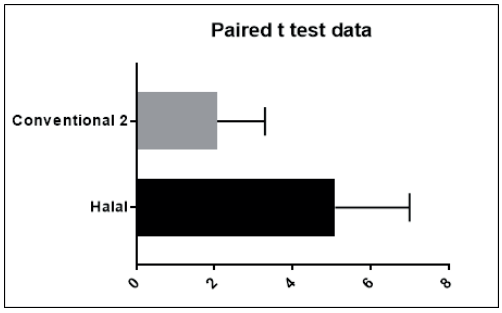


Figure 8. Mean value between batch 2 and batch 3

Batch 2 slaughtered halal and batch 4 slaughtered tradutionally obtained statistically insignificant differences ($P > 0.05$).

Both groups were slaughtered without stunning.

The increased level of cortisol is an indicator of the animals' stress response, resulting from the stimulation of the hypothalamic-pituitary-adrenal axis and the sympathetic and parasympathetic nervous system (Śmiecińska et al., 2011).

The mean cortisol value of batch 2 is 5.09 µg/dL, this batch being represented by cattle slaughtered halal in October.

Batch 4, represented by calves traditionally slaughtered in December, recorded an average value of 5.98 µg/dL (Figure 9).

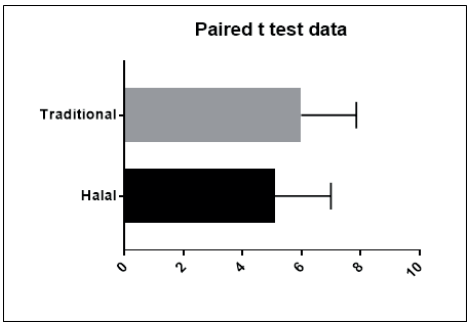


Figure 9. Mean value between batch 2 and batch 4

Batch 3 slaughtered conventionally in August and batch 4 slaughtered tradutionally obtained statistically significant differences ($P < 0.05$) (Figure 10).

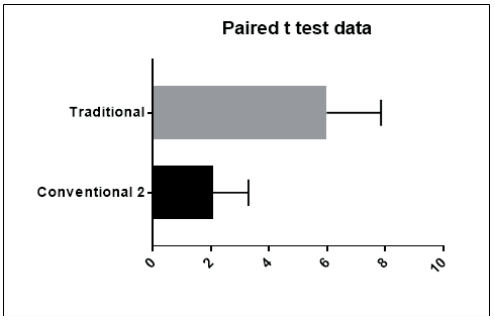


Figure 10. Mean value between batch 3 and batch 4

CONCLUSIONS

In the study slaughterhouses, all stages of the technological flow of slaughtering animals are respected, both for conventionally slaughtered (with stunning) and for traditionally and halal slaughtered (without stunning).

The refusal of the animals to enter the containment box and the accidental fall of the animals on the adduction corridor were not observed.

The method of stunning, practiced in conventional slaughter is mechanical stunning with a captive bolt gun.

Excessive handling of cattle induces their stress, therefore special attention must be paid to the rest period before slaughter, to physiologically balance the body, but also to the slaughtering process, to minimize stress levels and ultimately improve meat quality obtained.

Samples collected from cattle slaughtered in the conventional system obtained a lower average value of cortisol compared to the average value obtained for blood samples collected from traditionally and halal slaughtered cattle, a fact that most likely correlates with the way the animals are reared, with the individual reactivity of each animal, with the transport stress and with the season in which the animals were slaughtered.

Following the statistical analysis of the four batches, analyzed by the ANOVA one-way test it resulted that there were significant differences within the cortisol levels recorded for all the batches ($P < 0.05$).

REFERENCES

Apple, J.K., Kegley, E.B., Galloway, D.L., Wistuba, T.J., Rakes, K.L. (2005). Duration of restraint and isolation stress as a model to study the dark-cutting condition in cattle. *J Anim Sci*, 83(5): 1202-1214.

Banu, C., Alexandru, A., Bârsan, I.G., Bărăscu, E, Bordei, D., Bulancea, M., Croitor, N., Gyemant, A., Hopulele, T., Ionescu, A., Iordan, M., Jantea, C., Nour, V., Panuţuru, D., Păsat, G., Răsmeriţă, D., Săhleanu, V., Stoica, A., Stoicescu, A., Stroia, A., Tofan, I. (2009). *Tratat de indusrie alimentară. Tehnologii alimentare.* Editura ASAB, Bucureşti.

Barrasso, R., Ceci, E., Tufarelli, V., Casalino, G., Luposella, F., Fustinoni, F., Dimuccio, M., Bozzo, G. (2021). Religious slaughtering: Implications on pH and temperature of bovine carcasses. *Saudi Journal of Biological Sciences.* https://doi.org/10.1016/j.sjbs.2021.12.002, ISSN 1319-562X.

Blokhuis, H.J., Keeling, L.J., Gavinelli, A., Serratosa, J. (2008). Animal welfare's impact on the food chain. *Trends Food Sci Tech 2*, 19(Supl 1): S79-S87.

Casal, N., Manteca, X., Peña, R., Bassols, A., Fàbrega, E. (2017). Analysis of cortisol in hair samples as an indicator of stress in pigs. *Journal of Veterinary Behavior*, 19: 1-6.

Ciliberti, M.G., Albenzio, M., Inghese, C., Santillo, A., Marino, R., Sevi, A., Caroprese, M. (2017). Peripheral blood mononuclear cell proliferation and cytokine production in sheep as affected by cortisol level and duration of stress. *J. Dairy Sci.*, 100(1): 750–756.

D'Eath, R.B., Turner, S.P., Kurt, E., Evans G., Thölking, L., Looft, H., Wimmers, K., Murani, E., Klont, R., Foury, A., Ison, S.H., Lawrence, A.B., Mormède, P. (2010). Pigs' aggressive temperament affects pre-slaughter mixing aggression, stress and meat quality. *Animal*, 4(4): 604-616.

Deiss, V., Temple, D., Ligout, S., Racine, C., Bouix, J., Terlouw, C., Boissy, A. (2009). Can emotional reactivity predict stress responses at slaughter in sheep? *Applied Animal Behaviour Science*, 119(3–4):193-202.

Dhabhar, F.S., and B.S. McEwen. (1997). Acute stress enhances while chronic stress suppresses immune function in vivo: A potential role for leukocyte trafficking. *Brain Behav. Immun*. 11: 286–306.

Dhabhar, F.S. (2002). Stress-induced augmentation of immune function - The role of stress hormones, leukocyte trafficking and cytokines. *Brain Behav. Immun.*, 16: 785–798.

Ferguson, D.M., Bruce, H.L., Thompson, J.M., Egan, A.F., Perry, D., Shorthose, W.R. (2001). Factors affecting beef palatability – Farmgate to chilled carcass. *Australian Journal of Experimental Agriculture*, 41: 879–891.

Ferguson, D.M., Warner, R.D. (2009). Have we underestimated the impact of pre-slaughter stress on meat quality in ruminants? *Meat Science*, 80(1):12-9.

Fischer, K. (2001). Fleischfehler mussen nicht sein. *Fleischwirtschaftm* 10: 21-24.

Franco, D., Mato, A., Salgado, J.F., López-Pedrouso, M., Carrera, M., Bravo, S., Parrado, M., Gallardo, J.M., Zapata, C. (2015). Tackling proteome changes in the longissimus thoracis bovine muscle in response to pre-slaughter stress. *Journal of Proteomics*, 122: 73-85, ISSN 1874-3919, https://doi.org/10.1016/j.jprot.2015.03.029.

Gispert, M., Faucitano, L., Oliver, M.A., Guardia, M.D., Coli, C., Siggens, K., Harvey, K., Diestre, A. (2000). A survey of pre-slaughter conditions, halothane gene frequency in five Spanish pig commercial abattoirs. *Meat Sci.,* 55: 97-106.

Guerrini, V.H., Bertchinger, H. (1982). Effect of ambient temperature and humidity on plasma cortisol in sheep. *British Veterinary Journal*, 138(2): 175-182.

Ionescu, E., Diaconescu, C. (2010). *Procesarea şi conservarea unor produse de origine animală-aspecte chimice şi biochimice.* Editura Fundaţiei de mâine, Bucureşti.

Jackson, P.G.G., Cockcroft, P.D. (2002). Appendix 3 Laboratory Reference Values: Biochemistry. *Blackwell Science Ltd*, 303-305.

Lawrie, R. (1966). *Metabolic stresses which affect muscle.* Physio Biochem. Muscle Food, pp. 137-164.

Leyva-García, I.A., Figueroa-Saavedra, F., Sánchez-López, E., Pérez-Linares, C., Barreras-Serrano, A. (2012). Impacto económico de la presencia de carne DFD en una planta de sacrificio Tipo Inspección Federal. *Arch Med Vet*, 44: 39-42.

Linares, M.B., Bórnez, R., Vergara, H. (2008). Cortisol and catecholamine levels in lambs: Effects of slaughter weight and type of stunning. *Livestock Science*, 115(1): 53-61.

McNeill, S., Van Elswyk, M. E. (2012). Red meat in global nutrition, Meat Science, 92(3): 166–173.

Mihai, D.O,, Petcu, C.D., Tăpăloagă, D., Predescu, C., Ghiţă, M., Ghimpeţeanu, O.M., Murariu, O.C., Ciobotaru-Pîrvu, E. (2021). Comparative study on the variation of cortisol level in blood serum depending on swine slaughtering method. *The International Conference "Agriculture for Life, Life for Agriculture", Bucharest, 3-5 of June 2021, Scientific Papers. Series D. Animal Science.* Vol. LXIV, No. 2, 351-358. ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

Miranda De La Lama, G.C., Villaroel, M., María, G.A. (2014). Livestock transport from the perspective of the pre-slaughter logistic chain: a review. *Meat Sci*., 98(1): 9-20.

Mounier, L., Dubroeucq, H., Andanson, S., Veissier, I. (2006). Variations in meat pH of beef bulls in relation to conditions of transfer to slaughter and previous history of the animals. *J Anim Sci*, 84(6): 1567-1576.

Önenç, A., Kaya, A. (2004). The Effects Of Electrical Stunning And Percussive captive bolt stunning on meat quality of cattle processed by Turkish slaughter procedures. *Meat Sci*., 66: 809-815, DOI: 10.1016/S0309-1740(03)00191-8.

Pereira P.M.C.C., Vicente A.F.R.B. (2012). Meat nutritional composition and nutritive role in the human diet, *Meat Science*, 93(3): 586–592.

Pérez-Linares, C., Barreras, S.A., Sánchez, L.E., Herrera, B., Figueroa-Saavedra, F., (2015). The effect of changing the pre-slaughter handling on bovine cattle DFD meat. *Rev.MVZ* Cordoba, 20(3), ISSN 0122-0268.

Petcu, C.D., (2013). Researches concerning some meat products control in a specialized unit. *Scientific Papers. Series D. Animal Science*, Vol.LVI, pag. 323-325, ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN-L 2285-5750.

Petcu, C.D. (2015). *Calitatea și tehnologia cărnii.* Editura Granada, București.

Predescu, C., Papuc, C., Petcu, C., Goran, G., Rus, A.E., (2018). The Effect of Some Polyphenols on Minced Pork during Refrigeration Compared with Ascorbic Acid. *Bulletin UASVM Food Science and Technology*, 75(1): 36-42. ISSN-L 2344-2344; Print ISSN 2344-2344; Electronic ISSN 2344-5300

Salaberger, T., Millard, M., Makarem, S.E., Möstl, E., Grünberger, V., Krametter-Frötscher, R., Wittek, T., Palme, R. (2016). Influence of external factors on hair cortisol concentrations. *General and Comparative Endocrinology*, 233: 73-78.

Savu, C., Petcu, C.D. (2002). Igiena și controlul produselor de origine animală, Ed. Semne, București.

Sepúlveda, W., Maza, M.T., Mantecón, A.R. (2008). Factors that affect and motivate the purchase of quality-labelled beef in Spain. J Meat Sci., 80(4): 282-289.

Śmiecińska, K., Denaburski, J., Sobotka, W. (2011). Slaughter value, meat quality, creatine kinase activity and cortisol levels in the blood serum of growing-finishing pigs slaughtered immediately after transport and after a rest period. *Polish Journal of Veterinary Sciences*, Vol. 14(1): 47-54.

Sotelo-Flores, A.P. (2008). Evaluación de cambios en las prácticas de manejo y su asociación con la presencia de carne DFD en bovinos. [Tesis de maestría]. Mexicali (BC): Universidad Autónoma de Baja California.

Terlouw, E.M., Picard, B., Deiss, V., și col., (2021). Understanding the determination of meat quality using biochemical characteristics of the muscle: stress at slaughter and other missing keys. *Foods*, 10: 84, 10.3390/foods10010084.

Van De Water, G., Verjans, F., Geers, R. (2003). The effect of short distance transport under commercial conditions on the physiology of slaughter calves; pH and colour profiles of veal. *Livest Prod Sci*, 82(2-3): 171-179.

Williams P.G. (2007). Nutritional composition of red meat, *The Role of Nutrition and Dietetics*, 64(4): 113-119.

Williamson, C.S., Foster, R.K., Stanner, S.A., Buttriss, J.L. (2005). Red meat in the diet, *Nutrition Bulletin*, 30(4): 323-355.

***Europe's Growing Muslim Population (2017)

***GraphPad Prism Statistical Software

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

THE EFFECT OF VARIOUS TYPES OF FLOUR AS FILLER MATERIALS ON PHYSICAL, CHEMICAL AND ORGANOLEPTIC CHARACTERISTICS OF SALAMI CULLED LAYING HENS

Sofi Margritje SEMBOR, Hengkie LIWE, Nova Nancy LONTAAN, Merry Diana ROTINSULU

Faculty of Animal Husbandry, Sam Ratulangi University, Manado – Indonesia

Corresponding author email: semborsofi@yahoo.com

Abstract

The aim of this study was to determine the effect of various types of flour on the physico-chemical and organoleptic characteristics of salami as a filler. The study was conducted using a completely randomized design with 5 treatments P1 (corn flour), P2 (sorghum flour), P3 (sago flour), P4 (wheat flour) and P5 (tapioca flour) each treatment was repeated 4 times. Variables measured included physical properties (Water Holding Capacity, Cooking Loss, and Tenderness) chemical properties (proximate) as well as organoleptic tests. Data were analyzed using ANOVA and continued with the Honest Significant Difference Test. Based on the research results obtained physical quality such as the highest cooking shrinkage of corn flour 19.62; The highest water binding capacity of sago flour is 42.03; the highest tenderness of sago flour 52; chemical quality such as the highest water content using sago flour (47.11%), the highest protein in wheat flour 20.65%, the highest fat in tapioca flour 20.46%, the highest carbohydrate in corn flour 19.58%; The organoleptic score for color was 2.66 (liked) using sago flour, aroma 2.77 (like) corn flour, texture 2.90 (neutral) using sago flour and taste 2.30 (like) using wheat flour.

Key words*: corn flour, sorghum, sago, flour, tapioca, salami.*

INTRODUCTION

In Indonesia, foods such as tubers, corn, sago and sorghum have begun to be promoted and are an alternative to solving problems due to scarcity of food ingredients such as rice. Judging from the nutritional value of sorghum, it is quite adequate as a food ingredient, which contains about 83% carbohydrates; 3.50% fat, and 10% protein (dry basis). Although its use as a food ingredient is still very limited, in addition to the lack of production it is also because the general public is not used to consuming it. Sorghum is an alternative food ingredient to replace carbohydrates where the carbohydrate content according to the USDA (2001) reaches (74.63 gr/100 gr ingredient) higher than wheat (71.97 gr/100 gr ingredient) and ranks third after rice (79.15 gr/100 gr ingredient), and corn (76.85 gr/100 gr of material).

Sago flour is processed from the processing of thatch stems or sago tree trunks. It is pale white in color with a rather rough texture. Sago flour can be used as a filler material aimed at improving texture, increasing water holding capacity, and small shrinkage, increasing product weight at relatively low prices so as to reduce production costs (Harsanto, 1986).

Wheat flour is a food ingredient that is still imported as a supporting material for the processing of food products such as sausages as a filler, while domestically there are many other types of flour such as corn flour, sorghum flour and tapioca flour, sago flour and wheat flour for salami products, as well as indirectly giving appreciation and new innovations to sausage and even salami producers, in addition to reducing producers' dependence on imported products so that producers are not at a loss looking for substitutes.

The addition of various types of flour as a filler can affect the physico-chemical and organoleptic properties of food products.

Based on the above background, research has been carried out to determine the effect of various types of flour on the physico-chemical and organoleptic characteristics of salami.

In addition to determining the right formulation in the processing of fermented sausages (salami) from various types of flour as filler.

MATERIALS AND METHODS

Tools and materials

The equipment used is a Philips HR 7620 Food Processor used to grind meat and mix sausage dough, a thermometer to measure temperature. Harnir as a filling tool for sausage casings, sausage casings with a size of 30 cm to wrap salami. Casing straps (mattress thread), hand stuffer, smoker for smoking salami, basin for storing meat, plastic tray for storing spices, gloves, electric scale, knife and cutting board. The materials used included Isa Brown strain culled laying hens aged 96 weeks.

The culled laying hens used were obtained from a livestock company in the village of Tetey, Dimembe District, Minahasa Regency. Spices for making salami such as garlic, ginger, pepper, nutmeg, sugar and salt, flour, skimmed milk, fat, ice or ice water. The starter cultures for fermentation were *Lactobacillus plantarum* and *Lactobacillus acidophillus* which were obtained from Nutrition Food Science Study Program, Gadjah Mada University, Yogyakarta.

Research methods

The study was carried out using a completely randomized design (CRD) with 5 treatments and each treatment was repeated 4 times to obtain 20 treatment sets (Steel and Torrie, 1995). Data were analyzed using ANOVA and continued with the Honest Significant Difference Test (Steel and Torrie, 1995).

Research procedure

The main ingredients for making salami consist of culled laying hens meat and fat in a ratio of 80: 20. The meat and fat are ground together, then frozen for 24 hours. Then it is ground again using a food processor together with spices, salt, sugar, garlic, ginger, pepper, nutmeg, and starter cultures of *Lactobacillus plantarum and Lactobacillus acidophillus* with a ratio of 1: 1: and 2% yeast. As treatments, namely sorghum flour, tapioca flour, sago flour, corn flour, wheat flour each 15%. To the salami dough is added skim milk, fat, ice or iced water and vegetable protein (Pearson and Tauber, 1984). After being thoroughly mixed, the dough is put into a casing with a diameter of 30 mm, then tied. Then hung on a rack and left (conditioning) for 24 hours at room temperature (Arief et al., 2008). After the salami has gone through the conditioning process, it is then fermented for 6 days at room temperature. Fermentation is interspersed with the smoking process for 1 hour per day. The temperature during smoking is maintained at 30-35^{o}C. The fuel used is dry coconut shells.

Parameters measured

1. Physical quality, namely water holding capacity, cooking loss and tenderness 2. Chemical quality, namely proximate analysis (water content, protein, fat, carbohydrates) (AOAC, 2005) 3. Organoleptic Quality (color, aroma, texture and taste).

RESULTS AND DISCUSSIONS

Effect of Treatment on Physical Quality

Data from the results of the study influenced the treatment using various types such as corn flour, sorghum flour (*Sorghum bicolor* L.) sago flour; and wheat flour and tapioca flour as filler for Cooking Loss (%), Water Holding Capacity (%), and Tenderness (%) of salami are presented in Table 1.

Table 1. Mean Cooking Loss (%), Water Holding Capacity (%), and Tenderness (mm/g/10 second) culled laying hens salami Using corn flour, sorghum flour (*Sorghum bicolor* L.) sago flour; and wheat flour and tapioca flour as filler

Average (%)			
Treatment	**Cooking Loss**	**Water Holding Capacity**	**Tenderness** mm/g/10 second
P1	**19.62^{a}**	**32.49^{c}**	**50.51^{b}**
P2	**17.70^{b}**	**36.35^{b}**	**48.13^{d}**
P3	**15.70^{c}**	**42.03^{a}**	**52.00^{a}**
P4	**16.01^{c}**	**41.16^{a}**	**48.37^{c}**
P5	**17.78^{b}**	**34.94^{c}**	**42.67^{e}**

Note: Different letters in the same column indicate a significant difference ($P < 0.05$)

The data in Table 1 shows that the highest cooking losses were found in treatment P1 (corn flour) which was 19.62% while the lowest cooking losses were obtained in treatment P3 (sago flour) of 16.01%. thus sago flour is a type of flour that has the lowest cooking losses compared to other types of flour. This means that sago flour has the ability to bind water or the amount of water bound and between muscle fibers and even other compounds found in salami, cooking losses become small. Low cooking loss means that the quality of the salami using sago flour is better than other treatments, because if a food product has a low cooking loss, it means that the product is of good quality. This is supported by Soeparno (2005); Aberlie et al. (2001) that meat or processed meat products with low cooking losses have better quality than meat with large cooking losses because the loss of nutrients during cooking will be less.

According to Kusmajadi (2006), Lawrie (2003) meat with low cooking loss has relatively better physical quality than meat with greater cooking loss. The low cooking loss is caused by a decrease in the pH of the postmortem meat which results in many myofibrillar proteins being damaged, which is followed by a loss of the protein's ability to bind water which in turn increases the cooking loss. The variation in the amount of cooking shrinkage from the results of this study was due to the effect of treatment with various types of flour.

Based on Table 1, it was found that the highest water holding capacity was found in treatment P3 (sago flour) of 42.03% while the lowest was obtained in treatment P1 (corn flour) 32.49% and varied between 32.35-42.03%, it was seen that there was an effect of using various treatments This type of flour as a filler has the ability to bind free water, especially during the process of forming meat emulsions which can grow well in medium with sufficient water content (Fardiaz, 1992). The results of this study are supported by previous studies that when the water holding capacity increases, water is tightly bound by proteins so that water cannot escape as a result the water content becomes high (Hultin, 1985, Aberlie et al., 2001). Another factor is that starch flour can increase its water binding capacity because it has the ability to retain water during processing and heating (Ockerman, 1983). If the starch is heated, it will cause the starch granules to vibrate rapidly until finally the bonds between the molecules are broken and the hydrogen side will be able to bind more water (Boyer and Shannon, 2003; Bulkaini et al., 2020).

The addition of fillers to meat products mainly increases stability, water holding capacity, flavor and product slice characteristics as well as reduced formulation costs. The results of Ratna Yulistiani's research (2011) reported that the best fillers in the manufacture of mackerel sausages such as corn flour (Arjuno variety) with a concentration of 6% obtained a high WHC value of 88% with fillers (rice flour, wheat flour, corn flour). But Suradi (2006) in his statement that the decrease in water holding capacity was due to the influence of the pH of the meat because the pH of the meat was low as well as the holding capacity of water was low. The higher the water holding capacity, the lower the cooking losses. Likewise with the results of this study obtained high water holding capacity but low cooking losses (Table 1).

Based on Table 1, it was found that the highest tenderness was found in treatment P3 (sago flour, 52 mm/g/10 seconds) while the lowest was obtained in treatment P5 (tapioca flour, 42.67 mm/g/10 seconds). The variation in the rate of tenderness from the results of this study was due to the different types of flour used as fillers for each treatment which gave different responses of tenderness. This shows that the use of various types of flour in salami processing can increase tenderness, causing more bound water and thus increasing tenderness as well. According to Ockerman (1983, Lawrie, 2005) that high water holding capacity will also be followed by high tenderness. The high rate of tenderness of sago flour (52 mm/g/10 seconds) in this study was due to sago starch with 27% amylose and 73% amylopectin having the same concentration as sago starch with high viscosity compared to starch solutions from other cereals (Harsanto, 1986) .

Meat tenderness is influenced by three meat components such as myofibrillar structure and muscle contraction, connective tissue content and the binding capacity of water and meat juices (Soeparno, 2005). According to Aberle et al. (2001) and Lawrie (2003) stated that the main component of meat tenderness is

influenced by connective tissue, muscle fiber groups and fat groups. Connective tissue, especially collagen and the number of cross-links play a role in the tenderness of the meat.

Effect of Treatment on Chemical Quality

Fillers are materials added in the process of making processed meat products that must have the ability to bind a certain amount of water. Data The results of the proximate analysis of each flour used in this study are presented in Table 2.

Data from the results of the study influenced the treatment using corn flour, sorghum flour (*Sorghum bicolor* L.) sago flour; and wheat flour and tapioca flour as fillers for the content of water, protein (%), fat and carbohydrates in salami are presented in Table 3.

Table 2. Proximate analysis results of corn flour, sorghum flour, sago flour and wheat flour and tapioca flour

Average (%)				
Flour Type	**Content water**	**Fat**	**Protein**	**Carbohydrate**
Corn flour	**13.49**	**0.4**	**9.27**	**76.77**
Sorghum flour	**12.20**	**1.23**	**9.44**	**75.55**
Sago flour	**16.51**	**0.23**	**2.22**	**80.62**
Wheat flour	**13.43**	**0.65**	**13.92**	**71.47**
Tapioca flour	**12.57**	**0.38**	**6.34**	**80.64**

Dairy Animal Nutrition Laboratory, Department of Nutrition Science and Feed Technology. Faculty of Animal Science, IPB, (2021)

Table 3. Average Moisture Content (%), Protein (%), Fat (%) and Carbohydrates (%) Salami chicken laying hens using corn flour, sorghum flour (*Sorghum bicolor* L.) sago flour and wheat flour and tapioca flour as filler

Average (%)				
Treatment	**Content water**	**Protein**	**Fat**	**Carbohydrate**
P1	**41.51^{c}**	**19.39^{ns}**	**16.13^{bc}**	**19.58^{a}**
P2	**40.85^{c}**	**20.36^{ns}**	**18.09^{b}**	**17.45^{b}**
P3	**47.11^{a}**	**19.32^{ns}**	**14.41^{d}**	**15.35^{c}**
P4	**43.86^{b}**	**20.65^{ns}**	**17.15^{b}**	**14.17^{c}**
P5	**41.48^{c}**	**19.44^{ns}**	**20.46^{a}**	**14.86^{c}**

Description:
P1 = Salami using 15% corn flour ; P2 = Salami using sorghum flour (*Sorghum bicolor* L.) 15%
P3 = Salami using 15% sago flour ; P4 = Salami using 15% wheat flour; P5 = Salami using 15% tapioca flour;
Different letters in the same column indicate a significant difference ($P < 0.05$)

Based on the data in Table 3, the use of various types of flour such as corn flour, sorghum flour (*Sorghum bicolor* L.) sago flour; and wheat flour and tapioca flour as fillers (filler) produce a fairly low water content for all types of flour used. This data is supported by data (Table 2) from the analysis of the water content of corn flour 13.49%, sorghum flour with a moisture content of 12.2%, sago flour 16.51%, wheat flour 13.43% and tapioca flour 12.57%.

The low water content (Table 3) of salami using various types of flour as filler is not in line with the results of a study by Sing *et al*. (2001), that the moisture content of smoked chicken sausage with a smoking temperature of 50 ° C for 20 minutes without starter yeast and lactic acid bacteria, followed by smoking 50 ° C for 90 minutes obtained a moisture content of 56.53%, with a final internal temperature of the sausage 70° C. It is strongly suspected that the low water content in this study indicates the effect of *L. acidophyllus and L. plantarum* starters in reducing the moisture content of smoked chicken sausages. The results of this study were supported by Arief et al. (2014) which stated that the water content of fermented beef sausage with kefir paste starter produced a lower water content than fermented lamb sausage with *L. plantarum* IIA-2C12, namely 65.11%. Likewise with the results of research conducted by Sulaiman (2016) that fermented lamb sausages with *L. plantarum* IIA-2C12 starter produced a moisture content of 60.75% and sausages with *L. acidophilus* IIA-2B4 starter produced a moisture content of 59.06%. In contrast to Sembor et al. (2020) in a report that the water content (34.97%) decreased with increasing levels of sorghum as a filler in salami using *L. acidophyllus* and *L plantarum*. The low water content of the salami is due to the fact that during the processing it undergoes fermentation and smoking. The research product is salami in the category of fermented sausage (dry sausage).

According to Hui et al. (2001) fermented sausages or dry sausages (dry sausage) have a moisture content of 30%-40%, close to the results of this study.

Asma Nisa (2016) also reported that smoked sausages will cause the surface to dry due to water evaporation from the sausages. It is also said that at the time before fermentation some water molecules form hydrates with other molecules containing oxygen atoms, nitrogen, carbohydrates, proteins, salts and other organic compounds so that bound water turns into free water. The free water will evaporate when it is dried during fermentation, so the higher the activity of the enzyme to break the bound water bonds into free water. According to Buckle *et al.* (2009) water is needed by microorganisms to grow and function normally. The decrease in water content is also due to the low pH value of the product because the production of lactic acid by the starter culture results in water not being tightly bound by the meat so that the water easily escapes during smoking (Fardiaz, 1992).

The data in Table 2 based on the results of variance shows that the protein content of salami using various corn flour, sorghum flour (*Sorghum bicolor* L.) sago flour; and wheat flour and tapioca flour as fillers gave results that were not significantly different (P>0.05) on the protein content of salami. The highest protein content data was obtained in the P4 (wheat flour) treatment followed by P2 (sorghum flour) while the lowest protein content was obtained in the P3 (sago flour) treatment. The decrease in protein content was caused by protein denaturation which caused the protein to lose its secondary and tertiary structure due to external pressure. Although the protein content between treatments did not show a significant difference on average 19.39-20.65%, it was still above the SNI standard 01-3820-1995 which recommended a minimum protein content of 13%, so this result was still far above the recommendation.

Based on the data in Table 2 shows that the highest fat content was obtained in treatment P5 (15% tapioca flour) which was 20.46% followed by treatment P2 (18.09%), P4 (17.15%), P1 (16.13%) and the lowest was in treatment P3. (14.41%). The result of fat analysis for tapioca flour was 0.33% (Table 2). Data in Table 2, it shows that overall, the research results meet the National Standardization Agency standard 01-3820-1995, namely a maximum fat content of 25%, although there are no recommendations for salami products, such as sausages. The high fat content of the results of this study was due to the material used in the form of culled laying hens which had a high fat content. In addition, using lactic acid bacteria such as *L. acidophyllus* and *L. plantarum*. Lactic acid bacteria have secondary lipolytic activity, can break down fat into simple chemical compounds. Lipolytic activity is controlled by the lipase enzyme which is owned by lactic acid bacteria so that it can free fatty acids (Reported by Asma Nisa, 2016). In contrast to the results of the study by Bulkaini et al. (2020) showed that the treatment without the addition of tapioca flour (0%) actually gave the highest sausage fat content, namely 2.82 ± 0.24, followed by the addition of tapioca flour of 10% of the total Sausage-making materials gave a value of 1.63 ± 0.55%, sausages with tapioca flour 20% (0.70 ± 0.21%) and the lowest was the addition of 30% tapioca flour (0.67 ± 0.08%). Sembor et al (2020) reported that the use of sorghum (*Sorghum bicolor* L.) flour at different levels (0%, 5%, 10%, 15% and 20%) as a filler in processing salami obtained fat levels ranging from 13 – 18% and the lowest fat content at the level of 15%. Fat acts as a discontinuous phase in the sausage emulsion and the fat content affects the tenderness and juice of the meat

The data in Table 2, shows that the use of various types of flour as filler in chicken laying hens salami resulted in highly significant different carbohydrate levels between treatments, although the highest carbohydrate content was obtained in treatment P1 (19.58% using corn flour) while the lowest carbohydrate content was obtained. in treatment P4 (14.17% using wheat flour as a filler).

The results of this study are in line with Boyer and Shannon, (2003) who stated that the largest chemical component in corn is carbohydrates, which is about 72% of the seed weight, most of which are starch, such as amylose 25-30% and amylopectin around 70-75%. Differences in the treatment of various types of flour as a source of carbohydrates in the manufacture of salami produce different levels of carbohydrates. The

use of various types of flour as a filler with 2% each of *L. acidophyllus and L. plantarum* as a starter, plus 2% yeast resulted in a decreased but stable salami carbohydrate content. This means that sago flour, wheat flour and tapioca flour did not show significant differences in carbohydrate content. In addition, because in the manufacture of salami, a starter of lactic acid bacteria is used as a fermenter with the same amount, both the number and type of lactic acid bacteria used. The most important characteristic of lactic acid bacteria is that they are able to ferment sugar into lactic acid (Fardiaz, 1992).

The quality of flour increases with the addition of microbes such as lactic acid bacteria in flour causing the development of bread (Gerez et al., 2006). Lactic acid bacteria are a group of bacteria that produce lactic acid as the main product of carbohydrate or sugar fermentation. Among the lactic acid bacteria groups that are widely used are *L. casei and L. bulgaricus* (Buckle et al., 2010). In this study, *lactic acid bacteria L. acidophyllus and L. plantarum* were used as starters in the processing of salami (fermented sausages) and various types of flour as fillers. The composition of amylose and amylopectin affects the starch profile. Ratnayake et al. (2002) and Bulkaini et al (2020) stated that amylopectin affects the development process of starch granules. Amylose can inhibit the increased expansion of starch granules so that complexes with fat are formed which inhibit the increase in peak viscosity at high pasting temperatures (Sang et al., 2008; Singh et al., 2010). Suparti (2003) states that tapioca flour can function as an adhesive and filler for meatball or sausage dough, so that the number of meatballs or sausages produced increases.

Starch has two main components, namely amylose and amylopectin. The composition of starch generally consists of 80-90% amylopectin and the remaining 10-20% amylose. The content of crude fiber and amylose in corn starch can increase water absorption (Niken, Ayu, 2013). Starch with high amylose content has the ability to absorb water and expands larger because amylose has the ability to form hydrogen bonds while high amylopectin content affects the swelling properties of starch (Suarni, 2009).

Effect of Treatment on Organoleptic Quality

The results of statistical analysis of salami were chicken laying hens using corn flour, sorghum flour (*Sorghum bicolor* L.) sago flour; and wheat flour and tapioca flour as
fillers for the color, flavour, texture and taste of various treatments can be seen in Table 4.

Table 4. Average of Salami Organoleptic Test Results on Color, Flavour, Texture and Taste

Treatment	Number of panelist	Color	Flavour	Tekstur	Taste
Averate					
P1	30	2.77^{b}	2.27^{c}	3.23	3.10^{a}
P2	30	3.93^{a}	3.60^{b}	3.97	3.60^{a}
P3	30	2.66^{b}	5.37^{a}	2.90	3.53^{a}
P4	30	3.27^{a}	5.23^{bc}	3.17	2.30^{b}
P5	30	2.97^{a}	4.07^{b}	3.10	3.80^{a}

Information: Different letters in the same column indicate significantly different (P 0.05).
Score 1 = very much like, 2 = like 3 = somewhat like, 4 = neutral, 5 = somewhat dislike, 6 = dislike, 7 = very much dislike

The data in Table 4 shows that the organoleptic test for salami color used corn flour, sorghum flour (*Sorghum bicolor* L.) sago flour; and wheat flour and tapioca flour as fillers (filler) showed that treatment P2 (sorghum flour) was very significantly different ($P < 0.01$) from treatment P1 (corn flour) and P3 (sago flour), but not significantly different ($P > 0.05$) with P4, and P5. The most preferred panelists were salami products using sago flour (P3) with a score of 2.66 with henic like quality. The data from this study are almost the same as Sembor, et al (2019) with a hedonic score of 1.83 (likes) using sorghum flour as a filler.

In contrast to the research results of Djukrana Wahab et al. (2017) showed that the organoleptic assessment of the composition of fresh tempeh, sago flour and smoked fish in the

manufacture of tempeh sausage products was favored in the P3 treatment (30% fresh tempeh composition, 50% sago flour and smoked fish 20%) with an organoleptic score of color 3.93% (like), aroma 4.23% (like), taste 4.10% (like) and texture 4.58% (like). The results of Sofyan et al's research (2018) found that sago flour as a filler had the highest average value, namely 4.50 and tapioca flour as filler had the lowest average value, namely 3.83 in white oyster mushroom sausage.

The color of the product is influenced by the smoking process (Soeparno, 2005), as well as the salami product in this study after undergoing the smoking process, the color becomes more brown for all treatments. According to Lawrie (2003), during the smoking process, the smoke component is absorbed by the surface of the product and causes a brownish color due to the reaction between the carbonyl groups of the smoke and the proteins found in the meat. Arief et al, 2008; Fadda et al., 1998), the color of fermented sausage is classified as dark due to the presence of H_2O_2 produced by microorganisms resulting from aerobic metabolism. However, the color of the salami as a result of the study became a slightly dark brown color. The ability of H_2O_2 to oxidize causes changes in the enzyme system of microbial cells so that it is used as an antimicrobial. H_2O_2 can cause the red color of sausage to decrease due to the formation of brown metmyoglobin (Varnam and Sutherland, 1995). Color changes in fermented sausages can also be caused by biochemical reactions due to the direct influence of temperature, relative humidity, curing time, meat composition, salt and nitrite concentrations, as well as the addition of culture in sausage processing (Arief et al., 2008; Fadda et al., 1998).

Based on the data in Table 4, the organoleptic test for odor/aroma showed a very significant effect ($P < 0.01$). The highest average value was obtained in the treatment using tapioca flour (P3), which was 5.37 (rather disliked) and the lowest was in the corn flour treatment with an average value of 2.27 (liked). The aroma of salami is caused by the addition of spices, the level of acidity because it uses lactic acid bacteria such as *L. acidophyllus* and *L. plantarum* and yeast so that it gets a sour aroma plus the aroma of smoke during the smoking process so that it produces a distinctive aroma from salami which is acidic. Other volatile compounds determine the aroma of cooked meat such as pyrazine and other compounds such as sulfur and oxygen, ammonia, acetaldehyde, acetone, diacetyl, as well as several compounds in small amounts including formic, acetate, propionate, butyrate and isobutyrate, and dimethylsulfide acid. However, in the combustion process, phenolic compounds become high and Policyclyc Aromatic Hydrocarbon (PAH) compounds are formed which are carcinogenic; The PAH compound is produced through a pyrolysis process during the burning of meat using charcoal and when meat fat drips onto hot coals, the PAH compound increases during direct cooking of meat with charcoal (Terzi et al., 2008., Fadda et al., 1998).

Based on the data in Table 4, the organoleptic test on the texture of the diversity analysis test results showed that the results were not significantly different ($P>0.05$) for each type of flour used. The sample using sago flour with a value of 2.90 (like) was the most preferred texture by the panelists, while salami using tapioca flour was 3.10 (somewhat like) by the panelists although statistically it did not show a significant difference ($P>0.05$). There was no significant difference in the texture attributes in this study because the filler used was both flour. So each treatment gave the same response to the culled laying hens salami products.

Texture is closely related to the balance of addition of water, fat and protein. If it contains a little fat, it will produce a hard and dry product (Toldra, 2002; Lawrie, 2003; Soeparno, 2005). The use of the type of filler can affect the texture, so it has quite different preference values, especially in the content of amylopectin and amylose. This is supported by the opinion of Matz (1962) that starch with a high amylopectin content will form a non-rigid gel, whereas starch with a low amylopectin content will form a stiff gel. Meanwhile, according to Tjokroadikoesoemmo (1986), the addition of tapioca will affect the chemical composition and taste organoleptic properties. The greater the amylopectin content or the lower the amylose content, the more sticky the processed product will be.

The results of organoleptic test observations on salami taste used wheat flour (2.30) which was the most preferred, followed by corn flour (liked with a score of 3.10) as a filler. Taste is the main factor of a food product. Assessment of taste shows consumer acceptance of a food ingredient. Taste is influenced by flavors that can stimulate the sense of acceptance when tasting and the impression left on the sense of taste after someone ingests a food product (Winarno, 2002). The addition of spices to the manufacture of salami (fermented sausages) is the most dominant component in shaping the taste elicited in salami products (Arief et al., 2014). In this study, salami processing using various types of flour as filler with a fermentation time of 6 days using Lactic Acid Bacteria and yeast caused a decrease in pH and is thought to cause an increase in total acid so that the taste becomes more sour. So far, people are not very familiar with salami or fermented sausage products, so only a few panelists like it and most panelists prefer salami which tastes less sour.

CONCLUSIONS

Based on the research results obtained physical quality such as the highest cooking loss of corn flour 19.62; The highest water holding capacity of sago flour is 42.03; the highest tenderness of sago flour 52; chemical quality such as the highest water content using sago flour (47.11%), the highest protein in wheat flour 20.65%, the highest fat in tapioca flour 20.46%, the highest carbohydrate in corn flour 19.58%; The organoleptic score for color was 2.66 (liked) using sago flour, aroma 2.77 (like) corn flour, texture 2.90 (neutral) using sago flour and taste 2.30 (like) using wheat flour.

REFERENCES

Aberle E.D., J.C. Forrest, D.E. Gerrad, E.W. Mills, 2001. *Principles of Meat Science*. Kendall/Hunt Publishing Company.

AOAC, 2005. Official Methods of Analysis of AOAC International. 18th. Ed. Assoc. off. Anal. Chem., Arlington

Arief I. I., R. R. A. Maheswari., T. Suryati, Komariah dan S. Rahayu, 2008.Kualitas Mikrobiologi Sosis Fermentasi Daging Sapi dan Domba yang Menggunakan Kultur Kering *Lactobacillus plantarum* 1B1 dengan Umur yang Berbeda.

Arief, I. I., Z. Wulandari, E. L. Aditia, M. Baihaqi, Noraimah, and Hendrawan, 2014. Physicochemical and Microbiological Properties of Fermented Lamb Sausages using Probiotic Lactobacillus plantarum IIA-2C12 as Starter Culture. *Procedia Environ. Sci*. 20:352–356.

Asma Khoyrun Nisa; Agustin Krisna Wardani, 2016. Pengaruh Lama Pengasapan dan Lama Fermentasi terhadap Sosis Fermentasi Ikan lele (*Clarias gariepinus*)

Badan Standardisasi Nasional, 1995. *Syarat Mutu Sosis Daging* SNI 01-3820-1995. Jakarta. Hal. 1 – 9.

Boyer, C. D. dan J.C. Shannon, 2003. Carbohydrates of the Kernel. Di dalam White, P.J. dan L.A. Johnson (eds). *Corn: Chemistry and Technology, 2nd edition*. American Association of cereal Chemistry Inc., St. Paul, Minnesota, USA.

Buckle, K.A., R.A. Edwards., G.H. Fleet., M. Wootton, 2010. *Ilmu Pangan*. Penerjemah Hari Purnomo Adiono. Penerbit Universitas Indonesia.

Bulkaini, Djoko Kisworo, Sukirno, Rani Wulandani dan Maskur, 2020. Kualitas Sosis Daging Ayam Dengan Penambahan Tepung Tapioka. *Jurnal Ilmu dan Teknologi Peternakan Indonesia*, Volume 6(1) 10 - 15; Juni 2020 p-ISSN: 2460-6669 e- ISSN: 2656-4645

Djukrana Wahab, Ansharullah reka pangan, Abdu Rahman Baco, Asfianty, 2017. Pemanfaatan Tepung sagu (*Metroxylon* sp.) sebagai Bahan Pengisi Sosis tempe: Kajian Organoleptik dan Nilai Gizi) rekapangan*. Jurnal teknologi Pangan 2017* ISSN 1978-4163 (cetak)/ISSN 2654 – 5292 (online)

Fadda, S., G.Vignolo, A.P.R. Holgodo dan G. Oliver, 1998. Proteolytic Activity of *Lactobacillus* Strain Isolated from dry fermented sausage on Muscle sarcoplasmic Proteins.*Jurnal Meat Science*, Vol. 49, Issue 1. Page. 11 - 18

Fardiaz, S., 1992.*Mikrobiologi Pangan*. Penerbit PT Gramedia Utama. Jakarta.

Gaman, P. M. and K. B. Sherrington, 1994. Ilmu Pangan: Pengantar Ilmu Pangan Nutrisi dan Mikrobiologi (Terjemahan oleh M. Gardjito; S. Naruki; A. Murdiati; Sardjono). Edisi Kedua. Gadjah Mada University Press. Yogyakarta.

Gerez, L.C., Rollan, G.C. dan Font de Valdez, G., 2006. Gluten breakdown by *Lactobacilli andpediococci* strains isolated from sourdough. *Letters in Applied Microbiology*, 42(5): 459-464.

Hultin H.O., 1985. *Characteristics of Muscle Tissue*. Second edition. Marcel Dekker Inc., New York

Harsanto, B., 1986. Budidaya dan Pengolahan Sagu. Kanisius: Yogyakarta

Hui, Y. H., W. K. Nip, R. W. Rogers, danO. A. Young, 2001. *Meat Science and Application*. Marcel Dekker Inc., New York, USA.

Kusmajadi Suradi, 2006. Perubahan Sifat Fisik Daging Ayam Broiler Post Mortem Selama Penyimpanan Temperatur Ruang (Change of Physical Characteristics of Broiler Chicken Meat Post Mortem During Room Temperature Storage*). Jurnal Ilmu Ternak. Universitas Padjadjaran. Bandung*, Vol. 6, No.1

Lawrie R.A., 2003. *Ilmu Daging*. Penerbit Universitas Indonesia. Edisi Kelima.

Matz, 1962. Food Texture, The Avi Publishing Co. New York.

Niken, Ayuk, 2013. Isolasi Amilosa dan Amilopektin dari Pati Kentang. *Jurnal Teknologi Kimia dan Industri*, Vol. 2, No. 3. Hal. 57-62.

Ockerman, H.W., 1983. *Chemistry of Meat Tissue.* Depart. Of Animal Science. The Ohio State University and The Ohio Agriculture Research and Departemen Center Ohio. p. 1–38

Pearson A. M, and F. M Tauber, 1984. Processed Meat. Westport. The AVI Publishing Co Inc. Connecticut

Ratnayake, W.S., R. Hoover and W. Tom, 2002. Pea starch: composition, structure, and properties. Review. *J. Starch*, 54: 217–234.

Ratna Yulistiani, 2011. Evaluasi Penggunaan Tepung Sereal dan Asap Cair Tempurung Kelapa pada Daya Simpan Sosis Ikan Tenggiri. *Jurnal Teknologi Pangan*, Vol. 5, No.1, Juni 2011. Hal. 27 - 36

Sang, Y., S. Bean, P.A. Seib, J. Pedersen and Y.C. Shi, 2008. Structure and functional properties of sorghum starches differing in amylase content. *J. Agric. Food Chem. 56: 6680–6685*

Singh,H., N.S. Sodhi and N. Singh, 2010. Characterization of starches separated from sorghum cultivars grown in India. *J. Food Chem*., 119: 95 - 100

Sembor, S.M, Liwe, H dan Lontaan N. N., 2019 Karakteristik Fisiko Kimia dan Mikrobiologi Salami Ayam Petelur Afkir Menggunakan Tepung (*Sorghum bicolor* L.) sebagai Bahan Pengisi (Filler**).** Laporan Penelitian Riset Terapan Unggulan Unsrat tahun 2019.

Sembor S.M, Liwe, H dan Lontaan. N.N., 2020. Introduksi Kadar Tanin Salami Ayam Petelur Afkir Menggunakan Tepung Sorgum **(***Sorghum bicolor* L.) sebagai Bahan Pengisi (*filler*) dengan Level yang Berbeda. Laporan Penelitian Riset Terapan Unggulan Unsrat tahun 2020.

Soeparno, 2009. *Ilmu dan Teknologi Daging*.Gadjah Mada University Press.Yogyakarta.

Sofyan I, Yusep Ikrawan, dan Linda yani, 2018. Pengaruh Konsentrasi bahan Pengisi dan Sodium Tripolyphosphate ($Na_5P_3O_{10}$) terhadap karakteristik Sosis jamur Tiram Putih. *Pasundan Food Technology,* Vol. 5, No. 1

Suarni, 2009. Prospek Pemanfaatan Tepung Jagung Untuk Kue Kering (Cookies). *Jurnal Litbang Pertanian*, 28(2) hal 63-71.

Suparti, L., 2003. Membuat Bakso Daging dan Bakso Ikan. Kanisius. Yogyakarta.

Steel, R.G.D dan J. H. Torrie, 1992. Prinsip dan Prosedur Statistika. Terjemahan: B. Sumantri. PT Gramedia Pustaka Utama, Jakarta.

Terzi G, T.H Celik, C. Nisbet, 2008. *Determination of benzo(a)pyrene in Turkish Doner Kebab Sample Cooked with Charcoal or gas Fire. Irish*. *J. Agric. Food Res*., 47: 187-193

Tjokroadikoesoemo P., 1986. HFS dan Industri Ubi Kayu Lainnya. Penerbit PT. Gramedia Pustaka Utama, Jakarta.

Toldra,F, Hui, Y.H; Astiasaran I; Wai-Kit Nip; Sebranek J.G; Expedito-T.F. Silveira; Stahnke L.H; Talon, R., 2007. *Fermented Poultry Sausages in Fermented Meat and Poultry*. Handbook.Blackwell Publishing. Ltd. First Edition. Australia. 361-368

USDA (United State Food and Drug Administration) *Center for Food Safety*, 2001. Washington, D.C. (UK): Applied Nutrition National Science Teachers Assosiation. Hal. 70.

Varnam and Sutherland, 1995. *Meat and Meat Products*. Chapman and Hall. London.

EXPERIMENTAL MEDICINE

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

THE DEVELOPMENT OF A PRECLINICAL MODEL FOR OSTEOINTEGRATION OF DENTAL IMPLANTS - A PILOT STUDY

Diana-Larisa ANCUȚA[1, 2], Maria CRIVINEANU[2], Cristin COMAN[1, 2, 3, 4]

[1]"Cantacuzino" National Medico-Military Institute for Research and Development, Splaiul Independentei 103, Bucharest, Romania
[2]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, Splaiul Independenței 105, Bucharest, Romania
[3]Fundeni Clinical Institute, Center of Excellence in Translational Medicine, Fundeni Road 258, Bucharest, Romania
[4]"Spiru Haret" University, Faculty of Veterinary Medicine, Basarabia Boulevard, Bucharest, Romania

Corresponding author email: diana.larisa.ancuta@gmail.com

Abstract

Functional tooth replacement and bone regeneration are areas of interest in modern dentistry and dental implant research involves increased attention to osteointegration. The aim of the study was to develop a small, inexpensive and reproducible animal model for testing dental implants. Fifteen male Wistar rats, 20 weeks old, average weight of 400 grams were included in the study. They were subjected to a rigorous bone support preparation protocol so that the maxillary first premolar was extracted from the left half arch. After a period of 30 days, necessary for the bone refilling of the dental alveolus, the radiological examination was performed. Then a surgical intervention was performed to mount the titanium implants of an adapted size. Clinically, the evolution was favorable, with no signs of discomfort or oral infection. At the radiological evaluation, optimal bone regeneration could be observed. necessary to ensure a suitable place for implant mounting. The implantation procedure was laborious due to the limited working area. However, rats are proving to be suitable animal models for implant-related studies or innovative treatments administered under pathological conditions.

Key words*: implant, osteointegration, rat, tooth extraction.*

INTRODUCTION

Dental implants, in recent times, represent the life-saving solution for patients with compromised oral health, tooth decay or other conditions that make an alternative of tooth replacement impossible. The demand is increasing, which makes the producers become competitive and offer an increasingly effective product, with high quality in terms of osteointegration or its acceptance by the human body. For this, the implants must pass two big thresholds, before being used in the dental clinic: *in vitro* and *in vivo* tests (Pilawski, 2020). Through the latter, the safety and effectiveness of implants in a living organism is evaluated. For researchers, choosing a suitable animal model is still difficult because regulatory agencies require the validation of a preclinical animal model (Stadlinger, 2012), and the ISO 7405:2018 standard requires that dental implants be tested in their human form. Consequently, the testing of dental implants would be justified only on large animals, but the choice of an experimental animal model is essential to be able to obtain justifiable preclinical results in subsequent clinical research (Spicer, 2012). Therefore, the animal model must guarantee the reproducibility of the clinical condition for which an implant is tested (Li, 2015).

When we talk about dental implants, we can think that their most appropriate testing would be at the level of the oral cavity, but the segmental mandibular defects potentially created at this level represent the biggest challenge, due to their poor intrinsic healing capacity. Researchers in the field of implant testing prefer the choice of small animal models for the well-known economic reasons (housing, care), easy maneuverability and the many possibilities of surgical intervention (da Silva

Morais, 2018). The results of the experiments can be influenced if attention is not paid to the fact that there are species-specific differences related to remodeling, composition, and the process of bone regeneration (Pearce, 2007). In terms of bone remodeling, humans, pigs, dogs, sheep, and goats are moderately similar, while the rabbit is the least comparable. Bone composition, mechanical abilities and bone density have shown interspecies differences (Aerssens, 1998).

Some researchers provide evidence that bone remodeling in rodents is similar to humans, which represents an advantage in choosing a model for studying implants (Baron, 1984). Cellular and molecular indices, regulation of the growth process, and expressed chemokines or cytokines are comparable to humans (Vieira, 2015). Moreover, the morphology of the alveolar bone of rodents does not differ from that of the pig, an animal considered to be the closest to humans, histological and immunohistochemical data highlighting this fact in a comparative study between species (Pilawski et al., 2020).

The aim of the study was to develop an animal model for the study of dental implants. We considered that rats represent the appropriate animal model, considering the morpho-functional similarities of the alveolar bone, the economic advantages, the manipulation and the surgical approach, even if the size requires the adaptation of the size of the implant to be tested.

MATERIALS AND METHODS

The animal experiments were carried out at the Baneasa Animal Facility (BAF) of the Bucharest National Medical-Military Institute for Research and Development (IC). The study was approved by the Ethics Committee of the Faculty of Veterinary Medicine Bucharest and by the veterinary health authority, in accordance with EU Directive 63/2010 on the care, use and protection of animals used for scientific purposes.

The procedures developed to create the animal model for testing dental implants were performed on 15 Wistar rats, aged 20 weeks, from the SPF (Specific Pathogen Free) kennel of BAF. Throughout the experiment, the animals were housed, in groups of 5, in conventional conditions at a temperature of 20-22°C, a 12 hours light: 12 hours dark cycle and received water and feed *ad libitum*. The general health status of all animals was checked daily and the specific clinical status and body weight monitoring, were evaluated every 2 weeks after surgery. The exclusion criteria were established before the start of the experiment and included as a condition, weight loss of 20% or more at any time of the experiment, which would require the immediate euthanasia of the animal.

The experimental procedure

1. Extraction of the maxillary molar

Under general anesthesia with a mixture consisting of IP Ketamine (0,5mg/kg, Pasteur, Romania) and Medetomidine (0,5mg/kg, Biotur, Romania), the animals were positioned on the operating table in dorso-ventral decubitus. A spacer was positioned between the upper and lower incisors. With a dental take-off for human use, the gingiva near the left maxillary first molar was separated from the tooth, and by rotational movements in the axis, it was extracted. The roots that broke and remained attached to the alveolus after the extraction were also removed with surgical forceps so that the extraction site remained free of any dental remains. The gingiva was sutured in a single point with a 4/0 resorbable multifilament thread (Novosyn Quick). At the end of the operation, the animals received an antidote (Atipamezole SC, 0.02 mg/kg, Biotur, Romania) an antibiotic (Enrofloxacin SC, 5mg/kg, Pasteur, Romania), and an anti-inflammatory (Ketoprofen SC, 5 mg/kg, Dopharma, Romania) for 3 days. After 4 weeks of healing of the extraction socket, radiological analysis by the high-sensitivity bioluminescence technique (IVIS Lumina XRMS, Werner ROEDL–PerkinElmer, Austria) was performed to check the level of bone regeneration. The experimental extraction operation in rats is shown in Figure 1.

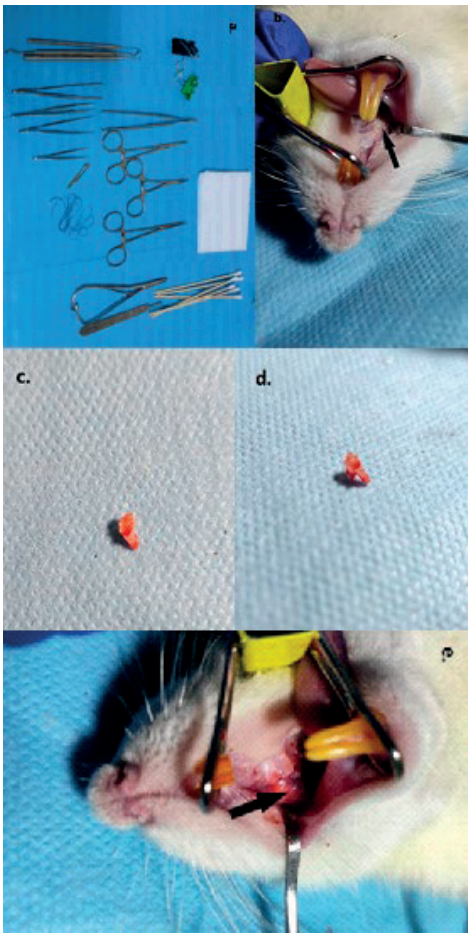


Figure 1: Dental extraction procedure (a - surgical instruments, b - maxillary left first molar, c, d - extracted teeth, e - tooth socket after extraction)

2. The implants mounting

After the 4 weeks necessary for the regeneration of the dental alveolus, the rats were anesthetized again using the same protocol as in the case of extraction and positioned in the same decubitus position. On the site of the extracted molar, the gingiva was sectioned with a scalpel blade, no. 15, followed by its detachment from the bone. After exposing the bone support, a 1.5 mm deep cavity was created with the help of a 1.2 mm diameter drill into which a 1.5 mm long and 1 mm diameter titanium implant was screwed (Figure 2). The gingiva was sutured over the implant with a 6/0 non-resorbable monofilament thread (Dafilon, Romania). After another 4 weeks, necessary for osteointegration, the radiological examination was performed.

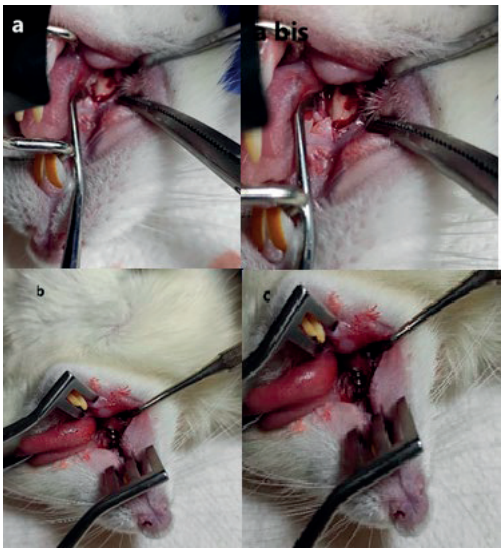


Figure 2: Experimental implantation operation in rats in (a, a bis - exposure of the bone support, b, c - mounting of the implant)

The animals were monitored daily by a veterinarian. On day 0, the animals were weighed, and blood was collected from the retroorbital sinus for hematological evaluation (complete hemoleucograms) after extraction. The monitoring of the weight of the animals was carried out every 2 weeks, and the hematological exams was repeated after the installation of the implants to evaluate the health status and also to follow the systemic immunoinflammatory index (SII). SII is frequently used in human medicine to predict several diseases, including bone inflammation, even in the absence of other specific signs. It is calculated based on the results obtained from complete blood counts by applying the formula (NEU×PLT)/LYM (NEU - neutrophil counts, PLT - platelet counts and LYM - lymphocyte counts). The radiological examination was performed to verify the regeneration of the bone support after extraction but also to evaluate the integration of the implants.

RESULTS AND DISCUSSIONS

Statistical analysis

Analyzes were performed using Prism 9 for Windows software (GraphPad LLC, USA). To compare the data, the One-way ANOVA function was used, and a value of $p < 0.05$ was considered statistically significant.

Clinically, the animals had a favorable evolution, but the post-extraction recovery, in the first 2 days, showed an alteration of the general state, represented by apathy, but as time went by and with the installation of analgesia after the institution of post-operative treatment, the rats returned to a good condition.

Body weight in the case of all animals registered a significant decrease ($p<0.05$) in the first 14 days post-extractive, following that until the day of mounting the implants this loss is recovered (Figure 3). Also, compared to day 0 and until day 74, weight increases were visible after each procedure applied to the animals, less pronounced after the installation of the implants, a sign that the animals tolerated these devices better.

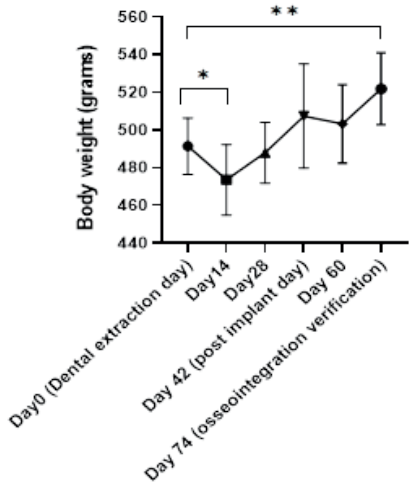


Figure 3: Evolution of body weight post-extraction-post implant

Following the SII analysis, surprising results were obtained, in the sense that it was significantly higher ($p = 0.0006$) after the installation of the implants, compared to the results obtained after extraction (Figure 4).

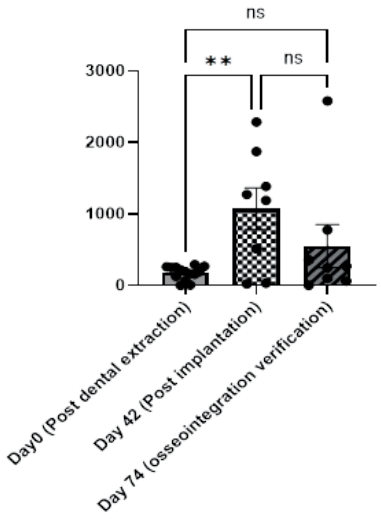


Figure 4: The difference between SII on the day of extraction, the day of implant mounting and the day of osteointegration verification

The radiological examination performed one month after the extraction showed an uniform bone support, the regenerative phenomena settling within the physiological limits (Figure 5).

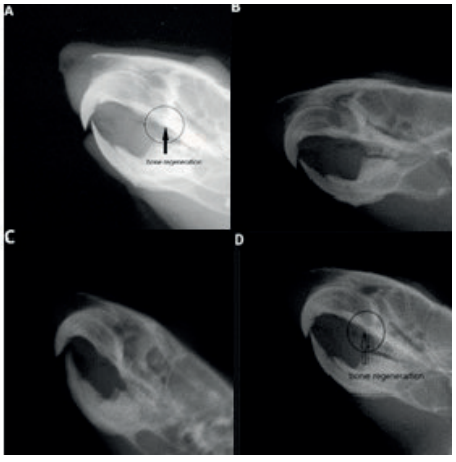


Figure 5: Bone bed appearance after extraction

At 74 days, when the osteointegration of the implants was checked, an optimal bone density could be observed around the implants (Figure 6), but out of the total of 15 mounted implants, 5 were lost, in these animals, cavity refilling was observed bones, shows other specific signs of device rejection.

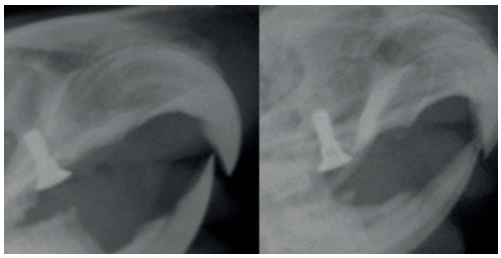


Figure 6: Appearance of dental implants (day 74)

The stability of an implant can be assessed both invasively and non-invasively.

Invasive methods include the pull/push test (Swami, 2016; Blazsek, 2009; Brunski, 2000), the disassembly test (Carvalho, 2010) or histological analysis (Bernhardt, 2012; Bissinger, 2017). These methods cannot be applied in clinical practice, therefore it is necessary to refine the non-invasive methods (Davies, 2007; Rodrigo, 2010) which refer to post implant radiological analysis (Atsumi, 2007), resonance frequency analysis (Huwiler, 2007) or clinical evaluation. For preclinical tests, the combination of both non-invasive and invasive methods could provide the best result, providing a safe basis for clinical applications.

Animal models seem to be the ideal solution to develop better devices for medical applications (Spicer, 2012; Van Griensven, 2015) because they offer the possibility of verifying osteointegration in a living organism. The medical world is still looking for the best animal model and testing method to increase the reliability of experiments (Hartung, 2010; Renaud, 2015), so that they are reproducible and reliable (Schmitz, 1986). The ISO/TS_22911:2016 guide provides indications for the preclinical evaluation of implants from a morphological, radiographic and histopathological point of view (ISO/TS_22911:2016, 2016)

Osteointegration refers to the direct contact between an implant and living bone tissue (Branemark, 1983). Moreover, the term also refers to the process of formation of this direct fixation which has a high dependence on the previous surgical procedure and preoperative circumstances (Trisi, 2009). Therefore, the implant-bone interface represents the area of major interest for researchers in the dental or orthopedic field. Through this study, we sought to create an animal model for testing dental implants that would approach the bone microstructure of the human jaw.

By extracting the maxillary left molar, we aimed to achieve the edentulous space of the human patient who needs an implant. Moreover, because this need for dental implants is more common in elderly people, the age of the rats was chosen accordingly, so that after 20 weeks, they are considered old. Aging influences numerous cellular processes, including immune responses, which may impact the outcome of bone injury healing, whether accidental or induced (Clark, 2017). Research's predominant use of young, healthy animals in preclinical models does not typically reflect the advanced age and potential comorbidities, such as impaired vascular function and reduced angiogenic responses, present in human patients (Stegen, 2015).

The systemic immuno-inflammatory index (SII) is a novel inflammation marker that is highly predictive of tumor prognosis and immune response status (Shui, 2021, Ji, 2020). Clear associations between IBS and inflammatory conditions have been observed. (Hamad, 2021), being also correlated with the loss of bone density (Du, 2021), in the case of our study, a much higher SII was observed in the condition of the loss of bone tissue following the creation of the implantation cavities but also of the secondary inflammatory reaction. The human equivalent for the bone healing process, in the case of rats, is 4-8 weeks (Hatt, 2022). Unlike histological and immunohistochemical analyses, which require animal euthanasia, radiographic imaging can be used to longitudinally assess bone healing in the same animal over time, which is an attractive means of reduction. the use of animals. New bone regeneration quantified from radiographic imaging is mostly expressed as bone volume/total volume (BV/TV), bone mineral density, new bone formation, or units. In this study, the X-ray analysis for evaluating bone

support regeneration post extraction or for evaluating the integration of implants was the ideal choice that allowed keeping the animals alive, thus making possible their transition to new stages of study. However, histology remains the main method of analysis and is used in all the studies presented. Histology is a powerful tool to assess native tissue infiltration within the construct, making it one of the most important outcome assessments. This is closely followed by CT/iCT, immunohistochemistry and radiography (Tcacencu, 2018).

CONCLUSIONS

Rats have proven to be suitable animal models for the study of dental implants. The implantation technique required additional attention, the working field being a limited one, the size of the implants being an adapted one. The body's response to the infamous post-implantation processes was an obvious one, but it was remitted through usual therapeutic protocols. The radiographic analysis completed the clinical picture so that through the technique approached on the chosen model, physio pathological conditions related to the implant, devices and innovative therapies can be tested.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Romanian National Authority for Scientific Research and Innovation, CCCDI-UEFISCDI, project number 89/2019 within PNCDI III, studies included in practical stages of Dr. Diana Larisa Ancuța 's doctoral thesis.

REFERENCES

Aerssens, J., Boonen, S., Lowet, G., & Dequeker, J. (1998). Interspecies differences in bone composition, density, and quality: potential implications for in vivo bone research. *Endocrinology*, 139(2), 663–670.

Atsumi, M., Park, S. H., & Wang, H. L. (2007). Methods used to assess implant stability: current status. The *International journal of oral & maxillofacial implants*, 22(5), 743–754.

Baron, R., Tross, R., & Vignery, A. (1984). Evidence of sequential remodeling in rat trabecular bone: morphology, dynamic histomorphometry, and changes during skeletal maturation. *The Anatomical record*, 208(1), 137–145.

Bernhardt, R., Kuhlisch, E., Schulz, M. C., Eckelt, U., & Stadlinger, B. (2012). Comparison of bone-implant contact and bone-implant volume between 2D-histological sections and 3D-SRμCT slices. *European cells & materials*, 23, 237–248.

Bissinger, O., Probst, F. A., Wolff, K. D., Jeschke, A., Weitz, J., Deppe, H., & Kolk, A. (2017). Comparative 3D micro-CT and 2D histomorphometry analysis of dental implant osseointegration in the maxilla of minipigs. *Journal of clinical periodontology*, 44(4), 418–427.

Blazsek, J., Dobó Nagy, C., Blazsek, I., Varga, R., Vecsei, B., Fejérdy, P., & Varga, G. (2009). Aminobisphosphonate stimulates bone regeneration and enforces consolidation of titanium implant into a new rat caudal vertebrae model. *Pathology oncology research: POR*, 15(4), 567–577.

Brånemark P. I. (1983). Osseointegration and its experimental background. *The Journal of prosthetic dentistry*, 50(3), 399–410.

Brunski, J. B., Puleo, D. A., & Nanci, A. (2000). Biomaterials and biomechanics of oral and maxillofacial implants: current status and future developments. *The International journal of oral & maxillofacial implants*, 15(1), 15–46.

Carvalho, C. M., Carvalho, L. F., Costa, L. J., Sa, M. J., Figueiredo, C. R., & Azevedo, A. S. (2010). Titanium implants: a removal torque study in osteopenic rabbits. *Indian journal of dental research: official publication of Indian Society for Dental Research*, 21(3), 349–352.

Clark, D., Nakamura, M., Miclau, T., & Marcucio, R. (2017). Effects of Aging on Fracture Healing. *Current osteoporosis reports*, 15(6), 601–608.

da Silva Morais, A., Oliveira, J. M., & Reis, R. L. (2018). Small Animal Models. Advances in experimental medicine and biology, 1059, 423–439.

Davies J. E. (2007). Bone bonding at natural and biomaterial surfaces. *Biomaterials*, 28(34), 5058–5067.

Du, Y. N., Chen, Y. J., Zhang, H. Y., Wang, X., & Zhang, Z. F. (2021). Inverse association between systemic immune-inflammation index and bone mineral density in postmenopausal women. Gynecological endocrinology. The official *Journal of the International Society of Gynecological Endocrinology*, 37(7), 650–654.

Hamad, D. A., Aly, M. M., Abdelhameid, M. A., Ahmed, S. A., Shaltout, A. S., Abdel-Moniem, A. E., Ragheb, A. M. R., Attia, M. N., & Meshref, T. S. (2022). Combined Blood Indexes of Systemic Inflammation as a Mirror to Admission to Intensive Care Unit in COVID-19 Patients: A Multicentric Study. *Journal of epidemiology and global health*, 12(1), 64–73.

Hartung T. (2010). Comparative analysis of the revised Directive 2010/63/EU for the protection of laboratory animals with its predecessor 86/609/EEC - a t4 report. ALTEX, 27(4), 285–303.

Hatt, L. P., Thompson, K., Helms, J. A., Stoddart, M. J., & Armiento, A. R. (2022). Clinically relevant preclinical animal models for testing novel cranio-maxillofacial bone 3D-printed biomaterials. *Clinical and translational medicine*, 12(2), e690.

Huwiler, M. A., Pjetursson, B. E., Bosshardt, D. D., Salvi, G. E., & Lang, N. P. (2007). Resonance frequency

analysis in relation to jawbone characteristics and during early healing of implant installation. *Clinical oral implants research*, 18(3), 275–280.

ISO/TS 22911:2016 (2016). Dentistry - Preclinical evaluation of dental implant systems - Animal test methods.

Ji, Y., & Wang, H. (2020). Prognostic prediction of systemic immune-inflammation index for patients with gynecological and breast cancers: a meta-analysis. *World journal of surgical oncology*, 18(1), 197.

Li, Y., Chen, S. K., Li, L., Qin, L., Wang, X. L., & Lai, Y. X. (2015). Bone defect animal models for testing efficacy of bone substitute biomaterials. *Journal of orthopaedic translation*, 3(3), 95–104.

Pearce, A. I., Richards, R. G., Milz, S., Schneider, E., & Pearce, S. G. (2007). Animal models for implant biomaterial research in bone: a review. *European cells & materials*, 13, 1–10.

Pilawski, I., Tulu, U. S., Ticha, P., Schüpbach, P., Traxler, H., Xu, Q., Pan, J., Coyac, B. R., Yuan, X., Tian, Y., Liu, Y., Chen, J., Erdogan, Y., Arioka, M., Armaro, M., Wu, M., Brunski, J. B., & Helms, J. A. (2021). Interspecies Comparison of Alveolar Bone Biology, Part I: Morphology and Physiology of Pristine Bone. *JDR clinical and translational research*, 6(3), 352–360.

Renaud, M., Farkasdi, S., Pons, C., Panayotov, I., Collart-Dutilleul, P. Y., Taillades, H., Desoutter, A., Bousquet, P., Varga, G., Cuisinier, F., & Yachouh, J. (2016). A New Rat Model for Translational Research in Bone Regeneration. Tissue engineering. Part C, Methods, 22(2), 125–131. https://doi.org/10.1089/ten.TEC.2015.0187.

Rodrigo, D., Aracil, L., Martin, C., & Sanz, M. (2010). Diagnosis of implant stability and its impact on implant survival: a prospective case series study. *Clinical oral implants research*, 21(3), 255–261.

Schmitz, J. P., & Hollinger, J. O. (1986). The critical size defect as an experimental model for craniomandibulofacial nonunions. *Clinical orthopaedics and related research*, (205), 299–308.

Shui, Y., Li, M., Su, J., Chen, M., Gu, X., & Guo, W. (2021). Prognostic and clinicopathological significance of systemic immune-inflammation index in pancreatic cancer: a meta-analysis of 2,365 patients. *Aging*, 13(16), 20585–20597.

Spicer, P. P., Kretlow, J. D., Young, S., Jansen, J. A., Kasper, F. K., & Mikos, A. G. (2012). Evaluation of bone regeneration using the rat critical size calvarial defect. *Nature protocols*, 7(10), 1918–1929.

Stadlinger, B., Pourmand, P., Locher, M. C., & Schulz, M. C. (2012). Systematic review of animal models for the study of implant integration, assessing the influence of material, surface and design. *Journal of clinical periodontology*, 39 Suppl 12, 28–36.

Stegen, S., van Gastel, N., & Carmeliet, G. (2015). Bringing new life to damaged bone: the importance of angiogenesis in bone repair and regeneration. *Bone*, 70, 19–27.

Swami, V., Vijayaraghavan, V., & Swami, V. (2016). Current trends to measure implant stability. *Journal of Indian Prosthodontic Society*, 16(2), 124–130.

Tcacencu, I., Rodrigues, N., Alharbi, N., Benning, M., Toumpaniari, S., Mancuso, E., Marshall, M., Bretcanu, O., Birch, M., McCaskie, A., & Dalgarno, K. (2018). Osseointegration of porous apatite-wollastonite and poly(lactic acid) composite structures created using 3D printing techniques. Materials science & engineering. C, Materials for biological applications, 90, 1–7.

Trisi, P., Perfetti, G., Baldoni, E., Berardi, D., Colagiovanni, M., & Scogna, G. (2009). Implant micromotion is related to peak insertion torque and bone density. *Clinical oral implants research*, 20(5), 467–471.

van Griensven M. (2015). Preclinical testing of drug delivery systems to bone. *Advanced drug delivery reviews*, 94, 151–164.

Vieira, A. E., Repeke, C. E., Ferreira Junior, S.deB., Colavite, P. M., Biguetti, C. C., Oliveira, R. C., Assis, G. F., Taga, R., Trombone, A. P., & Garlet, G. P. (2015). Intramembranous bone healing process subsequent to tooth extraction in mice: micro-computed tomography, histomorphometric and molecular characterization. *PloS one*, 10(5), e0128021.

Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

COST ANALYSIS OF TISSUE MICROARRAYS FOR CLINICAL DIAGNOSTIC

Alina Elena ȘTEFAN[1, 3], Daniela GOLOGAN[2, 3], Sorin MUȘAT[3], Matthew Okerlund LEAVITT[4], Raluca STAN[2], Manuella MILITARU[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăști Blvd, District 1, Bucharest, Romania
[2] Polytechnic University of Bucharest, 313 Splaiul Independenței, District 6, Bucharest, Romania
[3]Themis Pathology SRL, 56F 1 Decembrie 1918 Blvd, District 3, Bucharest, Romania
[4]LUMEA Inc., 2889 Ashton Blvd, Lehi, Utah, USA

Corresponding author email: sorin@lumea.net

Abstract

The need of issuing medical diagnoses with fast turn-around times (but without compromising accuracy) is generating technical challenges in all stages of histological processing. Because of the multiple variables related to tissue harvesting, processing, and sectioning quite often the resulting histological slides reach the pathologist with fragmented or incomplete tissue sections. In the present study we evaluated the feasibility of a new method of multiplexing tissue specimens with irregular shapes by placing them during grossing into sectionable matrices (BxFrame™ GRID). The working time required for a histotechnologist in obtaining multiplexed preparations as well as the costs of laboratory supplies was compared with conventional methods. Five different types of tissue (duodenum, brain, heart, tail, and skin) were placed in BxFrame™ matrices, and subjected to histological processing, sectioned, and stained. The new multiplexing method reduced the total working time with 45% to 70% when compared to conventional methods (depending on the type of tissue) while the cost of consumables was reduced with up to 70%.

Key words*: clinical diagnostic, clinical tissue microarray, cost reduction, laboratory consumables, sectionable matrix.*

INTRODUCTION

Delivering diagnoses with high accuracy and increasingly faster turn-around times have been a constant challenge in diagnostic laboratories all over the world. However, the use of lower-quality consumables, reductions in the budgets allocated to laboratories and the constraint of delivering medical findings in the shortest possible time sometimes can endanger the quality of diagnoses.

Multiple studies have shown that there are many categories of variations that can lead to diagnostic errors (Buesa, 2010):

a) Biopsy collection variations: sampling tools optimized for the type of excised biopsies (Wang et al., 2015), defining the minimum number of biopsies to be collected, type of fixative used (Varma et al., 2013)
b) Variations in the dehydration stage: different durations of the processing protocols, different reagents, different processors (specimen-transfer processors, fluid transfer processors, microwave assisted processors) (Gologan et al., 2021)
c) Variations at the embedding stage: various natural or synthetic waxes, paraffins with a melting range of 52°C to 64°C, epoxydic resins etc. (Suvarna et al., 2018)
d) Variations regarding microtome sectioning: sectioning one or multiple levels, sectioning blocks cooled in the freezer, sectioning blocks cooled on icy water, etc. (Xie et al., 2011).

Due to the rapid development of staining methods and molecular analysis new techniques for obtaining tissue microarrays (TMAs), for both research and diagnosis, are necessary and of major interest. In recent years, the main interest in the application of TMA methods has been directed towards clinical diagnostics. The idea of incorporating as many tissues as possible is aimed at reducing the use of consumables, specialized reagents for molecular testing, or to improve the quality of diagnosis by reducing/eliminating batch

variability in the many stages of histological workflow (Ștefan et al., 2020).

In 2013, a support matrix (BxChip™) was developed for parallel processing and sectionning of cylindrical biopsies of small diameters placed horizontally either directly by the surgeon in the operating room or transferred after arriving in the pathology laboratory. This sectionable matrix eliminates the fragmentation of the collected biopsies and increases the accuracy of the diagnosis (Farcaș et al., 2014; Jinga et al., 2012; Murugan et al., 2019; Mușat, 2013).

This paper presents a new sectionable matrix design that allows the multiplexing of tissue samples of irregular shapes and sizes and significantly decreases the required working time as well as the cost of consumables when compared with conventional histological methods.

MATERIALS AND METHODS

The first article describing the BxFrame™ sectionable matrix demonstrated that the formulation of this matrix withstands the decalcification solutions and dehydration protocols routinely used in pathology laboratories (Ștefan et al., 2021).

For this study, Themis Pathology SRL (Bucharest, Romania) provided two types of BxFrame™ GRID (Figure 1) sectionable matrices:

- Small matrices for regular histological cassettes: 22 mm x 16 mm x 2 mm (L x l x h)
- Matrices for large format cassettes: 30 mm x 22.6 mm x 2 mm (L x l x h)

The organs used for this experiment were:

- Heart, duodenum, brain, and tail from C57BL/6 mice provided by the Laboratory of Pathological Anatomy, "Ion Ionescu de la Brad" University of Agricultural Sciences and Veterinary Medicine, Iași, Romania.
- Pig skin provided by APOLLO slaughterhouse, Afumați, Ilfov, Romania.

Two methods were employed for grossing of the tissue samples, the “BxFrame™ method” in which multiple tissue samples were inserted in a single matrix (from each type of organ), and the conventional method where only one specimen was placed in each histological cassette (Table 1). The insertion of the samples into the sectionable matrices was conducted by breaking some dividing walls with the help of tweezers to accommodate them snugly but without any distortion.

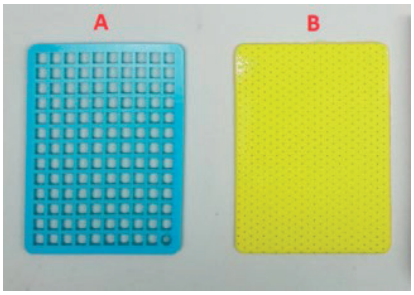


Figure 1. BxFrame™ GRID - (A) sectionable matrix; (B) sectionable perforated base

The processing protocol used had a total duration of 15 hours (Table 2) using a Sakura VIP 1000 tissue processor (Torrance, CA, USA). Before tissue processing, the mouse tails were subjected to decalcification with 5% formic acid (24 hours). All samples processed in this experiment were infiltrated and embedded in paraffin, using small and large metal moulds, and the resulting paraffin blocks were sectioned at 5 µm. During microtome sectioning, prior to facing the blocks, the yellow bases were completely removed (their role being strictly as a support for the tissues inserted in the matrix).

Table 1. Number of tissues and cassettes for each method

Method	Organs	Tissues	Cassettes
BxFrame	Duodenum	12	1
	Brain	8	1
	Tail	6	1
	Heart	4	1
	Skin	6	1
Conventional	Duodenum	12	12
	Brain	8	8
	Tail	6	6
	Heart	4	4
	Skin	6	6

Table 2. Tissue processing protocol schedule

Solvent	Time, (mins)	Temperature	P/V
NBF	60	37°C	Yes
70% Ethanol	90		
80% Ethanol	90		
95% Ethanol	60		
95% Ethanol	90		
100% Ethanol	60		
100% Ethanol	90		
Xylene	60		
Xylene	60		
Paraffin	60	60°C	Yes
Paraffin	60		
Paraffin	60		
Paraffin	60		

RESULTS AND DISCUSSIONS

Table 3 shows that the total working time required for six separate skin biopsies is almost three times longer than for biopsies processed simultaneously in a BxFrame™ GRID sectionable matrix. Although there is additional time needed during the step of loading the BxFrame™ GRID (cutting its walls and accurately loading biopsies), this extra-time is more than offset during paraffin embedding, sectioning, staining and examination of the resulting slides. In Figure 3 (Image C) it can be observed that the tissues maintain very well their position and orientation, and the use of consumables is significantly reduced.

Regarding the tail specimens it was observed that the period for processing independent biopsies is three times longer when compared to those multiplexed in the sectionable matrix. Although all tissues underwent a decalcification protocol, the sectionable matrix BxFrame™ GRID did not undergo any change in size or resilience keeping the tissues properly oriented. For heart tissue samples, the time required to process independent biopsies compared to a sectionable matrix was twice as long. The savings in terms of working time was slightly less than for the previous organs (due to the extra steps of cutting out the walls of the matrix and loading it with biopsies - since the hearts were small, they required more attention during their placement and orientation).

The working time benefit between independently processed brain samples and those placed in the BxFrame™ GRID was only 17 minutes. Similarly with heart biopsies, there was a longer time required for trimming appropriately the matrix but also for loading it with brain tissues because of its intrinsic fragile consistency. However, the time needed for sectioning a large paraffin block was half the time required for sectioning independent brain tissue. For duodenum the total working time was six times longer when processing independent biopsies versus the BxFrame™ GRID. Although it took nine minutes on average to load an array with twelve biopsies, there was no need to cut the matrix walls since the size of the BxFrame™ GRID cavities were perfectly matched to the size of the duodenum samples. Figure 2 is centralizing all the data regarding the working time needed for both methods (conventional and BxFrame™).

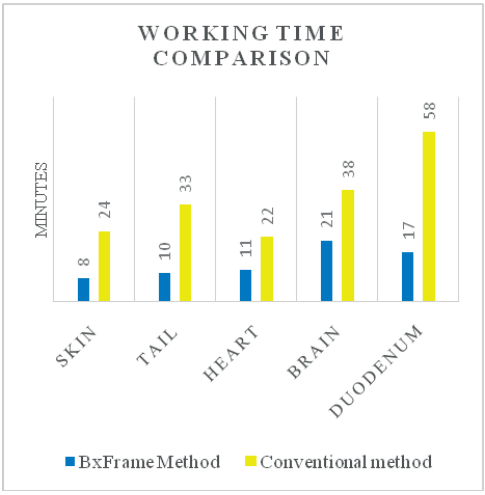


Figure 2. Comparison of total working time for the BxFrame™ technique vs the conventional method

For heart and brain samples, the total working time when sectionable matrices were used was reduced by half when compared with conventional methods.

Table 3. Detailed comparison between working time for BxFrame™ technique (A) and conventional method (B)

STEP	SKIN		TAIL		HEART		BRAIN		DUODENUM	
	A	B	A	B	A	B	A	B	A	B
Labeling tissue cassettes	10	60	10	60	10	40	10	80	10	120
Loading tissue cassette(s) with yellow base and BxFrame™GRID	15	-	17	-	23	-	25	-	20	-
Cutting walls of BxFrame™GRID	40	-	45	-	41	-	82	-	0	-
Loading tissues in BxFrame™GRID	100	-	105	-	160	-	435	-	557	-
Loading tissue cassettes with tissues	-	90	-	110	-	60	-	135	-	270
Embedding	60	209	84	260	51	146	95	270	120	539
Paraffin block cleaning	16	96	16	96	16	64	16	128	16	192
Microtome sectioning	199	695	315	1116	343	787	577	1232	280	1680
Labeling slides	6	36	6	36	6	24	6	48	6	72
Floating paraffin sections on the flotation bath	15	90	15	90	15	60	15	120	15	180
Loading the slides in the staining rack	4	25	4	25	4	16	4	32	4	48
Coverslipping	30	180	30	180	30	120	30	240	30	360
Total Time (seconds)	495	1481	647	1973	699	1317	1295	2285	1058	3461
Total Time (minutes)	**8**	**24**	**10**	**33**	**11**	**22**	**21**	**38**	**17**	**58**
Decrease (%)	**66.7**		**69.7**		**50.0**		**44.7**		**70.7**	

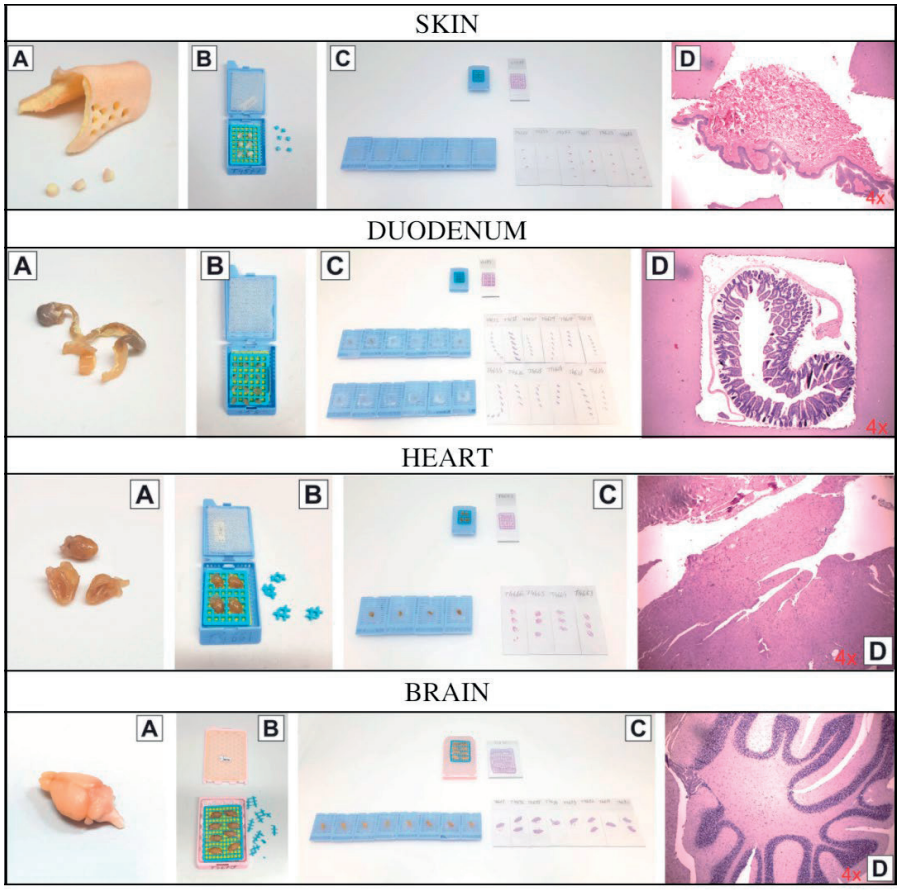


Figure 3. BxFrame™ GRID vs. conventional method: (A) Organ, (B) Loaded matrix, (C) Paraffin blocks and slides, (D) Microscopic image, 4x objective, haematoxylin-eosin stain

In the case of the tail and skin, total working time duration when employing the BxFrame™ was 3 times less than the conventional method while for small cylindrical tissue samples such as the duodenum, the savings in labour were the most spectacular since these samples can be placed directly in the receptacles of the sectionable matrix.

The analysis of the cost savings (consumables) offered by the sectionable matrix BxFrame™ GRID is presented in Table 4. The comparison was made only for tail, heart, duodenum, and skin. Mouse brains were excluded from this statistic because these tissue samples were larger in size and required sectionable matrices with different manufacturing costs, larger amounts of dehydration solvents, paraffin, etc. Prices for consumables were updated according to the 2021 Romanian market and are detailed for each histological step separately.

Tissue cassettes, sectionable matrices, histological sponges and containers with neutral formalin are consumables considered for the actual harvesting of biopsies (in the operating room) or during grossing (in the pathology laboratory). For tissue processing the working volume of reagents (dehydrants, clarifier and paraffin) was 30 ml, the minimum required for an adequate processing of the tissues, regardless of the protocol or specific tissue processor used. Paraffin block casting was estimated to require an average of 3 g of paraffin per block. Regarding glass slides all calculations were made assuming 3 slides per paraffin block will be used (2 levels for the actual diagnostic and a spare for eventual additional stains).

The skin and tail tissues required lower material costs for the BxFrame™ method versus conventional method (€13.95 versus €23.85, respectively). For heart samples, the differences are less spectacular. The material costs for the multiplexing method (€13.95) are quite similar to the costs incurred when using conventional methods (€15.90), due to the additional cost of the matrix. The advantage of using the new method in this case resides mainly in the superior preservation of the orientation of tissue samples within the matrix and the shorter processing time.

The largest cost saving is evident in the case of duodenum samples (€13.95 versus €47.70, for the BxFrame™ method versus conventional method, respectively). Figure 4 summarises the comparative costs of both methods, for all tissues examined.

A limitation of our study is the use of a single type of tissue processor and of a single dehydration protocol with predetermined conditions. However, preliminary results are suggesting that the previous observations are valid even when employing very different working situations, such as microwave-assisted tissue processors (where the protocols are much harsher: shorter dehydration protocols, solvents and paraffin heated to almost 70 degrees Celsius, and the pressure and vacuum can vary between 150 to 900 mBar).

One important aspect to be considered in the case of the BxFrame™ GRID technique is regarding the stage of loading the tissue samples within the sectionable matrix. The qualified person who performs this procedure must be careful that the matrix material does not dry out during loading, otherwise, the matrix becomes fragile, and it can become difficult to section after paraffin infiltration. In our experiments, a team of two skilled histotechnologists performed the loading of specimens in the matrices, but the learning curve for a novice who must carry out this step is quite short.

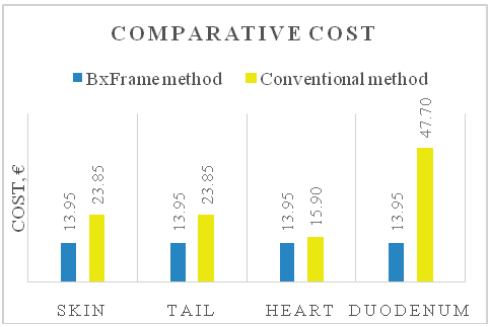


Figure 4. Comparative cost: BxFrame™ technique versus conventional method

Table 4. Cost comparison of consumables for BxFrame™ versus conventional method

Consumable	Price (€)	per	Skin		Tail		Heart		Duodenum	
			BxFrame	6 Biopsies	BxFrame	6 Biopsies	BxFrame	4 Biopsies	BxFrame	12 Biopsies
Tissue cassette	0.1	Cassette	0.10	0.60	0.10	0.60	0.10	0.40	0.10	1.20
BxFrame™ GRID	10	Unit	10.00	0.00	10.00	0.00	10.00	0.00	10.00	0.00
60 ml NBF container	2	Unit	2.00	12.00	2.00	12.00	2.00	8.00	2.00	24.00
Histological sponges	0.025	Unit	0.03	0.30	0.03	0.30	0.03	0.20	0.03	0.60
Processing cost for 1 cassette	0.25	30 ml	0.25	1.50	0.25	1.50	0.25	1.00	0.25	3.00
Paraffin (3g for one block)	0.045	Gram	0.14	0.81	0.14	0.81	0.14	0.54	0.14	1.62
Slides (2 levels and 1 spare)	0.3	Slide	0.90	5.40	0.90	5.40	0.90	3.60	0.90	10.80
Staining for 2 slides	0.25	10 ml	0.50	3.00	0.50	3.00	0.50	2.00	0.50	6.00
Coverslipping for 2 slides	0.02	Coverslip	0.04	0.24	0.04	0.24	0.04	0.16	0.04	0.48
TOTAL COST, €			**13.95**	**23.85**	**13.95**	**23.85**	**13.95**	**15.90**	**13.95**	**47.70**
Cost Decrease, %			**41.5**		**41.5**		**12.3**		**70.8**	

CONCLUSIONS

Multiplexing tissue specimens using the BxFrame™ GRID sectionable matrix reduces the working time required for histological preparations by more than half compared with conventional methods. The costs for consumables are considerably reduced up to 5 fold when compared with the costs needed for conventional methods. Tissue specimens inserted in the sectionable matrices maintain their orientation in a single plane throughout processing and embedding, inking is no longer necessary for traceability and microtome sectioning is performed without difficulty so that the histological section includes all the tissues surrounded by the BxFrame™ GRID walls.

ACKNOWLEDGEMENTS

Conflicts of interest: Alina Elena Ștefan, Daniela Gologan and Sorin Mușat are Themis Pathology SRL employees. Matthew Okerlund Leavitt is a shareholder of LUMEA Inc (the sole proprietor of Themis Pathology SRL). The first and the second authors had equal contribution in this study.

REFERENCES

Buesa, R. J. (2010). Productivity standards for histology laboratories. *Ann Diagn Pathol*, *14*(2), 107–124.

Farcaș, C.P., Spînu, A.D., Petrescu, A., Dumitrescu, M., Vasilescu, F., Dinu, M., Mischianu, D., Mușat, S. (2014). High Throughput processing and analysis of prostate core biopsies for research applications. *Revista Română de Urologie*, *13*, 9–16.

Gologan, D., Ștefan, A. E., Stan, R., Militaru, M., Mușat, M. (2021). Factors influencing the quality of formaldehyde fixed paraffin embedded tissue samples – Review. *Revista Română de Medicină Veterinară, 31*, 87–92.

Jinga, V., Petrescu, A., Berdan, G., Tănase, F., Braticevici, B., Amuzescu, B., Mușat, S., Moișanu, D., Rădăvoi, D. (2012). C174 A comparison between classical and simultaneous processing of prostate core biopsies using Histobest Biopsy Chip™. *European Urology Supplements, 11*(4), 124.

Murugan, P., Shukla, D., Morocho, J., Smith, D., Sciacca, D., Pickard, Wahlsten, M., Gunderson, A., Konety, A., Konety, B., Khalifa, M. A., Warlick, C. (2019). Prostate Biopsy Processing: An Innovative Model for Reducing Cost, Decreasing Test Time, and Improving Diagnostic Material. *American Journal of Clinical Pathology, 152*(6), 757–765.

Mușat, S. (2013). Matrix for receiving a tissue sample and the use thereof. US Patent 9,851,349

Ștefan, A. E., Gologan, D., Leavitt, M. O., Mușat, S., Pleșea, I. E., Stan, R., Pleșea, R. M., Militaru, M. (2020). Tissue MicroArrays, brief history, techniques and clinical future. *Romanian Journal of Morphology & Embryology, 61*(4), 1077–1083.

Ștefan, A. E., Gologan, D., Mușat, S., Stan, R., Sanda, C. A., Militaru, M. (2021). Biomimetic matrices for histological processing. *Revista Română de Medicină Veterinară, 31*(3), 12–16.

Suvarna, K. S., Layton, C., Bancroft, J. D. (2018). *Bancroft's Theory and Practice of Histological Techniques* (8 ed.): Elsevier Health Sciences.

Varma, M., Berney, D. M., Algaba, F., Camparo, P., Comperat, E., Griffiths, D. F., Kristiansen, G.,

Beltran, A. L., Montironi, R., Egevad, L. (2013). Prostate needle biopsy processing: a survey of laboratory practice across Europe. *J Clin Pathol, 66*(2), 120–123.

Wang, J., Wan, B., Li, C., Wang, J., Fu, Q., Zhao, W., Zhang, K. (2015). Diagnostic yield and complications using a 20 gauge prostate biopsy needle versus a standard 18 gauge needle: A Randomized Controlled Study. *Urol J, 12*(5), 2329–2333.

Xie, R., Chung, J.-Y., Ylaya, K., Williams, R. L., Guerrero, N., Nakatsuka, N., Badie, C., Hewitt, S. M. (2011). Factors influencing the degradation of archival formalin-fixed paraffin-embedded tissue sections. *Journal of Histochemistry & Cytochemistry, 59*(4), 356–365.

ISSN 2065 – 1295
ISSN-L 2065 – 1295