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SUMMARY

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COMPARISON OF ZINC HAIR MINERAL LEVELS IN CATS WITH LIVER AND KIDNEYS DISORDERS

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Abstract

This research's main objective was the assessment of zinc levels in hair from cats with different liver and kidneys disorders, compared to control group. Zinc (Zn) hair content analysis of the cats with liver disorders (n = 6), cats with kidneys disorders (n = 9), and clinically healthy cats as control (n = 6), was performed by inductively coupled plasma-optic emission spectrometry (ICP-OES). Zn concentrations in hair were significantly different (p = 0.015) between males with liver disorders (22.10 mg kg^{-1}) and males with kidneys disorders (6.181 mg kg^{-1}), and no significant differences have been observed compared to zinc mean concentrations in clinically healthy males (10.60 mg kg^{-1}). Significant differences were observed when comparing Zn mean levels in males with different kidneys disorders to Zn levels in clinically healthy males. No significant differences depending on health status or age were found in either studied category. The highest zinc mean value was found in hair samples from male cats with liver disorders, and the lowest zinc mean value was found in male cats with kidneys disorders. This research on zinc assessment from hair, an easy and non-stressfully collected sample, shows that this kind of sample could be appropriate for the evaluation of the mineral status of cats with different organ dysfunctions in urban areas. Hair zinc levels found in this study may contribute to the database of reference concentrations of minerals in cats from Romania.

Key words*: hair, cats, zinc, liver disorders, kidneys disorders.*

INTRODUCTION

Zn is an essential metal necessary for the proper functioning of organisms. Zinc is relatively abundant in nature and is widely used in industry and agriculture (Sloup *et al*., 2017).

Zn is a cofactor of over 3000 proteins or enzymes (Beyersmann & Haase, 2001; Andreini *et al.*, 2006; Mohommad *et al.*, 2012) being the only metal that is part of all six classes of enzymes: oxidoreductase, transferase, hydrolase, lyase, isomerase, ligase (Webb, 1992; Mohommad *et al*., 2012; Cornish-Bowden, 2014). Zn is also essential for cell proliferation and differentiation processes, especially for DNA synthesis and mitosis (Beyersmann & Haase, 2001; Mohommad et al., 2012).

Unlike other transition metals, such as Fe or Cu, Zn has no redox activity (Berg & Shi, 1996).

In patients with chronic kidney disease, Zn deficiency/altered metabolism is well documented, especially in those with nephrotic disease and uremia (Mahajan, 1989), and is also observed in patients with many types of liver disease (Mohommad *et al*., 2012).

The change in Zn metabolism can be determined by the decreased dietary and intestinal absorption levels, increased endogenous secretion and urinary excretion of Zn (Mahajan, 1989; Mohommad et al., 2012), activation of certain zinc transporters, and induction of hepatic metallothionein (Mohommad *et al.*, 2012). Zn is excreted mainly through urine, but also through perspiration, saliva, and is incorporated into the hair (Goran & Crivineanu, 2016c; Sloup *et al.*, 2017).

The liver and kidneys' roles in xenobiotics metabolism and excretion expose them to high levels of toxic substances and their metabolites (Osweiler, 1996a; 1996b; Goran & Crivineanu, 2016a; 2016b).

The mineral composition of the hair reflects the mineral content of the body's tissues. If there is a mineral deficiency or excess in the hair, it usually indicates a mineral deficiency or excess inside the body, although sometimes it can mean the opposite.

Thus, the concentration of minerals and heavy metals in animal hair reflects the presence of these elements in the surrounding forage and soil and varies from area to area, providing information on pollution in the area (Rashed & Soltan, 2005).

Because of hair's easy and non-stressful sampling way, it is used for evaluation of the mineral content of the animal organisms. Hair mineral analysis has become a routine analysis since the early 1970s and it is increasingly used, both for the assessment of pollution in the area, as well as for the evaluation of the involvement of minerals/metals in various pathologies in animals and humans (Combs *et al.*, 1982; Foo *et al.*, 1993; Poon *et al.*, 2004; Adams *et al.*, 2006; Goran & Crivineanu, 2007; Długaszek *et al.*, 2008; Skibniewska *et al.*, 2011; Kosla *et al.*, 2011; Kolachi *et al.*, 2012; Skibniewski *et al.*, 2013; Panhwar *et al.*, 2013; Wołowiec *et al.*, 2013; Roug *et al.*, 2015; Badea *et al.*, 2016; Badea *et al.*, 2017; Goran *et al.*, 2017a; Goran *et al.*, 2017b).

The goal of this study was to evaluate the levels of zinc in hair samples from cats with different liver and kidneys disorders, compared to control samples, using inductively coupled plasma-optic emission spectrometry (ICP-OES).

MATERIALS AND METHODS

Sampling and samples preparation

Analysis of hair Zn content of cats with liver disorders (n = 6), cats with kidneys disorders (n = 9), and clinically healthy cats as control (n = 6) was performed by ICP-OES.

The number of animals in this study broken down into categories depending on age and sex in each studied group are presented in Table 1.

The cats with liver and kidneys disorders showed symptoms that led to the suspicion of organ injuries, and the diseases were confirmed by biochemical blood tests and ultrasound examination.

For all studied animals, the hair samples were collected as close to the skin as possible from the flank region, placed in labelled paper envelopes, and transported to the laboratory, where the hair samples were stored in a dark, dry place, with a constant temperature.

The samples were prepared initially by degreasing, washing, rinsing, and drying, and then they were weighed and mineralized. All hair samples were digested using a Speedwave MWS-2 Berghof microwave oven as following: Step 1: 120ºC, power 50%; Step 2: 180ºC, power 75%; Step 3: 100ºC, power 40%. The samples were then analyzed to assay the Zn levels by ICP-OES.

Table 1. Studied cats depending on health status, age and sex

HA	6	**< 8**	4	**M**	1
				F	3
		> 8	2	**M**	1
				F	1
LD	9	**< 8**	2	**M**	1
				F	1
		> 8	7	**M**	4
				F	3
KD	6	**< 8**	1	**M**	0
				F	1
		> 8	5	**M**	1
				F	4

*HA - clinically healthy animals; LD - animals with liver disorders; KD - animals with kidneys disorders; < 8 - animals below the age of 8; > 8 - animals above the age of 8; M - male cats; F - female cats.

For all studied animals, the hair samples were collected as close to the skin as possible from the flank region, placed in labelled paper envelopes, and transported to the laboratory, where the hair samples were stored in a dark, dry place, with a constant temperature.

The samples were prepared initially by degreasing, washing, rinsing, and drying, and then they were weighed and mineralized. All hair samples were digested using a Speedwave MWS-2 Berghof microwave oven as following: Step 1: 120ºC, power 50%; Step 2: 180ºC, power 75%; Step 3: 100ºC, power 40%. The samples were then analyzed to assay the Zn levels by ICP-OES.

Spectrometric analysis

For mineralization all hair samples were treated with 5 mL HNO_3, 0.8 mL HCl and 1 mL H_2O_2, then diluted to 10 mL with ultrapure water and analyzed using a Thermo iCAP ICP-OES spectrometer (RF1100 W; reading time 30 s, washing time 30 s, nebulizer gas flow 0.5 L/min; auxiliary gas flow 0.5 L/min; sample injection pump flow 50 rpm). Calibration curves were developed using standard solutions of 0.001 mg kg^{-1}, 0.01 mg kg^{-1}, 0.1 mg kg^{-1}, 1 mg kg^{-1}, 5 mg kg^{-1}, 10 mg kg^{-1}, 50 mg kg^{-1} obtained by dilution

from a multi-element ICP MERCK standard containing 1000 mL/L of Zn.

Statistical analysis

VassarStats software: Website for Statistical Computation (http://vassarstats.net/) was used for performing One-Way ANOVA for all samples' mineral concentrations. In order to verify the ANOVA assumptions, Student's t-test (Microsoft Excel) with unequal sample sizes, unequal variances (Welch's *t*-test), was used.

RESULTS AND DISCUSSIONS

The mean Zn contents of hair samples from clinically healthy cats and those with different liver and kidneys disorders are presented dependent of age and sex in Table 2 and expressed as mg kg^{-1}.

Mean Zn levels found were higher in HA and LD cats compared to the cats with KD but with no significant differences ($p = 0.168$).

The highest mean Zn concentration was found in the group of LD cats (11.916 mg kg^{-1}).

The Zn hair concentrations reported by other authors were significantly different in some studies such as Skibniewska *et al.* (2011) who evaluated the Zn levels in pet and feral cats' hair. They reported much greater values compared to those found in our study. They determined that in feral cats were determined greater values (250.52 mg kg^{-1}) than in pet cats (227.28 mg kg^{-1}). Other studies on cats' hair mineral content from Bucharest (Romania) also reported mean hair Zn values comparable to those found in all these study groups (Badea *et al.*, 2016; Goran *et al.*, 2017a).

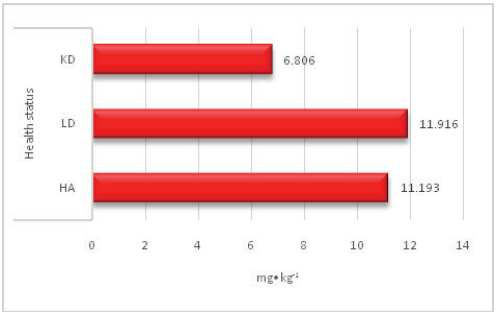
Table 2. Mean Zn levels in cat hair samples depending on health status, age and sex (mg kg^{-1})

Health status						
Element	**Health status**	**Mean**	**SD**	**Std err mean**	***p*-value**	**Welch t-test**
Zn	HA	11.193 [a]	2.839	1.159		HA/LD=0.289
	LD	11.916 [a]	2.517	0.839	*0.168*	HA/KD=0.009
	KD	6.806 [b]	8.472	3.459		LD/HD=0.05
Age						
Element	**< 8 years**	**Mean**	**SD**	**Std err mean**	***p*-value**	**Welch t-test**
Zn	HA	11.290 [a]	3.414	1.707		HA/LD=0.312
	LD	6.779 [a]	1.158	0.819	*0.334*	HA/KD=0.060
	KD	13.000 [a]	0	0		LD/HD=0.059
	> 8 years	**Mean**	**SD**	**Std err mean**	***p*-value**	**Welch t-test**
	HA	11.000 [a]	0.9	0.636		HA/LD=0.018
	LD	11.700 [b]	9.266	4.144	*0.442*	HA/KD=0.009
	KD	6.814 [b]	2.786	1.053		LD/HD=0.089
Sex						
Element	**Males**	**Mean**	**SD**	**Std err mean**	***p*-value**	**Welch t-test**
Zn	HA	**10.600** [a]	1.3	0.65		HA/LD=0.382
	LD	**22.100** [a]	0	0	***0.015***	HA/KD=0.039
	KD	**6.181** [ab]	3.075	1.375		LD/HD=0.265
	Females	**Mean**	**SD**	**Std err mean**	***p*-value**	**Welch t-test**
	HA	11.490 [a]	3.313	0.919		HA/LD=0.373
	LD	9.880 [a]	7.826	3.501	*0.662*	HA/KD=0.049
	KD	7.589 [ab]	1.154	1.537		LD/HD=0.111

*Levels not connected by the same letter are significantly different.
**LD - liver disorders; KD - kidneys disorders; HA - healthy animals.

Although a higher mean Zn hair content was observed in the group of younger KD cats (13.0 mg kg^{-1}), the age of cats did not influence the Zn hair level in a statistically significant way. As it is presented in Figure 2, the lowest mean Zn content was found in the group of LD cats, independent of age (6.779 mg kg^{-1} in cats below 8 years of age, and 6.814 mg kg^{-1} in cats above 8 years of age).

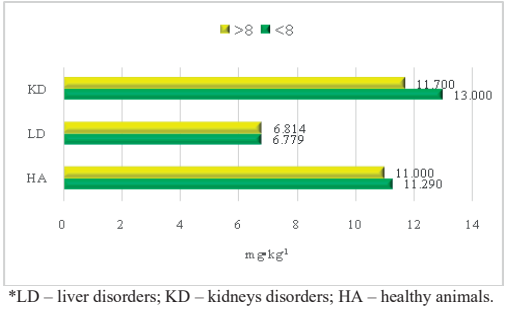
Another study on hair mineral content also has reported that in both studied groups below and above 5 years of age, mean Zn levels were higher than in the cats with kidneys failure (Badea *et al.*, 2016).



*LD - liver disorders; KD - kidneys disorders; HA - healthy animals

Figure 1. Mean Zn levels in hair samples from cats with LD or KD compared to HA

In a study on female cats' hair mineral content was reported a significantly lower mean Zn hair level in clinically healthy animals (10.1 mg kg^{-1}), compared to its value found in cats with chronic hepatitis (18.4 mg kg^{-1}) (Goran *et al.*, 2017a). Another study on cats' Zn hair content reported higher values in older cats (242.14 mg kg^{-1}) (Skibniewska *et al.*, 2011).



*LD – liver disorders; KD – kidneys disorders; HA – healthy animals.

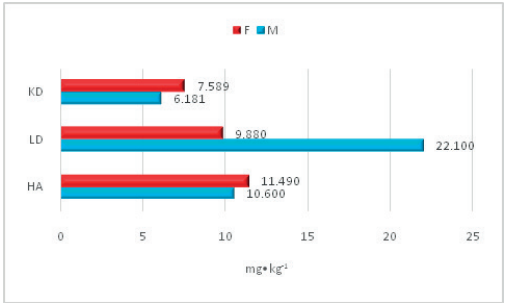
Figure 2. Mean Zn levels in hair samples from cats with LD or KD compared to HA, depending on age

Zn hair content provides useful information about the Zn body's levels, but the interpretation can be complex, as both high and low hair Zn levels may indicate low Zn body levels (Cutler, 2004). These Zn levels could be influenced by the disturbed capacities of liver metabolization in LD animals, and increased excretion in KD animals, correlated to the organism low capacity of Zn compounds use, which are also reflected in hair mineral content. Zn hair levels recorded significant differences between its levels ($p = 0.015$), the highest concentrations being found in male cats from LD group hair samples (22.1 mg kg^{-1}), followed by those in female HA cats, which were almost 2 times lower (Figure 3). The significant differences correlations between Zn hair levels could be made between hair samples from HA and LD cats vs. those from KD cats ($p < .05$). The Zn hair levels' differences between HA cats and LD cats were not statistically significant.

Skibniewska *et al.* (2011) have reported Zn hair levels much higher than those found in the present study, independent of health status, with higher levels in female feral cats (268.09 mg kg^{-1}), and lower in pet female cats (214.49 mg kg^{-1}).

Even the Zn hair levels found in females, independent of health status, had no significant differences ($p > 0.05$), it can be observed that Zn hair values were lower in LD and KD cats compared to HA animals.

Other researches on hair mineral content showed that Zn hair level in male cats (6.18 mg kg^{-1}) and female cats (7.59 mg kg^{-1}) with renal failure, were lower than clinically healthy animals (Badea *et al.*, 2016), which was not the case in hair samples of cats with chronic hepatitis (Goran *et al.*, 2017a).



*LD - liver disorders; KD - kidneys disorders; HA - healthy animals.

Figure 3. Mean Zn levels in hair samples from cats with LD or KD compared to HA, depending on sex

Some toxic metals and essential metals have common chemical characteristics, which could lead to interactions (Goyer, 1997). There was reported that interactions between essential and toxic metals indicate that toxic elements compete with the essential metals, but at the same time the mineral status assay needs to be realized on a specific tissue or organ (López Alonso *et al.*, 2004). Also, choosing potential target organs for the metals' concentrations assessment needs to be carefully analyzed for interfering elemental interactions (Elsenhans *et al.*, 1987).

CONCLUSIONS

This study presents investigations of the hair use as a mineral biomarker for the cats' hepatic and renal pathology in a Romanian urban area.
Generally, the highest Zn mean level was found in cats with liver disorders, and the lowest in cats with kidneys disorders.
The highest Zn mean value recorded significantly difference in hair samples from male cats with liver disorders compared to the lowest Zn mean value, found in male cats with kidneys disorders hair samples.
This research on Zn assessment from hair, an easy and non-stressfully collected sample, shows that this kind of sample could be appropriate for the evaluation of the mineral status of cats with different organ dysfunctions in urban areas.
Zn recorded insignificant differences between its levels in hair samples from female cats independent of the health status, and all animals independent of age and health status.
Mean Zn hair concentrations were below the determined levels in cats reported in studies in other countries.
Zn hair levels reported in the present research may *contribute* to a database of reference levels of minerals in cats in Romania.

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STUDY REGARDING THE VARIATIONS OF SOME HAEMATOLOGIC, ELECTROLYTIC, ENZYMATIC AND BIOCHEMICAL PARAMETERS IN EARLY LACTATION IN EWES

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Abstract

The aim of this study is to highlight the influence of early lactation physiological stage on the haematological, electrolytic, enzymatic and biochemical profiles in ewes. The studied parameters (WBC, RBC, Hb, Ht, MCV, MCH, MCHC, Ca, P-inorganic, K, Na, Cl, ALT, AST, CK, GGT, LDH, glucose, urea, cholesterol, triglycerides, creatinine, total proteins, albumin, total bilirubin) were determined in the 20th day of the lactation period, the results obtained being compared to those of the witness group of non-pregnant nor lactating ewes. Important variations of the studied parameters were observed. The WBC value (7.21 ± 0.29 m/mm^3) decreased significantly ($p<0.05$); the calcium and phosphorus values (9.20 ±2.56 ;4.25± 0.60 mg/dL) decreased non-significantly ($p>0.05$); the AST (21.60 ± 2.41 U/L) and GGT (12.10 ± 1.45 U/L) activity increased significantly ($p<0.05$); the glucose (66.1 ± 8.43 mg/dL) and cholesterol (47.90 ± 7.01 mg/dL) values decreased significantly ($p<0.05$). The results are correlated with the high energy demand in order to support the mammary gland intense activity of milk synthesis in the first third of the lactation period.

Key words*: biochemical parameters, ewes, haemoleucogram, lactation.*

INTRODUCTION

Ewes' breeding is closely correlated with the productive and reproductive potential, the outstanding adaptability and resistance to the various environmental conditions and the superior bio economical characteristics of this species. In order to increase the profitability and efficiency of the ewes' exploitation, it is necessary to know each individual's necessities imposed by the physiological stage in which it is found.

In this context, the haematology and blood biochemistry analyses allow monitoring the animal health status in various physiological stages. The importance of the main blood parameters determination is highlighted by the fact that the wide variations of their mean values may cause disturbances of the homeostasis and, implicitly, systemic disorders, even if the animal's general condition remains unchanged (Crivineanu et al., 2010; Turcitu et al., 2012).

The first third of lactation period, considered by most authors the interval between the first and the 60th day of lactation, as the average lactation period in ewes is about 180 days long, represents a metabolically demanding physiological stage (Anwar et al., 2012). Thus, the main objective of this study was monitoring a series of blood parameters (haematological, electrolytic, enzymatic and biochemical) in the 20th day of lactation in order to reveal their fluctuations, therefore the influence of this physiological stage, the research being also focused on two main aspects: maintaining the ewes' health and maintaining a high level of productivity.

MATERIALS AND METHODS

The study was carried out between August 2017 and April 2018 in a livestock of ewes belonging to a household in the village of Comosteni, Dolj County. The experimental group consisted of 10 healthy individuals, with no history of pathologies, approximately equal body mass and multiparous.

The blood sampling was performed on: August 15, 2017 for non-pregnant ewes and February 25, 2018 for lactating ewes, on the 20th day of lactation. The haematological parameters were determined by using whole blood and the MS-45™ haematology analyser (Melet Schloesing

Laboratoires, France). The biochemical and enzymatic parameters were determined by using plasma and the Spotchem EZ 4430 ARK RAY analyser. For the electrolytic parameters, plasma was collected in sterile syringes, which were then labelled, refrigerated and dispatched to a commercial laboratory according to the standard laboratory procedures, the samples being processed by automatic analysers using the spectrophotometric (colorimetric) method.

RESULTS AND DISCUSSIONS

Haematological profile

The haematological parameters variations in the first third of the lactation period were predominantly not significant from a statistic point of view ($p>0.05$) and the mean values shown in Table 1 and Figure 1 were found within the limits of the reference ranges of this species for all studied parameters.

Table 1. The haematological profile' mean values in non-pregnant ewes and in the 20th day of the lactation period

PARAMETER	PHYSIOLOGICAL STATUS	
	Non-pregnant	20th day of lactation
WBC (m/mm^3)	8.32 ± 0.44	7.21 ± 0.29**
RBC (M/mm^3)	11.04 ± 0.77	10.17 ± 1.38*
Hb (g/dL)	12.07 ± 1.11	11.20 ± 0.94*
Ht (%)	36.1 ± 3.32	34.3 ± 2.95*
MCV(fl)	32.7 ± 2.11	33.7 ± 2.01*
MCH (pg)	10.9 ± 0.78	11.0 ± 0.57*
MCHC(g/dL)	33.4 ± 2.01	32.6 ± 1.97*

*p>0.05 - statistically non-significant differences
**p<0.05 - statistically significant differences

The analysis of the main haematological parameters (WBC, RBC, haemoglobin, haematocrit, MCV, MCH, MCHC) in correlation with the physiological status in ewes revealed that in the 20th day of the lactation period can be observed a statistically significant decrease ($p<0.05$) of the WBC mean value and a non-significant decrease ($p>0.05$) of the RBC and haemoglobin compared to the mean values obtained in non-pregnant ewes, that also represented the control group. Other studies regarding the haematological parameters in sheep or cattle reported similar results, the main cause incriminated by most authors being the migration of the leukocytes from blood to milk in order to protect the mammary gland from the pathogens' action by phagocytosis, but also in order to provide the necessary load of antibodies for the new-born lamb (Antunovic et al., 2011). The high concentration of white blood cells in ewes' milk was demonstrated and highlighted using milk cytological investigations in other research studies (Ognean et al., 2016). Also, a RBC mean value ($p>0.05$) decrease was observed, this results being consistent with those recorded by other authors and mentioned in the specialized literature. MCV, MCH and MCHC remained unchanged throughout the study not showing any statistically significant variations ($p>0.05$).

The overall results regarding the haematological profile are in accordance with various studies on this topic, present in the speciality literature (Anwar et al., 2012; Khaled. & Illek, 2012; Bamerni, 2013).

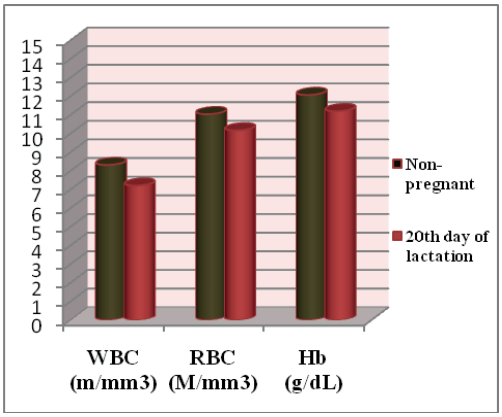

Figure 1. The dynamics of the WBC, RBC and Hb mean values in non-pregnant ewes and in the 20th day of the lactation period

The variations of the haematological parameters observed in the first third of the lactation period, respectively on the 20th day of lactation in ewes, were predominantly not significant from a statistical point of view ($p>0.05$). The analysis of the haematological parameters in correlation with the physiological status revealed that at the beginning of the lactation period, there can be observed a significant decrease of the mean values of the white blood cells count ($p<0.05$) and a non-significant decrease ($p>0.05$) of the mean

values of erythrocytes count and haemoglobin concentration compared to the mean values obtained in non-pregnant ewes. The main cause of the white blood cells count mean values significant decrease in the 20th day of lactation (*p>0.05*) was the massive migration of the leukocytes from the mother's blood into milk in order to provide the necessary antibodies for the new-born lamb and also to protect the mammary gland by phagocytosis.

Electrolytic profile

There have been observed important variations of the electrolytic parameters mean values, this findings being most likely associated with the increased milk synthesis in the early stages of lactation. The evolution of the mean values of the electrolytic profile parameters is presented in Table 2. The variations of the electrolytic parameters are considered by the speciality literature to be expected in this metabolically demanding period, in this species, not being associated, normally, with pathological stages (Codreanu, 2016).

Table 2. The electrolytic profile' mean values in non-pregnant ewes and in the 20th day of the lactation period

PARAMETER	PHYSIOLOGICAL STATUS	
	Non-pregnant	20th day of lactation
Ca (mg/dL)	9.45 ± 2.72	9.20 ± 2.56*
P-inorganic (mg/dL)	4.31 ± 0.64	4.25 ± 0.60*
K (mmol/L)	5.98 ± 0.49	6.28 ± 0.69*
Na (mmol/L)	153.97 ± 2.09	159.70 ± 2.15*
Cl (mmol/L)	102.55 ± 3.41	117.30 ± 0.95*

*p>0.05 – statistically non-significant differences

The decrease of the calcium blood concentration in ewes after parturition, at the beginning of the lactation period can be associated with the increased calcium secretion through milk and, implicitly, its rearrangement in bones (Liesegang et al., 2007). Calcium and phosphorus are mobilized in similar ways, but larger quantities of calcium are excreted through milk. Phosphorus isn't secreted in milk in the same amount as calcium, therefore its blood concentration being higher.

The potassium mean values increased non-significant (*p>0.05*) in the 20th day of the lactation period compared with the results obtained in non-pregnant ewes. The fluctuations of the main electrolytes concentrations, especially potassium, may lead to structural and functional imbalances and also to tissues and organs dysfunctions. For example, skeletal and cardiac muscle dysfunctions may occur when the potassium level fluctuates, therefore maintaining a constant potassium level is necessary in order to maintain the homeostasis.

The electrolytic parameters variations in the first third of the lactation period, respectively on the 20th day of lactation, are associated with the increased milk synthesis and their excretion through milk.

Enzymatic profile

The blood enzymes activity of the studied group of ewes is presented in Table 3 and Figure 2. All the studied enzymatic parameters fell between the reference range values, except for the GGT activity that slightly exceeded the upper limit of the reference interval in the 20th day of lactation. Significantly elevated concentrations of AST and GGT were detected in lactating ewes compared to not pregnant ewes ($p<0.05$). An opposite trend - decreasing - was observed in the ALT concentration but with no statistically significant differences ($p>0.05$).

The significant increase of the AST and GGT activity in lactating ewes indicates, most likely, an increase of the hepatic metabolism (Codreanu & Călin, 2018). The results are consistent with those recorded in various other studies regarding the influence of the physiological status on the enzymatic activity in ewes and cattle (Antunovic et al., 2011; Anwar et al., 2012; Caldeira et al., 2007). The high AST values observed on the 20th day of lactation when the highest milk production is expected to occur indicate a stimulation of the liver function, phenomenon associated most of the time with high productivity periods.

CK mean activity showed a moderate increase but with no statistically significant variations ($p>0.05$).

LDH activity was also visibly increased at the beginning of the lactation period, but with no statistically significant differences (*p>0.05*).

All the enzymatic variations are due to an intense liver activity in lactating ewes, in order

to sustain an increased energy and protein demand imposed by the milk production.

Table 3. The enzymatic profile' mean values in non-pregnant ewes and in the 20th day of the lactation period

PARAMETER	PHYSIOLOGICAL STATUS	
	Non-pregnant	20th day of lactation
ALT (U/L)	19.90 ± 2.58	16.44 ± 3.73*
AST (U/L)	15.30 ± 3.74	21.60 ± 2.41**
CK (U/L)	87.50 ± 7.19	84.20 ± 6.28*
GGT (U/L)	8.88 ± 2.31	12.10 ± 1.45**
LDH (U/L)	412.50 ± 44.75	486.60 ± 69.19*

*p>0.05 – statistically non-significant differences
**p<0.05 – statistically significant differences

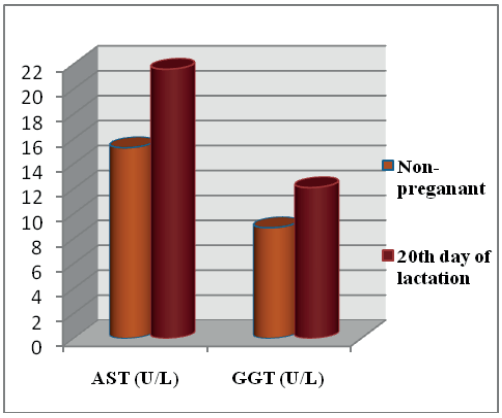

Figure 2. The dynamics of the AST and GGT activity in non-pregnant ewes and in the 20th day of the lactation period

The mean blood enzymatic activity of the studied group of ewes intensified in the first third of the lactation period. The mean activity of AST showed a significant increase ($p<0.05$) at the start of the lactation period, not exceeding the upper limit of the reference range. GGT activity also increased significantly ($p<0.05$) in the 20th day of the lactation period, even exceeding the upper limit of the reference range for this species. The enhanced enzymatic activity indicates an intensification of the hepatic functions in lactating ewes, in order to support the milk production and, implicitly, the increased energy and protein demand.
The overall results obtained in the enzymatic profile review indicate that no pathological entity was involved in this case, according to the speciality literature, the variations of the enzymatic profile being associated with the physiological stage, not with a pathological event (Codreanu, 2016).

Biochemical profile

The mean values of the studied biochemical parameters are shown in Table 4 and Figure 3.
There have been observed important variations of the studied parameters, their mean values generally fell within the range of the reference values for this species. Variations of the blood levels of some biochemical indicators such as glucose, cholesterol, triglyceride and urea - indicate an energy shortage in ewes at the beginning of the lactation period.

Table 4. The biochemical profile' mean values in non-pregnant ewes and in the 20th day of the lactation period

PARAMETER	PHYSIOLOGICAL STATUS	
	Non-pregnant	20th day of lactation
Glucose (mg/dL)	90.3 ± 13.03	66.1 ± 8.43**
Urea (mg/dL)	21.00 ± 2.80	23.77 ± 3.11*
Cholesterol (mg/dL)	61.90 ± 8.96	47.90 ± 7.01**
Triglycerides (mg/dL)	10 ± 1.34	10.5 ± 1.51*
Creatinine (mg/dL)	0.88 ± 0.09	0.78 ± 0.05*
Total proteins (g/dL)	6.73 ± 0.46	5.90 ± 0.31*
Albumin (g/dL)	3.41 ± 0.38	2.80 ± 0.31*
Total bilirubin (mg/dL)	0.20 ± 0.02	0.41 ± 0.09**

*p>0.05 – statistically non-significant differences
**p<0.05 – statistically significant differences

Glucose mean values decrease statistically significant ($p<0.05$) on 20th day of lactation as compared to the values obtained in not pregnant ewes. The decrease of this parameter's mean values at the onset of the lactation period can be the result of the constant loss of energy due to the increased milk production. These changes suggest that the intensive use of glucose for the lactose synthesis in the early lactation period may be incriminated for the body's inability to maintain the glucose homeostasis. The statistically significant ($p<0.05$) decrease of the glucose concentration may also be associated with the increased milk production during the

first part of the lactation period and with the intense activity of the mammary gland.

The slightly elevated urea levels ($p>0.05$) in lactating ewes may be caused by the mobilization of large amounts of body reserves, translated into an intense muscle protein catabolism.

Decreased total protein and albumin concentrations ($p>0.05$) can be a consequence of the intense mobilization of plasma immunoglobulin in order to form the colostrum in the mammary gland, this process taking place during the last months of gestation.

The mean values of cholesterol decreased significantly ($p<0.05$), being consistent with the increased energy demand in the first third of the lactation period. The slightly increase of the triglycerides mean value ($p>0.05$) in early lactation can be associated with an increased mobilization of fat from the adipose tissue due to the negative energy balance in this period.

There was also observed a significant increase ($p<0.05$) of the total bilirubin mean value, indicating an intensification of the hepatic metabolism, along with the results obtained in the enzymatic profile parameters.

The mean value of the blood creatinine showed little variations that were not statistically significant ($p>0.05$).

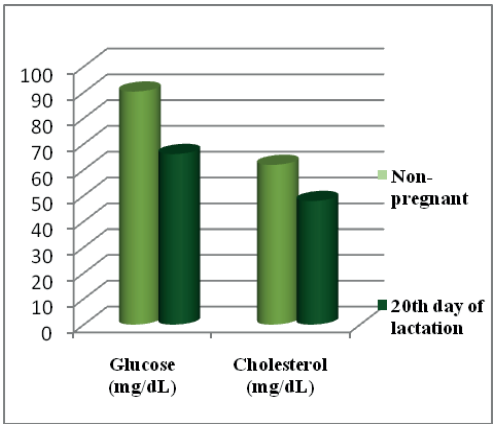

Figure 3. The dynamics of the glucose and cholesterol mean values in non-pregnant ewes and in the 20th day of the lactation period

The results obtained in the study of the biochemical parameters are correlated with the high energy demand in this physiological stage. Therefore, the glucose mean values decreased significantly ($p<0.05$) on the 20th day of lactation as a result of the increased energy loss due to milk synthesis and intense activity of the mammary gland. These changes suggest that the increased utilization of the glucose for the synthesis of lactose during the first third of the lactation period can lead to the body's inability to maintain the glucose homeostasis. Slightly elevated levels of urea in lactating ewes may be a consequence of the muscle protein catabolism intensification when large amounts of body reserves are mobilized. The decreased albumin and total protein mean values on the 20th day of lactation are due to the intense mobilization of plasma immunoglobulin during the last months of gestation when the colostrum is formed in the mammary gland.

CONCLUSIONS

The variations of the studied parameters in the first third of the lactation period in ewes indicate an energy deficiency and an important intensification of the metabolism overall. The analysis of the haematological parameters revealed a significant decrease of the white blood cells count mean values due to the migration of the leukocytes from the mother's blood into milk in order to provide antibodies for the new-born and for the protection of the mammary gland, results that are also correlated with the decreased albumin and total protein mean values due to the immunoglobulin's mobilization in the last months of gestation when the colostrum is formed. The variations observed in the biochemical profile were the result of the increased energy loss due to milk synthesis and mammary gland intense activity and also a consequence of the muscle protein catabolism intensification due to the increased mobilization of body reserves.

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DIAGNOSIS AND TREATMENT OF CANINE APPENDICULAR OSTEOSARCOMA. A CASE REPORT

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Abstract

Canine osteosarcoma is an agressive tumour that has both a locally invasive and highly metastatic biologic behavior. Osteosarcoma is the most important primary bone tumour in dogs, being the most common. Usually, it's occurrence is in the appendicular skeleton and the most frequently involved are large and giant breeds, being the cancer of elderly, large dogs. The aim of this case report is to describe the diagnostic methods, the therapeutic targets and the evolution of a canine osteosarcoma. This case highlights that the diagnosis is made by corroborating the results of the blood tests, the imaging methods (radiographs, CT scans) and the cytological evaluation. Multimodal therapy should be considerd to provide patients with maximum survival time and to improve the quality of life. The therapeutic management for a canine osteosarcoma case should include surgery, adjuvant chemotherapy and palliative care. Despite these therapies, the prognosis is, for the most part, negative with a great metastatic occurrence, mostly to the lungs. This report is an example of how diagnostics and therapeutics can be used in the management of a canine appendicular osteosarcoma.

Key words*: canine osteosarcoma, cancer, chemotherapy, palliative treatment.*

INTRODUCTION

Osteosarcoma (OSA) is a malignant mesenchymal tumour of primitive anaplastic bone cells, these cells being characterized by an extracellular production of osteoid (matrix) (North and Banks, 2009). Osteosarcoma is by far the most frequent primary bone tumour in dogs, accounting for approximately 85% of all bone tumours and 5 % of all malignant tumours in dogs (Dobson and Lascelles, 2019). It mostly affects the limbs (appendicular skeleton) but it can also develop in the skull, spine and the ribs (axial skeleton). Approximately 75% of osteosarcomas affects the appendicular skeleton with the remaining of 25% affecting the axial skeleton. The most common locations for the appendicular osteosarcomas are the distal radius or proximal humerus followed by the proximal and distal femur and tibia (Klopfleisch, 2016).

Canine osteosarcoma can occur in dogs of all ages but it is most common in 7-9 year old dogs. It usually develops in older, large and giant breeds, and there is no gender predisposition confirmed. Osteosarcomas appear to affect large breed dogs more commonly than the small breeds (Klopfleisch, 2016; Tuohy et al., 2019). Reported predisposed breeds include Great Danes, Saint Bernards, Rottweilers, Doberman Pinschers, Golden Retrievers, German Shepherd Dogs, Greyhounds, Irish Setters, Labrador Retrievers (Szewezyk et al., 2015). Nevertheless, higher weight and larger size are reported to be a stronger predisposition factor than breed (Klopfleisch, 2016).

Osteosarcoma is a highly aggressive and metastatic tumour, therefore an early diagnosis is desired. Diagnosis is based on clinical history, clinical and paraclinical examination including imaging methods of investigation (X-rays, MRI, CT), and the confirmation is made by cytology, histopathology. At the time of diagnosis, usually, the cancer cells have already spread in the body even though they are not detectable (about 90-95% of dogs have micrometastasis) (Liptak et al., 2004; Morello et al., 2011). Prognosis is based on many factors like age, large tumour volume, tumour location, elevated alkaline phosphatase (ALKP), high tumour grade, and presence of

metastasis (Boerman et al., 2012). The prognosis is not a favorable one, usually the median survival times (MST) is between 6 and 12 months, depending on the treatment received (Dobson and Lascelles, 2019).
The most effective management of canine appendicular osteosarcoma involves multimodal therapy, to address both the primary tumour and metastatic disease (Tsuji et al., 2019). Surgery (amputation, limb-sparing surgery) with adjuvant chemotherapy is the main treatment option for canine osteosarcomas (Davis et. al., 2013; Kozicki et al., 2015). Even if the the primary goal is local tumour and metastatic control, a very important objective that should not be ignored, is represented by the alleviation of pain and improving the quality of life - the palliative treatment (Duffy et al., 2018; North and Banks, 2009).
In the presented case we described the clinical signs, the diagnostic methods, the treatment but also the evolution of a classic case of canine appendicular osteosarcoma.

MATERIALS AND METHODS

In October 2019, a 10 years old, female, Presa Canario was presented at the clinic for evaluation, with signs of generally lameness and swelling of the left posterior limb. After the anamnesis, it was found that there was no trauma before the onset of clinical symptoms. The owner noticed the swelling of the leg four months ago and treated the dog, at the recommendation of a veterinarian from another clinic, with non-steroidal anti-inflammatory drugs (NSAIDs) and analgesics. Initially, an improvement was observed after one month of Meloxicam (orally administered for one month with an initial dose of 0.2 mg/kg followed by a maintenance dose of 0.1 mg/kg q 24 h). But, in the last month, the patient presented a lack of appetite and severe lameness. Also, the owner noticed that the swelling of the posterior limb is no longer localized but the entire limb is swollen (Figure 1). As the lameness worsens, there was no noticeable response to rest and non-steroidal anti-inflammatory drugs. Following the clinical examination, the visible symptoms such as limping or swelling were observed, and also the palpation revealed that the left posterior limb was mildly painful. No changes were detected at the level of the regional lymph nodes. In order to make a diagnosis, it was necessary to perform blood tests (complete blood count - CBC and serum chemistry), radiographs of the affected limb, chest and abdominal radiographs (in order to detect possible metastases). After the radiographs evaluation, it was recommended to perform a CT scan and a fine-needle aspiration for cytological evaluation. Also, a specialized cardiological consultation was recommended, after which the patient was diagnosed with dirofilariosis and a therapeutic scheme was performed.

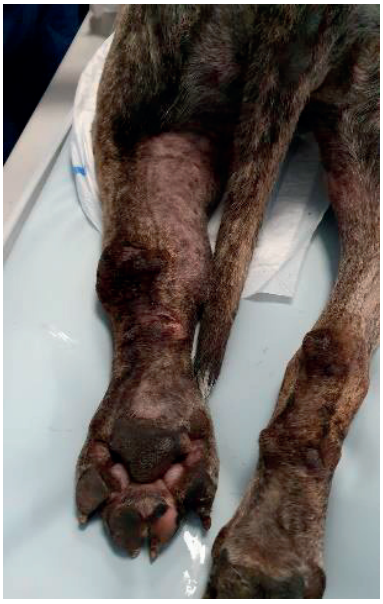

Figure 1. Swollen posterior limb

RESULTS AND DISCUSSIONS

The complete blood count (CBC) was normal but there were changes in the biochemical parameters as shown in table 1. Although most blood tests were within normal limits, increasing alkaline phosphatase was a cause for concern. In patients with osteosarcoma, routine blood work like haematology, biochemistry and urinalysis are often normal. However, an evaluation of alkaline phosphatase may appear, which is a negative prognostic factor (North and Banks, 2009).

Table 1. Biochemical parameters of the dog presented in this paper

Parameter	Value	Reference range
TP	7.9	5.2-8.2 g/dL
ALB	3.5	2.2-3.9 g/dL
GLOB	4.2	2.5-4.5 g/dL
CRE	0.8	0.5-1.8 mg/dL
UREA	11	7-27 mg/dL
ALKP	295 ↑	23-212 UI/L
ALT	78	10-125 UI/L
GLU	88.5	70-143 mg/dL

TP: Total protein, ALB: Albumin, GLOB: Globulin, CRE: Creatinine, ALKP: Alkaline Phosphatase, ALT: Alanine aminotransferase, GLU: Glucose

Radiographs of the affected limb revealed an osteolytic and proliferative lesion of the femur. Radiographic features displayed that both the diaphysis and the epiphysis of the femur were afected.

Cortical lysis and perpendicular radiating new bone formation from the cortical bone into the surrounding soft tissue (sunburst pattern) were observed.

Even if at the clinical examination it was observed that the tibia region was swollen, there were no changes of the tibia visibile on x-ray. Radiographs of the affected bones are of particular revelance for the diagnosis of canine osteosarcomas.

Usually, the radiographic appearance in osteosarcoms is variable, it presents either loss of bone structure (lysis), or proliferative lesions, or a mixture of both patterns (Klopfleisch, 2016).

Three view thoracic radiographs revealed no pulmonary pattern consistent with lung metastasis. Even if less than 15% of dogs have clinically detectable metastasis at the time of initial diagnosis, approximately 90% will die with metastatic disease, usually to the lungs.

Metastasis occurs primarily through hematogenous routes, particularly to the lungs, but on rare occasions extension to regional lymph nodes may occur (Dobson and Lascelles, 2019).

The CT scan revealed the appearance of lysis, cortical erosion and bone proliferation of the proximal femur.

The CT scans allowed a better evaluation, and were essential to improve visualization of tumour margins (Figures 2, 3, 4).

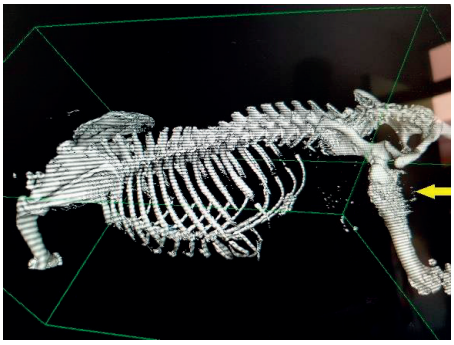

Figure 2. A 3-D reconstruction of the proximal femur with osteosarcoma of the dog presented in this paper, the yellow arrow indicates both lysis and bone proliferation

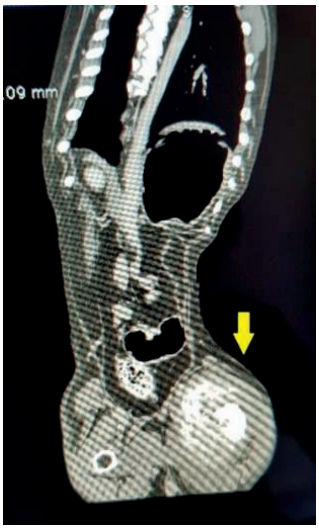

Figure 3. Transverse view of a dorsal 2-D reconstructed CT scan with contrast, yellow arrow shows the extension of the appendicular osteosarcoma of the dog presented in this paper

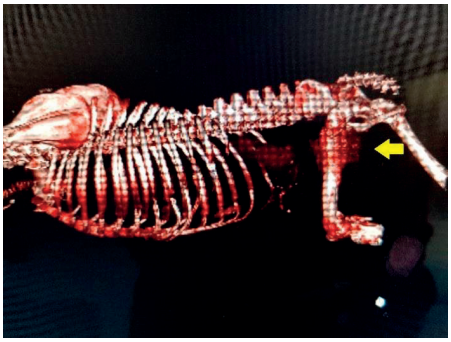

Figure 4. A 3-D reconstruction of the appendicular osteosarcoma of the dog presented in this paper, the yellow arrow shows the vast vascularisation of the tumour

The fine-needle aspiration from the mass and the cytological examination were performed, being less invasive than a bone biopsy. Fine needle aspiration entails using a very thin needle to collect a sample of a lesion for microscopic examination. In order to perform the microscopic evaluation, the aspirate smear was stained using the DiffQuik staining procedure. The Diff-Quick stain consists of a fixative agent (methanol), solution I (eosinophilic) and solution II (basophilic). The smear was dipped sequentially into each solution 5 times, followed by a water rinse and drying. The cytological evaluation revealed a group of osteoblasts, anisocytosis, anisokaryosis, binucleated cells and osteoid extracellular protein matrix among the cells (Figure 5). Based on these findings and taking into account the rest of the results (radiographs, CT scans, biochemestry analysis) the certainty diagnosis of osteosarcoma was made.

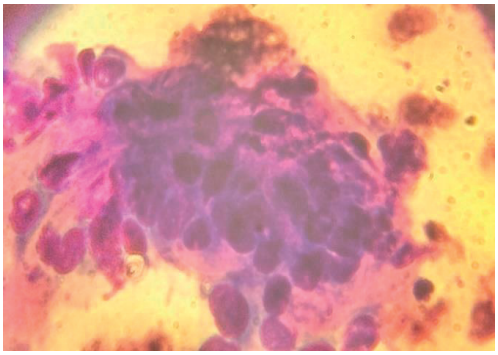

Figure 5. Cytology of fine needle aspirate of the osteosarcoma of the dog presented in this paper. Diff-Quik ×100

The patient's prognosis and treatment options were discussed in detail with the owner. A multimodal therapy was recommended, including both the treatment of the local disease (primary tumour) and systemic disease (micrometastasis). The therapy was primarily focused on local tumour control, so amputation of the affected limb was recommended. Surgery is the standard treatment for canine appendicular osteosarcoma, even large and giant breed dogs can function well after limb amputation (Dobson and Lascelles, 2019). In order to control the micrometastasis adjuvant chemotherapy was recommended (even if metastasis were not detected, canine osteosarcoma is a very agresive and metastatic tumour, so we assumed it already spread in the body).

The owner refused the surgery, considering that the pacient is a very large and old dog that will not adapt to the loss of a limb, but he agreed with the administration of the systemic treatment.

Subsequently, carboplatin chemotherapy was initiated and given IV (300 mg/m^2) once every 3 weeks for a total of four seassions.

During the administration of chemotherapy the patient was constantly monitored and also blood tests were performed. Adverse reactions following therapy were in the gastrointestinal system, clinically represented by vomiting and diarrhea. To reduce side effects, when needed, the patient was treated with antiemetics, antidiarrheal drugs.

Considering the aggressiveness of the tumour and the patient's condition, palliative treatment was recommended to maintain the patient comfort. It was administred Meloxicam in a dose of 0.1 mg/kg daily with a gastrointestinal protector (omeprazol). The goal of palliative treatment is to alleviate pain and improve quality of life.

The patient was monthly evaluated, and unfortunately, after 5 months pulmonary metastases were detected on radiographs. The patient's condition worsened, became dyspneic, lethargic and had inappetence. Because the quality of life of the animal decreased euthanasia was recommended.

Canine osteosarcoma represents a challenge for veterinarians being a highly malignant and metastatic tumour, as reported in this case.

The prognosis is often negative, with frequent euthanasia in patients due to the extension of the neoplastic lesion and the appearance of metastases. Being able to make an early diagnosis and starting a multimodal treatment as soon as posible, contributes to providing a longer median survival time.

Radiographs and cytologic examination were essential to diagnose, but CT images contributed to the assessment of the tumour extension and the blood tests contributed to the assessment of the patient's health.

In order to obtain the best result, the owners' compliance is required. In the present case, we consider that if the owner had accepted the

amputation of the affected limb, the patient's survival time would have been extended.
If a patient diagnosed with osteosarcoma is treated only with analgesics and non-steroidal anti-inflammatory drugs the survival time rarely increase beyond 3 months.
When treated only surgically, MST is increased, but when surgery is associated with adjuvant chemotherapy MST is considerably increased with a 12 month survival rate (North and Banks, 2009).
In the present case, in which the treatment consisted only of chemotherapy, the survival time after diagnosis was 5 months, being obtained a longer interval than if no treatment had been received at all, but less than if the amputation had been performed.

CONCLUSIONS

The diagnosis of osteosarcoma is based on the corroboration of anamnetic data, clinical data, and mainly on the results obtained in the imaging methods and on the cytological evaluation.
In the treatment of canine osteosarcoma is recommended both a local approach for tumour (surgery) and a systemic approach (chemotherapy) to control or prevent metastasis. The palliative treatment is essential in any type of cancer, in order to provide comfort to the patient.

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FERTILITY IMPROVEMENT IN LACTATING DAIRY COWS USING A PRESYNCHRONIZATION PROTOCOL BEFORE OVSYNCH

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Abstract

Pregnancy rates obtained after artificial insemination following Ovsynch in lactating dairy cattle are currently around 40-45%. Therefore, various fertility programs have been established in an attempt to increase these percentages. The aim of the present research was to use a simple and cost-effective presynchronization protocol, that would allow maximization of ovulatory response after the first GnRH of Ovsynch, and therefore improve fertility of treated animals, in comparison to those in which Ovsynch was used alone. A total of 240 non-pregnant and lactating dairy cows were divided in two groups (n = 120). Group 1 was synchronized with Ovsynch alone, while in group 2, PGF2α and GnRH were administered almost simultaneously, in different injection sites, 7 days before the initiation of Ovsynch. Ovarian structures were observed by ultrasonography on the day when the first GnRH injection of Ovsynch was made and 4 days later. Results showed a 42.5% pregnancy rate in group 1 and a 57.5% pregnancy rate in group 2. Therefore, presynchronization treatments allowed an improved pregnancy rate of 15%, which totally covers the supplemental costs implied by such measures.

Key words*: dairy cows, fertility, presynchronization, Ovsynch.*

INTRODUCTION

Estrus synchronization protocols are nowadays considered to be almost mandatory in dairy cattle farms, for the enhancement of reproductive function and optimal organization of artificial insemination activity. One of the most common methods is currently Ovsynch (Pursley et al., 1995), which involves synchronization of follicular wave development and ovulation using GnRH and PGF2α. On the other hand, the classical techniques tend to be replaced by more complex fertility programs, which yield better results, and therefore allow for superior pregnancy rates as they involve a pre-synchronization, before the classical Ovsynch is performed. There are currently several fertility programs which are generally accepted to be the most efficient: Presynch-10, Presynch-11, Presynch-12, Presynch-14, Double Ovsynch, G6G etc. (Bello et al., 2006; Astiz et al., 2013; Dirandeh et al., 2015; Souza et al., 2008; Ayres et al., 2013; Herlihy et al., 2012).

Presynchronization brings significant advantages, as it was shown that fertility of cows is improved if the first GnRH of Ovsynch induces ovulation of a pre-existing dominant follicle. This leads to a new corpus luteum formation and initiation of a new follicular wave, that produces a new dominant follicle. Administration of Ovsynch's PGF2α induces luteolysis of an active corpus luteum and subsequent estrus due to the dominant follicle reaching the mature stage.

Beside the advantages that such fertility programs bring, there are also several drawbacks, that result from their complexity and necessity of multiple hormonal treatments, and therefore multiple farm visits.

It was shown by several authors (Stevens et al., 1993; Peters et al., 2003) that if PGF2α and GnRH are administered together, they do not adversely influence each other in what induction of luteolysis (by PGF2α) or ovulation (by GnRH) are concerned.

Therefore, presynchronization before Ovsynch, using a simultaneous administration (in two

different injection sites) of PGF2α and GnRH was attempted in lactating dairy cattle (Yousouf et al., 2016; Martins et al., 2017) with good results.
Thus, the aim of the present research was to use a simple and cost-effective presynchronization protocol, that would allow maximization of ovulatory response after the first GnRH of Ovsynch, and therefore improve fertility of treated animals, in comparison to those in which Ovsynch was used alone.

MATERIALS AND METHODS

Our research was carried out in a private dairy farm from Cluj County, Romania.
All animals included in the experiments were multiparous Holstein cows, 3.5-6 years of age, housed in free stalls and fed a combined fixed ration, made up of corn and alfalfa silage as well as concentrates. Salt and water were offered ad-libitum.
Production of these cows averaged approximately 30 liters of milk/day/cow.
Females were randomly chosen, without establishing the phase of the estrous cycle that they were into.
Farm records were consulted and they confirmed that all cows that were included in the experiment were either between 55 and 65 days post-partum, waiting for their 1st insemination or were confirmed non-pregnant by ultrasounds 40 days after the previous AI.
All hormonal products that were used were licensed for cattle, while the producer's recommended dose was respected, as follows: PGF2α - 25 mg dinoprost tromethamine (Dinolytic, Zoetis) and GnRH - 50 µg D-Phe 6-gonadorelin (Gonavet 50, Veyx Pharma).
All treatments were performed as intramuscular injections, using sterile single use syringes and needles.
A total of 240 non-pregnant and lactating dairy cows were divided in two equal groups (n = 120). Group 1 was synchronized with Ovsynch alone, while in group 2, PGF2α and GnRH were administered almost simultaneously, in different injection sites, 7 days before the initiation of Ovsynch (Figure 1).
In both groups Ovsynch was performed as follows:

- Day 0 - GnRH,
- Day 7 - PGF2α,
- Day 8 - PGF2α,
- 32 hours later - GnRH,
- 16 hours later - artificial insemination.

Ovarian structures were observed by ultrasonography on the day when the first GnRH injection of Ovsynch was made, as well as 4 days later, in order to confirm any modifications that occurred.
All ultrasound examinations were performed using a Mindray DP-10 Vet ultrasound scanner and a 4-6 MHz linear transducer, by the usual trans-rectal approach.

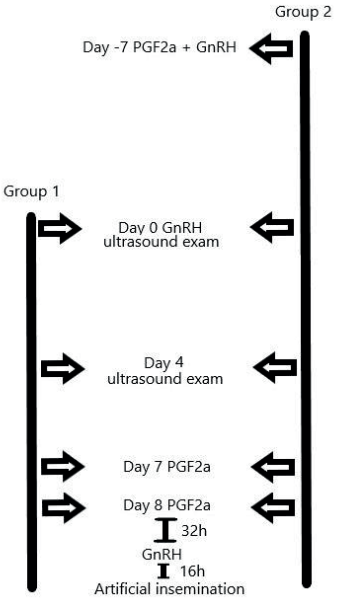

Figure 1. Graphical representation of the two estrus synchronization protocols

Artificial insemination was performed 16 hours after the second GnRH administration of Ovsynch, using frozen/thawed bull semen, using the classical transcervical technique.
Pregnancy diagnosis was performed 35 days after artificial insemination by ultrasonography. Re-confirmation of pregnancy, as well as fetal sexing was also performed by ultrasonography 55 days after insemination.
Cows were closely monitored throughout the entire experiment, regarding their health status, behavioral estrus-associated modifications as well as general welfare.

RESULTS AND DISCUSSIONS

One of the first differences that was observed between the two groups of cows was related to the percentage of females that had a functional corpus luteum at the time of the first GnRH injection of Ovsynch, and therefore did not ovulate following this treatment.

In group 1, which had no presynchronization treatment, only 73 out of 120 cows (60.83%) had a large follicle (Figure 3) on their ovary when the first GnRH administration of Ovsynch was performed, and were confirmed to have ovulated 4 days later, by ultrasounds. The other 47 cows (39.17%) had an active corpus luteum on their ovary on day 0 (Figure 4), when the first GnRH was administered, and only small follicles (below 0.8 cm in diameter), which became luteinized after the treatment (Table 1, Figure 2).

In group 2, in which PGF2α and GnRH were administered 7 days before the beginning of Ovsynch, 98 out of 120 cows (81.66%) had a dominant follicle at the time of the first GnRH injection of Ovsynch, as confirmed by ultrasounds and ovulated following this treatment, as the follicle had disappeared and a new active corpus luteum was identified by ultrasounds 4 days later.

Table 1. Results of ovarian ultrasound examination on the day of first GnRH administration of Ovsynch

	Group 1 No. (%)	Group 2 No. (%)
Cows with large follicle at the first GnRH of Ovsynch	73 (60.83%)	98 (81.66%)
Cows with active corpus luteum at the first GnRH of Ovsynch	47 (39.17%)	22 (18.33%)

Table 2. Results of pregnancy ultrasound examination and fetal sexing

	Group 1 No. (%)	Group 2 No. (%)
Pregnant cows	51 (42.5%)	69 (57.5%)
Identified sex of fetuses	22 males 29 females	32 males 37 females

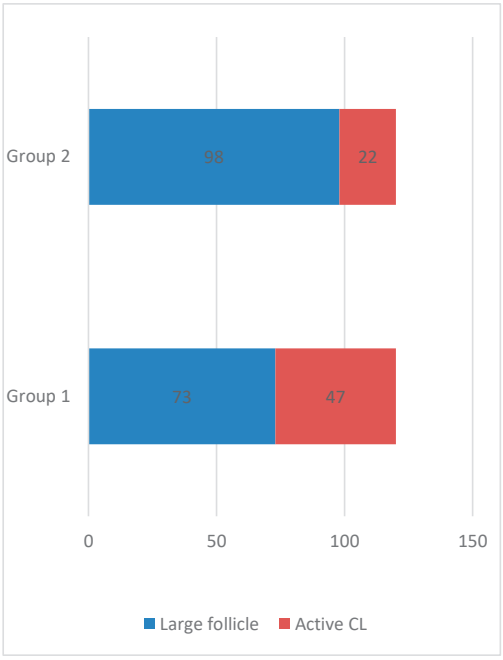

Figure 2. Graphical representation of the results obtained on ultrasound examination on the day of first GnRH administration of Ovsynch

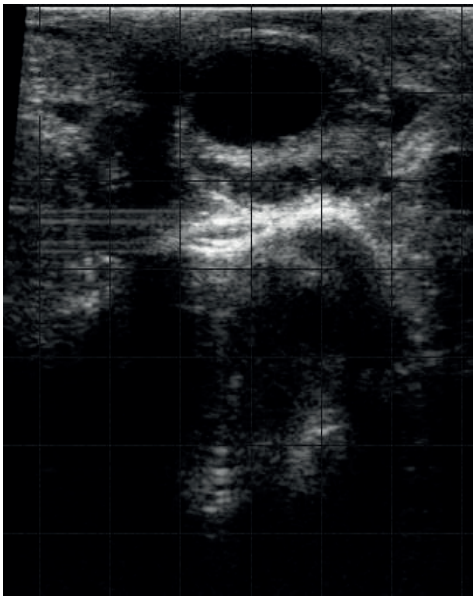

Figure 3. Ultrasound image of a large follicle

The other 22 cows (18.33%) had an active corpus luteum on their ovary and small follicles that became luteinized after the first GnRH administration of Ovsynch (Table 1, Figure 2). Therefore, the presynchronization treatment significantly increased the number of cows that ovulated after the first GnRH of Ovsynch.
After the second PGF2α administration of Ovsynch, estrus behavior was monitored and only 53 out of 240 cows were shown to display behavioral estrus, without significant differences between the two groups (27 cows from group 1 and 26 cows from group 2).
Nevertheless, timed artificial insemination (TAI) was performed in all cows, 16 hours after the second GnRH of Ovsynch in all females.

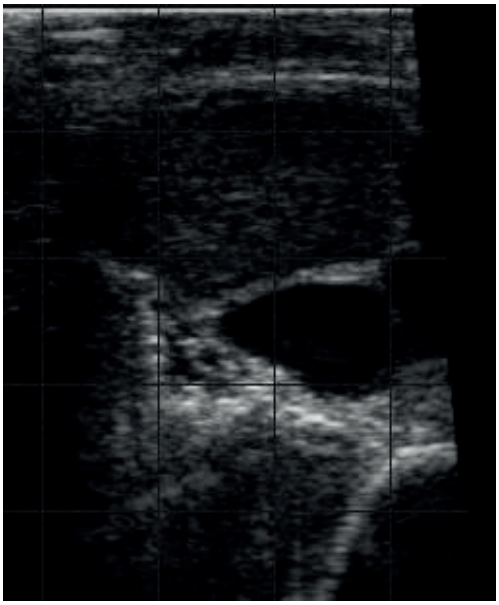

Figure 4. Ultrasound image of an ovary with a corpus luteum

Out of the 120 cows from group 1, 51 (42.5%) were diagnosed pregnant by ultrasounds 35 days after artificial insemination and all of them were reconfirmed pregnant at 55 days after insemination. Upon fetal sexing, 22 males and 29 females were found (Table 2, Figures 5, 6).
Out of the 120 cows from group 2, 69 (57.5%) were diagnosed pregnant by ultrasounds 35 days after artificial insemination and all of them were reconfirmed pregnant at 55 days after insemination. Upon fetal sexing, 32 males and 37 females were found (Table 2, Figures 5, 6).

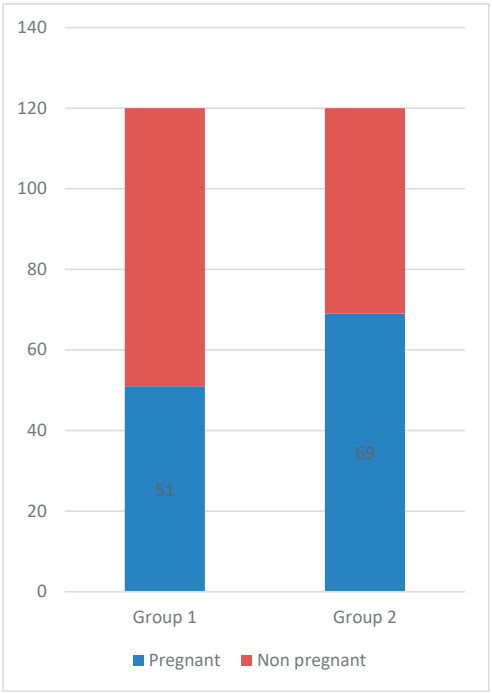

Figure 5. Graphical representation of the results obtained on pregnant diagnosis

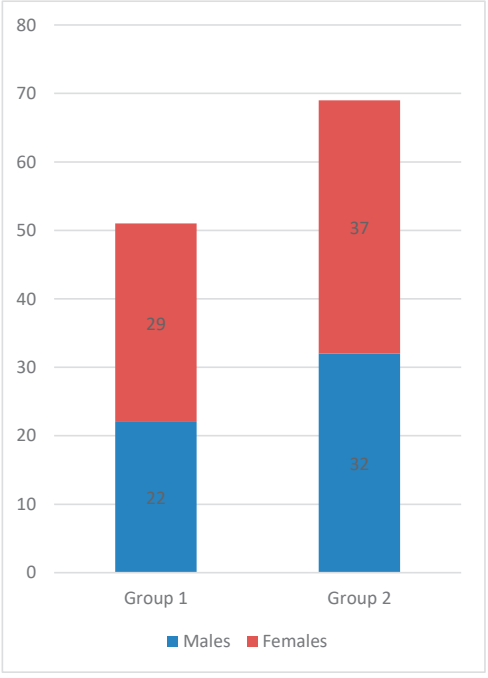

Figure 6. Graphical representation of the results obtained on fetal sexing

As shown above, the results obtained were significantly better in group 2, where a superior pregnancy rate following estrus synchronization was obtained, due to the presynchronization treatment, which allowed a better response to Ovsynch.

This improved response was mainly due to an increased number of ovulations that occurred after the first GnRH injection of Ovsynch.

The fact that a significantly number of cows presented a large follicle after pre-synchronization was due to the positive effect of the combined administration of PGF2α and GnRH.

The former induced luteolysis of any luteal structure that was present on the ovary while the latter produced luteinization of any follicular structure. Therefore, a new follicular wave was initiated, which selected a dominant follicle, that was ready to ovulate when the first GnRH of Ovsynch was administered. This also led to the presence of an active corpus luteum, which was lysed under the influence of the two PGF2α injections of Ovsynch, and this allowed growth and ovulation of the new dominant follicle.

In group 1, where no presynchronization protocole was performed, the cows presented various ovarian structures and therefore not all responded to the first GnRH injection of Ovsynch. As the percentage of ovulations was lower, the amount of luteal tissue on the occasion of Ovsynch's PGF2α administration was diminished, as was luteolysis induced by it, and therefore ovulation rate before artificial insemination was also low. Thus, the pregnancy rate was obviously lower too.

These observations are supported by the research of Vasconcelos et al., 1999, who showed that the ovulation rate after the first GnRH injection of Ovsynch is much higher, if this administration is performed on days 6-7 of the estrous cycle, as compared to a random moment.

This presynchronization protocol involves less work and also less costs than other such methods, as it only needs one single extra farm visit, before the onset of Ovsynch. As the pregnancy rate is significantly higher (15%), the costs of labor and hormonal products are easily covered by the extra profit obtained.

This technique allows an adequate management of reproductive activity in dairy farms, allows timed artificial insemination to be performed, without the need of estrus detection, and also involves no risks for the cows and neither for the consumer of dairy products obtained from treated animals.

CONCLUSIONS

Results showed a 42.5% pregnancy rate in group 1 and a 57.5% pregnancy rate in group 2. Therefore, presynchronization treatments allowed an improved pregnancy rate of 15%, which totally covers the supplemental costs implied by such measures. Also, the presynchronization protocol was easy to apply, as only one supplemental farm visit was needed as compared to the classical Ovsynch.

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INTRASCLERAL PROSTHESIS IN KITTEN AS AN OPTION FOR EYEBALL ENUCLEATION

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Abstract

Feline herpesvirus is associated with complex ocular disorders: buphthalmia, descemetocele, deep corneal ulcers or penetrating corneal wound, in many cases the enucleation of the eyeball being the unique surgical option. During a period of 2 years (2016-2018), in 11 kittens with the age between 4 weeks and 6 months, intrascleral prosthesis was performed as an alternative to eyeball enucleation. Seven cases needed corneal reconstruction: we performed corneal-conjunctival flap in 4 patients and applied Vetshield® collagen contact lens in the other 3 patients, followed by a third eyelid flap in all patients. Periodic ophthalmologic examinations revealed a postoperative evolution without major complications. The silicone prosthesis is well tolerated by the tissue. Patients are receiving longterm local treatment with hyaluronic acid gel. Intrascleral prosthesis is an aesthetic option for keeping the eyeball into the orbit.

Key words*: enucleation, intrascleral prosthesis, kitten, silicone prosthesis.*

INTRODUCTION

Feline herpesvirus (FHV-1) is widespread in the feline population worldwide, with reported exposure rates of up to 97% (Gould, 2011) and with reports of active infection rates ranging from 5 to 20% (Bol et al., 2015).

FHV-1 has been associated with a wide range of ocular disorders: ophthlamia neonatorum, blepharoconjunctivitis, corneal ulcers (superficial or deep), uveitis, keratoconjunctivitis sicca, symblepharon, corneal sequestrum. Severe cases present with descemetocele, endophtalmitis and buphthalmos (Ionascu, 2017).

Cases of feline congenital or early-onset glaucoma are associated with various ocular malformations, including aphakia microphakia, ectopia lentis, iridoschisis, pectinate ligament dysplasia, iridociliary cysts and persistent pupillary membranes. The secondary glaucomas, constituting 95-98% of feline glaucoma cases, are associated with antecedent ocular or systemic disease processes (McLellan & Miller, 2011).

All patients presented in this study had clinical sigs of herpesvirus (blepharoconjunctivitis, ocular and nasal discharge), prior or at the time of the first examination.

Cases with buphthalmos that is refractory to medical therapy, descemetocele, perforated ulcers, eyes with no realistic possibility for vision, may require enucleation of the eyeball as the only surgical option.

This paper aims to present an alternative to eyeball enucleation: intrascleral prosthesis. In cases with severe corneal damage, intrascleral prosthesis was combined with corneal surgical treatment (island conjunctival flap or Vetshield® collagen contact lens, followed by third eyelid flap).

The evisceration procedure requires the removal of the intraocular contents through a scleral incision, maintaining the corneoscleral shell, into which a silicone prosthesis is inserted and the scleral wound apposed (Gelatt & Gelatt, 2011). In contrast with eyeball enucleation, the intrascleral prosthesis results in a cosmetic repair, keeping the corneoscleral shell in place.

This study reports a postoperative evolution with no major complications, the silicone prosthesis being well tolerated by the tissue. The novelty of this study is represented by the young age of the patients, as well as the use of this surgical procedure in cases with disease previously reported as contraindication.

MATERIALS AND METHODS

Medical records of 11 cats were analysed. Patients that were included in this study had a clinical diagnostic of glaucoma or buphthalmos, associated with secondary corneal disease and underwent intrascleral prosthesis implantation and corneal repair surgery (Table 1).

Patients underwent complete ophthalmological and physical examination. Additional diagnostic tests were performed, such as ultrasound, complete blood count and serum biochemistry.

Anterior to posterior axial globe length was determined for each eye using B-mode ultrasonography, with a 12.5-MHz ultrasound transducer.

Complete blood count and serum biochemistry showed no significant changes.

Evisceration of the eyeball and the implant of an intrascleral prosthesis (An-Vision Inc, Hennigsdorf, Germany) were decided.

The evisceration procedures requires the removal of the intraocular contents through a scleral incision, maintaining the corneoscleral shell, into which a silicone prosthesis is inserted and the scleral wound apposed (Gelatt & Gelatt, 2011).

The procedure involves exposing the bulbar dorsal conjunctiva and application of 4 stay sutures using nylon 4/0 (Ethilon 4/0, Ethicon, Johnson & Johnson, Germany). Lateral canthotomy for additional exposure was not necessary to perform. The conjunctival incision of approximately 120°-160° is performed between the stay sutures using a scalpel blade of 15 mm, at 3-4 mm distance from the limbus and parallel to it. For the scleral incision a tenotomy scissor was used. Two small hemostatic forceps were applied on both end of the scleral incision. The lens and the vitreous are easily removed, the uveal tract and the retina are removed by gentle traction using a hemostatic forceps. A lavage with saline is performed inside the corneascleral shell, to remove the blod clots and the remaining intraocular tissue. The sterile silicone prosthesis, after being flushed with saline, is implanted using a Carter injector (An-Vision Inc, Hennigsdorf, Germany). Silicone prosthesis's size was recorded for each patient. The size of the prosthesis was chosen after measuring ultrasonographically the diameter of the fellow eye minus 1-2 mm. The sclera and the overlaying conjunctiva are sutured separately using 5/0 vicryl (Vicryl 5/0, Ethicon, Johnson & Johnson, Germany), in a simple continuos suture, starting from the external angle. Seven patients needed corneal repair surgery after the prosthesis implantation. We performed an island conjunctival flap in 4 cases and applied a Vetshield® collagen bandage lens (Oasis Medical Inc, USA) in the other 3 cases, followed by a third eyelid flap in all 7 patients. The island conjunctival graft was harvested from the bulbar conjunctiva. The island conjunctival graft is sutured on the corneal defect using vicryl 8/0 (Vicryl 8/0, Ethicon, Johnson & Johnson, Germany) simple interrupted sutures. The Vetshield® collagen lens was applied in 3 cases with deep corneal ulcer. After the collagen bandage lens is hydrated with saline and applied on the corneal defect, a third eyelid flap is performed using a simple interrupted suture. The complete third eyelid flap was performed in order to mantain the collagen lens on the surface of the cornea after the surgery. The sutures are maintained for 3 weeks. Postoperative medications were similar for all patients and included meloxicam (Loxicom 0.5 mg/ml, Norbrook, Ireland) 0.1 mg/kg SID for 5-7 days, doxicycline (Ronaxan 20 mg, Merial, Lyon, France) 10 mg/kg SID 14 days, local antibiotics (Ofloxacin, Floxal, Bausch & Lomb Rochester, NY, SUA) TID for 14 days and artificial tears with hyaluronic acid TID for 30 days (Diferion®, Micromed Vet, Austria). After 30 days, the local treatment consisted only of artificial tears gel BID a la long (Optixcare Eye Lube Plus®, CLC Medica, Ontario, Canada). Patients that underwent corneal repair surgery and third eyelid flap received hyaluronic acid gel BID 6 months following sutures' removal. Histopathology performed on the eviscerated tissue excluded the presence of neoplasia and infection.

RESULTS AND DISCUSSIONS

In the study were included 11 intact cats: 9 Domestic Shorthair and 2 British Shorthair, 3 females and 8 males. The age of the patients ranged was from 1 to 6 months. The right eye

was affected in 5 cats and the left eye in 6 cats. All 9 cases presented buphthalmos with high intraocular pressure with an IOP range from 30 mm Hg to 72 mm Hg (Figures 1 and 2).

Table 1. Patients' data included in the study

Case number	Sex	Age	Name, Breed	Diagnosis	Prosthesis size	Corneal repair surgery	Complications
1	M	1 month	Piki, DSH	OS Buphthalmos, aphakia, secondary corneal ulcer	17	-	-
2	M	4 months	Amir, British Shorthair	OD Congenital glaucoma, buphthalmos, posterior lens luxation, mature cataract	18	-	-
3	M	1 month	Stelu, DSH	OS Buphthalmos, superficial corneal ulcer	17	-	-
4	F	2 months	Maxi, DSH	OS Buphthalmos, aphakia	17	-	-
5	M	4 months	Chiorete, DSH	OD Buphthalmos, aphakia, central desmetocele	19	Island conjunctival flap + 3rd eyelid flap	-
6	M	6 months	Thomas, DSH	OS Buphthalmos, aphakia, central desmetocele	19	Island conjunctival flap + 3rd eyelid flap	-
7	M	5 months	Berlioz, DSH	OS Buphthalmos, aphakia, central desmetocele	19	Island conjunctival flap + 3rd eyelid flap	-
8	F	6 months	Freia, DSH	OD Buphthalmos, central desmetocele	18	Island conjunctival flap + 3rd eyelid flap	OD Entropion, 2 months postoperatively
9	F	4 months	Matoosh, British Shorthair	OD Buphthalmos, secondary corneal ulcer	17	Vetshield® + 3rd eyelid flap	-
10	M	6 months	Tufi, DSH	OS Congenital glaucoma, secondary corneal ulcer	18	Vetshield® + 3rd eyelid flap	OS Superficial corneal ulcer, 7 days postoperatively
11	M	5 months	Emi, DSH	OD Buphthalmos, secondary corneal ulcer	18	Vetshield® + 3rd eyelid flap	-

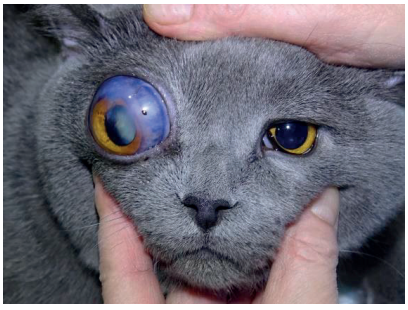

Figure 1. OD Buphthalmos, posterior cataract lens luxation (case 2)

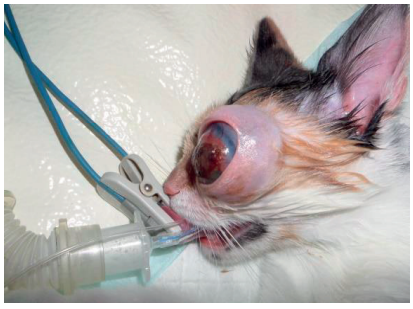

Figure 2. OS Buphthalmos (case 1)

Eight cases presented corneal disease. Three cases presented secondary corneal ulcer with positive fluorescein test (Figures 3 and 4) and one case presented vascularized corneal ulcer (Figure 5).

Four cases presented central descemetocele (Figures 6 and 7). In 5 cases the ultrasound examination revealed aphakia.

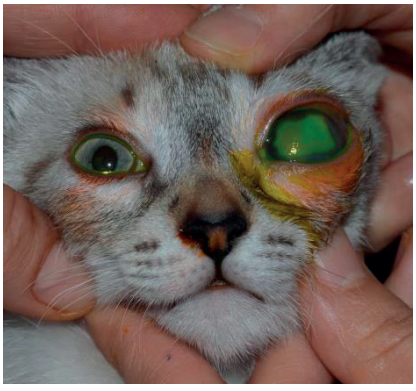

Figure 3. OS Buphthalmos, secondary corneal ulcer (case 3)

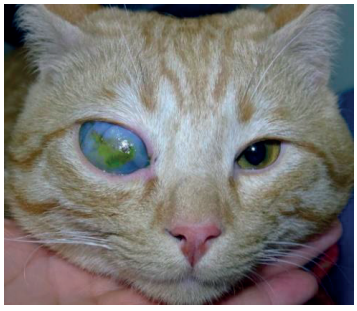

Figure 4. OD Buphthalmos, secondary corneal ulcer (case 11)

Figure 5. OS Buphthalmos, secondary vascularized corneal ulcer (case 1)

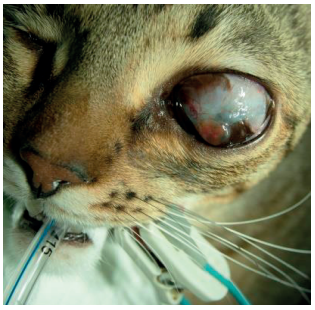

Figure 6. OS Buphthalmos, central descemetocele (case 6)

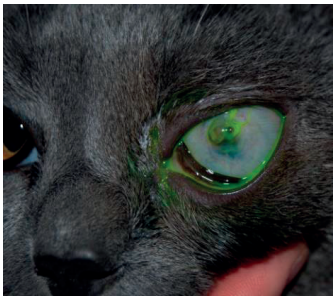

Figure 7. OS Buphthalmos, central descemetocele (case 7)

The third eyelid flap was performed (Figures 9 and 11) in 7 cases: 3 cases with corneal ulcer and Vetshield® collagen lens application (Figure 8) and 4 cases with descemetocele and island conjunctival flap (Figure 10).

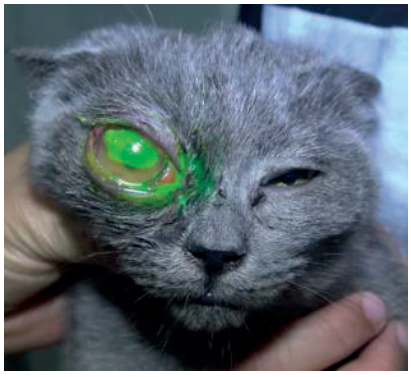

Figure 8. OD Buphthalmos and secondary corneal ulcer. Selected case for Vetshield® collagen lens aplication (case 9)

Figure 9. OD Third eyelid flap after Vetshield® collagen lens aplication (case 9)

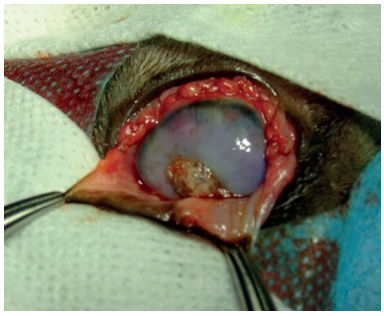

Figure 10. Postoperatively aspect of the island conjunctival flap and conjunctival suture following intrascleral prosthesis (case 6)

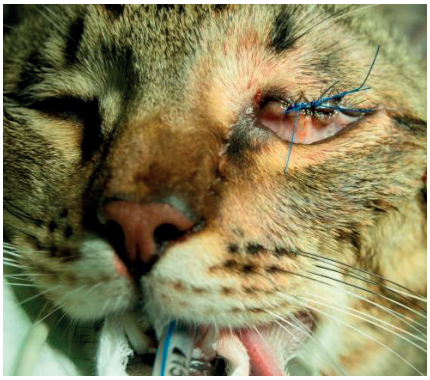

Figure 11. OS Third eyelid flap, island conjunctival flap and conjunctival suture (case 6)

Even the studies (Cook, 1997; Gelatt & Gelatt, 2011) recommend the sphere size should be +/- 1 mm of the horizontal corneal diameter of the affected eye. In our study, the silicone prosthesis size was determined by ultrasonographic measurement of the antero-posterior diameter of the fellow ocular globe minus 1-2 mm. This method was chosen because 7 cases also had concurrent corneal disease as well, and a larger sphere would have put more tension on the cornea and impaired the corneal healing under the graft or under the Vetshield® collagen lens.

The immediate postoperative aspect of the patients that underwent only intrascleral prosthesis implant reveals hemorrhage in the corneoscleral shell and chemosis of the bulbar dorsal conjunctiva (Figure 12).

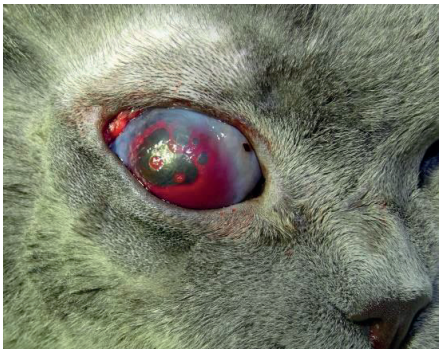

Figure 12. OD Immediate postoperatively aspect, hemorrhage in the corneoscleral shell (case 2)

Following surgery, the patients were kept under observation for a mean period of 21 months, with a range between 9 months and 36 months.

The first re-check was after 24 hours postoperatively, then every week for a month, and after that, every 3 months.

The eyes were examined assessing surgical wound healing, the ocular discharge, the Schirmer Tear Test value and the corneal fluorescein uptake. One case developed corneal ulcer 7 days after the surgery (Figure 13).

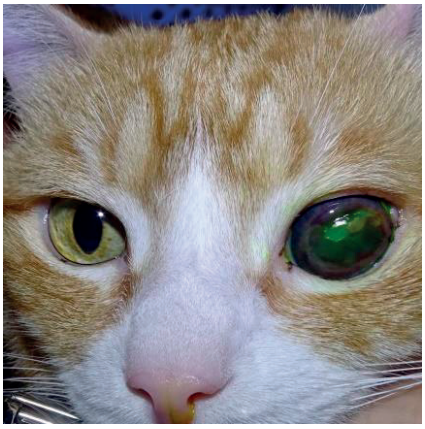

Figure 13. OS Corneal ulcer 7 days after the surgery (case 10)

Clinical aspect 30 days following surgery reveals non painful eyes, with an opaque cornea. Eyes with large corneal lacerations, otherwise considered contraindication for the intrascleral implant, responded well to the implant. After the suture removal at 21 days postoperatively the fluorescein test was negative.

One patient presented mild entropion two months postoperatively. The cornea did not uptake fluorescein and surgery for the correction of the entropion was performed 6 months after the intrascleral prosthesis surgery (Figure 14).

The reason for the entropion occurrence might be the growth of the facial bones along with its structures, including the eyelids, while the elastic corneoscleral shell reduces in size over 1-3 months to conform to the implant size (Gelatt & Gelatt, 2011; Hamor et al., 1994)

Clinical aspect 3 months following surgery reveals the normal size of the eye; the cornea is opaque, has a blue color (Figures 15 and 16), normal value of the Schirmer Tear Test and negative fluorescein test.

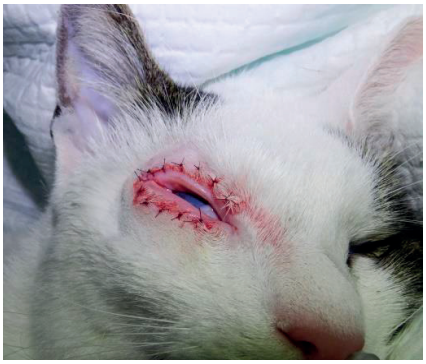

Figure 14. Postoperatively aspect of the entropion surgery performed 6 months after the intrascleral prosthesis (case 8)

None of the patients in this study developed pigmentation of the cornea, which may be the case in dogs with intrascleral prosthesis (Wilkie et al., 1994).

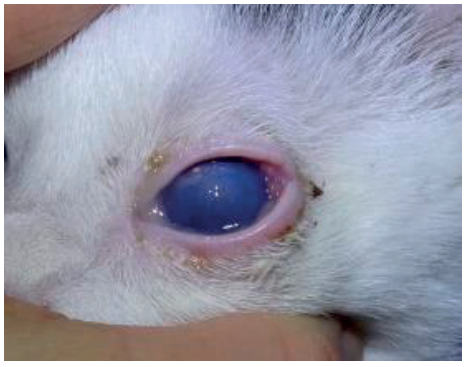

Figure 15. Clinical aspect 3 months after intrascleral prosthesis for buphthalmos and aphakia (case 4)

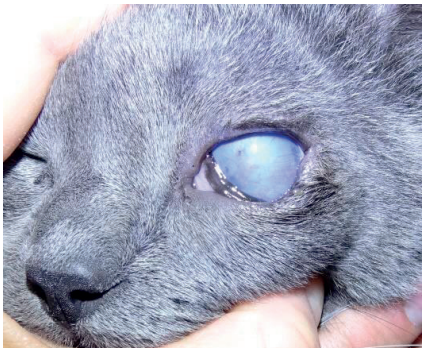

Figure 16. Clinical aspect 3 months after intrascleral prosthesis for buphthalmos and descemetocele (case 7)

Clinical aspect 6 months following surgery in the cases with large corneal injuries reveals non painful eye with normal size; the cornea has blue color (Figure 17), normal value of the Schirmer Tear Test and negative fluorescein test.

Figure 17. OS Clinical aspect 6 months after intrascleral prosthesis for buphthalmos, afakia and corneal ulcer (case 1)

Entropion (1/11) and superficial corneal ulcer (1/11) were the only complications that occurred in this case-series.

Following evisceration with intraocular prosthesis, several adverse effects or complications can occur: corneal surface disease, entropion, keratoconjunctivitis sicca (KCS), dehiscence of the scleral suture, implant extrusion, endophthalmitis, and regrowth of undiagnosed intraocular neoplasia (Blocker et al., 2007; Ruoss et al., 1997, Cook, 1997, McLaughlin et al., 1995, Naranjo et al., 2014).

Blocker et al. stated that 52% of dogs in their study developed a superficial ulceration within the central cornea at one of the time points postoperatively. Their study also shows that the presence of buphthalmos appeared to be a factor in globes developing decreased corneal sensitivity. Given the fact that corneal diseases are the most common complication that occurs, even in the 14 days following surgery (Lin et al., 2007) and taking into consideration the decreased corneal sensitivity and the reduction in aqueous tear production, artificial tear supplements with hyaluronic acid were long term prescribed for all 11 patients.

Other reports recommend that intraocular prosthesis should be delayed or not performed in eyes with major corneal diseases, including deep ulceration and low tear production, because of the weakened cornea (Blocker et al., 2007; Gelatt & Gelatt, 2011). One study reports that intraocular silicone prosthesis were

implanted in the eyes of a horse and a dog with traumatic corneal lacerations and protrusion of intraocular contents and that they were well tolerated (Riggs et al., 1990).

Kim et al. (2015) describes in a case-report an intrascleral prosthesis with penetrating keratoplasty on perforated corneal ulcer secondary to KCS in a Shih Tzu Dog.

However, exposure of silicone ball occurred at 9 months after the surgery due to the irritation of implant, thus enucleation was performed (Kim et al., 2015).

Our study also contradicts earlier reports that have cited corneal disease as a contraindication for implantation of intraocular silicone prosthesis.

In the follow up period, all patients in this study tolerated the implant well; all patients are pain-free at the follow-up recheck examinations.

The minor complications that occurred, entropion and superficial corneal ulcer, were well controlled with hyaluronic acid as well. All owners were satisfied with the surgical outcome and the cosmetic appearance.

CONCLUSIONS

Intrascleral prosthesis was performed as an alternative to eyeball enucleation in severe, complicated cases of increased intraocular pressure, associated with corneal ulcer and descemetocele.

Placement of an intraocular prosthetic provides comfort to the patient and a cosmetic effect to the globe.

The silicone prosthesis was well tolerated by the tissue, with no major complications.

The novelty of this study is represented by the young age of the patients, as well as the use of this surgical procedure in cases with disease previously reported as contraindication.

Postoperative treatment requires longterm administration of hyaluronic acid gel.

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RADIOLOGICAL ASPECTS ASSOCIATED WITH OSTEOCHONDRODYSPLASIA IN A SCOTTISH FOLD CAT: CASE REPORT

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Abstract

Scottish Fold cats have an unique appearance due to their forward-folded ears, being considered a defining feature of the breed. It is considered that the folded ears are a result of an underlying cartilage disorder, a genetic abnormality that predisposes the affected cats for another genetical condition of the musculo-skeletal system, termed osteochondrodysplasia. Osteochondrodysplasia is a painful and progressive syndrome characteristic only to the Scottish Fold breed due to a mutation in the TRPV4 gene. This condition is characterised by skeletal deformities, such as shortened feet and splayed phalanges, affecting primarily the hindlimbs, short, thick and inflexible tails, shortened caudal vertebras and ankylosing polyarthropathy of the affected joints. This paper has the objective of characterising the main bone and joint problems observed in a 1 year-old female Scottish Fold cat which presented for lameness and postural abnormalities of the hindlimbs.

Key words: *cat, Scottish Fold, radiography, osteochondrodysplasia, congenital anomaly.*

INTRODUCTION

The Scottish Fold cat has been developed as a breed starting the 1970s in Scotland, after the mating of a farm cat with naturally occurring folded ears to local cats and British Shorthairs (Wastlhuber, 1991).

Although the breeding of these cats started as early as 1961, the first mention of skeletal abnormalities and deformities was not made until 10 years later, when osteochondrodysplasia was recognised as a painful clinical syndrome, characterised by progressive bony abnormalities and a crippling lameness (Robinson & Pedersen, 1991; Wastlhuber, 1991).

Genetic analyses have designated the gene responsible for the defective endochondral ossification as Fd, suggesting that this is the gene that also causes the articular cartilage to fold and is inherited as a simple autosomal dominant trait (Robinson & Pedersen, 1991). Over several years of genetic testing the conclusion is that kittens that show radiological lesions are only a product of mating Fold-to-Fold cats (Jackson, 1975).

The breed has been banned in the United Kingdom starting as early as 1974 and breeding in the United States and other countries has been frowned upon ever since by many organisations focused on the welfare of cats (Malik et al., 1999; Brocklehurst, 2017).

Recent DNA testing of 44 Scottish fold cats and 54 control cats suggested that there is a variant of the TRPV4 gene that is associated with ear folding (Selting et al., 2019). TRPV4 is expressed in chondrocytes and other mesenchymal cells and has a great impact in tissue homeostasis and cellular differentiation (Gandolfi et al., 2016) .

The first description of osteochondrodysplasia of the Scottish fold cat was made by Jackson, in 1975 (Jackson, 1975). The main radiological findings are abnormally thick and inflexible caudal vertebrae, short feet and splayed phalanges, resulting in reduced ability to support weight, an abnormal gait, reluctance to jump and lameness (Malik et al., 1999; Chang et al., 2007). The joint lesions progress until the

cats become non-ambulatory. The radiographical lesions are evident in cats as young as 7 weeks of age (Jackson, 1975). The metacarpal and metatarsal bones, as well as the phalanges have distorted metaphyses, with widened physes, resulting in decreased length and abnormal shape of the affected bones and shortening of the hindlimbs (Malik, 2001). The caudal vertebrae have reduced length and widened endplates. Cats older than 6 months develop gross plantar exostoses of the tarsal and metatarsal bones, which become evident both clinically and radiographically. Diffuse osteopenia of the bones is also present (Chang et al., 2007).

The histopathological examination of affected tissues shows defective bone formation at the level of the growth plates, affected chondroblast proliferation and irregular groups of cells arranged in a disorganized manner. The osseous physes are grossly expanded, with deficient ossification, irregular mineralisation and remodelling and multiple centres of ossification (Malik et al., 1999).

Due to the fact that the underlying disease has a genetic trait, the methods of therapy used have the primary aim of reducing clinical signs and the accompanying pain (Hubler et al., 2004). One study shows the effects of pentosane polysulfates and complex glycosaminoglycans which provided reduction in lameness and discomfort in some cats (Malik et al., 1999). Another study focuses on the effect of the surgical approach in which bilateral ostectomies and pantarsal arthrodesis of both hindlimbs resolved the lameness in a cat (Mathews et al., 1995). A study from the year 2000 had obtained the same results only by surgical removal of the tarsal exostoses (Simon, 2000). The latest studies involving therapy for cases with osteochondrodysplasia focus on the effects of radiation therapy in conjunction with phosphonic acid, which showed immediate, whole-body improvement in mobility (Selting et al., 2019).

MATERIALS AND METHODS

A one year old, entire female Scottish fold cat was presented to a private practice near Pitesti, Romania in 2019 due to progressive lameness in the hindlimbs over the past weeks, abnormal gait and misshapen distal hindlimbs.

There was no previous medical history associated with the patient. On clinical examination, the cat was bright, alert and responsive. The heart rate was 160, with no murmurs or arrhythmias present. Body condition score was 4/9 and the patient was ambulatory, although it had a stiff gait. The tail was observed by the owners to be moving only at the tip ever since it was a kitten. When palpating the hind limbs, the tarsal region appeared firm on the plantar part and elicited pain upon palpation. The region was stiff and could not be manipulated easily. The hind paws had an abnormal shape, with the first and last toes being in front of the other. The middle part of the tail was fused and could not be flexed easily in any direction. The abdomen was within normal limits.

Conscious radiographs of the hindlimbs, forelimbs and tail were obtained in latero-lateral and dorso-palmar projections.

RESULTS AND DISCUSSIONS

The changes observed as a result of the radiological examination were consistent with osteochondrodysplasia, including shortened coccygeal vertebrae with widened endplates (Figure 1), malformed tarsal and metatarsal bones, exuberant exostoses of the tarsal region (Figure 2 A, B) and shortened and splayed phalanges (Figure 3). The interphalangeal joints spaces were irregular and widened (Figure 3). The forelimbs presented radiological signs of incipient bilateral elbow osteoarthritis, although not clinically significant at the time of the examination (Figure 4 A, B).

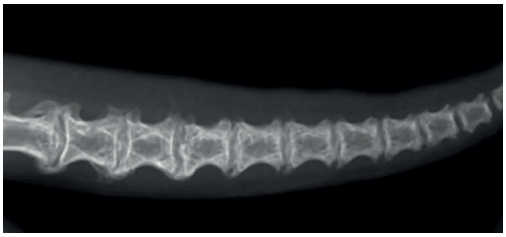

Figure 1. Lateral radiograph of the tail. The middle caudal vertebrae are short, thickened and the intervertebral spaces are reduced. There is periarticular new bone formation observed

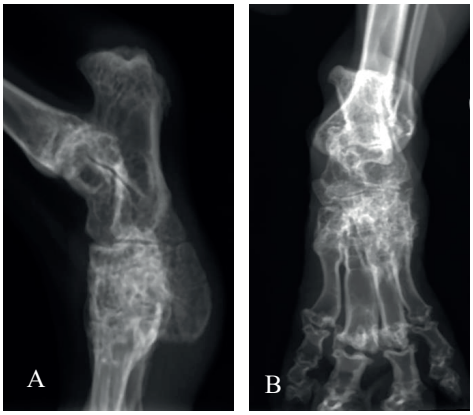

Figure 2. Lateral (A) and dorso-palmar (B) radiographs of the left hindlimb. Extensive new bone formation originating from the tarsus and extending to the metatarsus is present. The metatarsal bones are short, thick and misshapen

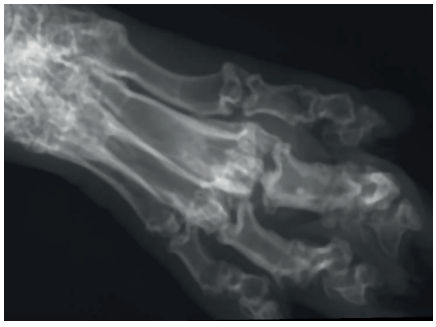

Figure 3. Dorsopalmar radiograph of the paw of the right hindlimb. The phalanges are short, misshapen and there is diffuse reduced opacity of the bones. The interphalangeal joints are irregular and widened

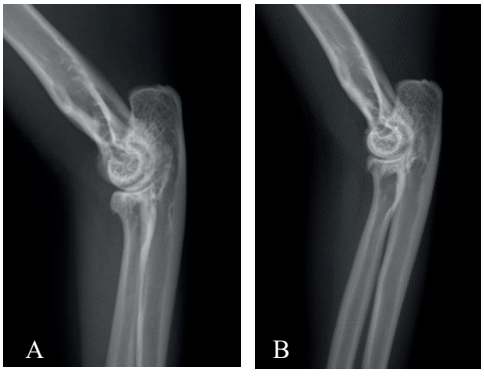

Figure 4. Medio-lateral radiographs of the right (A) and left (B) forelimbs. There are reduced signs of elbow osteoarthritis present

Metacarpals were somewhat shortened and with an abnormal shape compared to the distal limb of a domestic shorthaired cat (Figure 5 A, B).

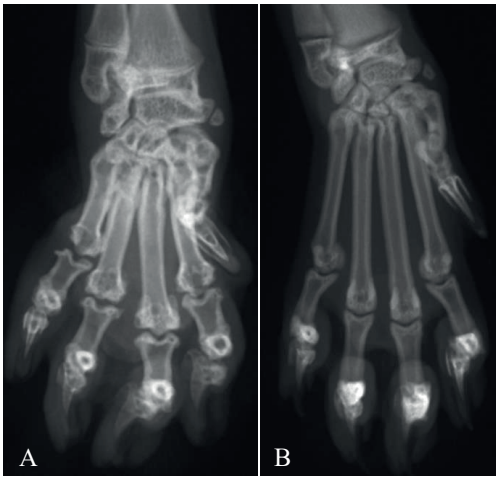

Figure 5. Dorsopalmar radiograph of the distal forelimb region of the cat in this study (A) and a domestic shorthair cat (B) for comparison. There is evident shortening and thickening of the metacarpal bones and phalanges in A compared to B and widening of the interphalangeal joints

The changes observed were bilaterally symmetric and affecting primarily the tarsal region. There was massive new bone formation extending from the proximal part of the calcaneus to the metatarsus, leading to bone fusion and tarsal ankylosis.

The new bone was smoothly marginated, with a typical trabecular pattern. There was diffuse decreased opacity of the bones affected (osteopenia). The metatarsal bones were short, thickened, malformed and had a splayed appearance. The interphalangeal joints were irregular. In the shortened and thickened tail, several caudal vertebrae were shorter than normal, with widened end plates, reduced intervertebral joints and new bone formation, tending towards ankylosis.

The forelimbs were thought to have minor changes related to osteochondrodysplasia in the distal parts and mild changes of degenerative joint disease of both elbows – not clear if related to osteochondrodysplasia or not, further studies needing to be conducted.

The defining phenotypic feature which identifies a cat as being part of the Scottish fold breed is characterised by the forward folding of the ears, suggesting a developmental cartilage defect (Malik et al., 1999).

It has been previously reported that osteochonddrodysplasia of the Scottish Fold cat can be diagnosed easily through survey radiographs in animals as young as 7 weeks old, older individuals developing ankylosis, as can be seen in the tarsal region of the cat in our study (Chang, 2007; Malik et al., 1999).

Treatment for cats suffering from this disease has not been determined convincingly to date, although some studies have shown the benefits of using polysulphated glycosaminoglycans, chondroprotective agents or non-steroidal anti-inflamatory agents safe for cats (Chang, 2007). Other studies have shown the benefits of osteotomies or arthrodeses (Matthews et al., 1995) and of using palliative irradiation (Hubler et al., 2004) or novel radiation therapies (Selting et al., 2019), but to this date the main proposed solution would be removal of the affected individuals from the mating pool and to restrict breeding to cats with a normal ear conformation (Allan, 2000).

CONCLUSIONS

The clinical presentation, the onset of clinical signs and the radiological changes observed make the diagnosis of Scottish fold osteochondrodysplasia certain for the cat in our study.

Prospective owners should be warned about the possibility of Fold developing musculoskeletal abnormalities and about the implications that this disease has on the quality of life of their animal.

Additional studies should be conducted using serial computed tomography or magnetic resonance imaging in order to obtain further information regarding bone and joint changes in a non-invasive manner.

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COAGULOPATHY AS A COMPLICATION OF BABESIOSIS IN A DOG WITH HEMOTHORAX: CLINICAL CASE REPORT

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Abstract

Canine babesiosis is a protozoan tick-borne disease affecting dogs worldwide, caused by intra-erythrocytic large and small piroplasms of the genus Babesia (Apicomplexa: Piroplasmida). Clinical manifestations are polymorph and evolution of the disease depends to a large extent on the virulence of the causative piroplasm species but also to host-related factors and as well as to the time of animal presentation for consultation, the early diagnostic and specific therapy administered in due time. The most frequent pathological changes of canine babesiosis are anemia and different degrees of thrombocytopenia. Additionally, the systemic inflammatory response syndrome and disseminated intravascular coagulation are described as possible complications. Here we present a clinical case of babesiosis complicated by coagulopathy in a 11 months-old male Beagle dog, which was presented at a veterinary clinic with dyspnoea and apathy. The clinical, radiological, and ultrasound examinations showed an active bleeding in the thoracic cavity, prolonged coagulation time and post-haemorrhagic anemia (packed cell volume of 22.2%). Based on these primary registered aspects, intoxication with anticoagulant rodenticides was suspected. As it was considered an emergency, the dog was transferred to a specialized clinic for hemo-transfusion and stabilized. However, a week later, the dog returned to the clinic with a febrile syndrome. Based on the hemo-parasitological investigations, the dog was diagnosed with babesiosis. Initially, a therapeutical protocol, based on clindamycin was administered, but without a satisfactory evolution; therefore, a babesiicid treatment, using imidocarb dipropionate, was administered, after which the clinical status of the dog rapidly improved. By this case report, the challenges for diagnostic and therapeutical management of canine babesiosis and its impact on the disease evolution, including potential complications, are emphasized.

Key words*: babesiosis, thrombocytopenia, clinical case, dog.*

INTRODUCTION

Canine babesiosis is a tick-borne protozoan disease that affects dogs all around the world. It is caused by intra-erytrocytic small and large piroplasms (Apicomplexa: Piroplasmida). Knowledge about the prevalence and the clinico-pathological aspects of this disease in dogs are of significant epidemiological and veterinary medical interest (Casapulla et al., 1998).

Infected dogs displays polymorphic clinical signs depending to a large extent on the virulence of the causative *Babesia* species, but also to the general status of animal, as well as the time of presentation at the consultation and the early administration of the specific therapy. Babesiosis in dogs is usually characterized by lethargy, anorexia, fever, pale mucous membrane, hemolytic anemia, icterus, and hemoglobinuria (Bourdoiseau, 2006).

Additionally, the most frequent pathological changes are anemia, ranging from mild, moderate to severe, and various degrees of thrombocytopenia (Irwin et al., 2010).

Systemic inflammatory response syndrome (SIRS) and disseminated intravascular coagulation (DIC) are two possible complications that lead to the syndrome of single and/or multiple organ dysfunction in canine babesiosis (Moore, 1979; Mathe et al., 1998; Welzl et al., 2001).

SIRS is commonly found in canine babesiosis. In veterinary medicine, several authors have proposed different SIRS criteria (Purvis and Kirby, 1994). Limit values for SIRS criteria are a major problem in veterinary medicine because normal values for temperature, heart

rate and respiratory rate vary in dogs due to major size differences (Huston and Radostitis, 2000). Use of SIRS criteria it is proposed by Okano et al. (2000), because this tested model showed the best prognostic value in canine babesiosis.

The therapeutic regimen used for treatment of of dogs with babesiosis differs depending on the clinical form they present, uncomplicated or severe, complicated disease, respectively.

In addition to the treatment administered to dogs with mild disease, rehydration, heparin, and blood transfusion along with other supportive therapy are recommended to dog with severe clinical babesiosis. Mortalities are reported primarly in dogs with severe disease and were attributed also to disseminated intravascular coagulation (Gopegui et al., 2007; Leica et al., 2019).

In addition to the directly pathogenic piroplasms (intraerythrocytic parasites' development, division and finally the cell lysis) resulting in hemolytic anaemia, parasitic antigens also act on lipoprotein metabolism, and on the complement system with the formation of anti-erythrocytic antibodies and immune complexes, all of which resulting in intra- and extravascular hemolysis (Ginger et al., 2005; Mitrea, 2011).

Therefore, it is suggested that a great importance must be given to the second pathogenetic mechanism, that of parasitic antigens on the reticulo-histiocytic system in which cell hyperplasia occur (hyperplasia associated with the agglutination and adhesion to the vascular endothelium of the parasitized erythrocytes, platelets, and antigens from plasma) causing capillary blockage in different organs with varied consequences (Irwin, 2010).

Here, we present a case report on canine babesiosis complicated by coagulopathy.

MATERIALS AND METHODS

Case presentation

In October 2018, a 11-months old male Beagle dog was presented in a veterinary clinic with dyspnea and apathy, for a radiological examination, being suspected for pleural effusion.

The dog spent several days outdoors in a private yard (in Constanta county), and thereafter in Tulcea county (Isaccea), for a few days, where it was suspected for eating suspected food, as the owner stated. After that, the dog showed dyspnea, apathy, and the owner presented him at veterinary clinic.

The dog was subjected for a routine clinical examination followed by paraclinical investigations, including a count blood cells (hemoleucogram), radiological, and ultrasound investigations.

RESULTS AND DISCUSSIONS

Clinical history and investigations

At clinical examination, the dog displayed dyspnea and severe apathy.

The laboratory, radiological and ultrasound investigations revealed free fluids in the thoracic cavity (Figures 1, 2), prolonged coagulation time, and pronounced post haemorrhagic anemia (PCV of 22.2%).

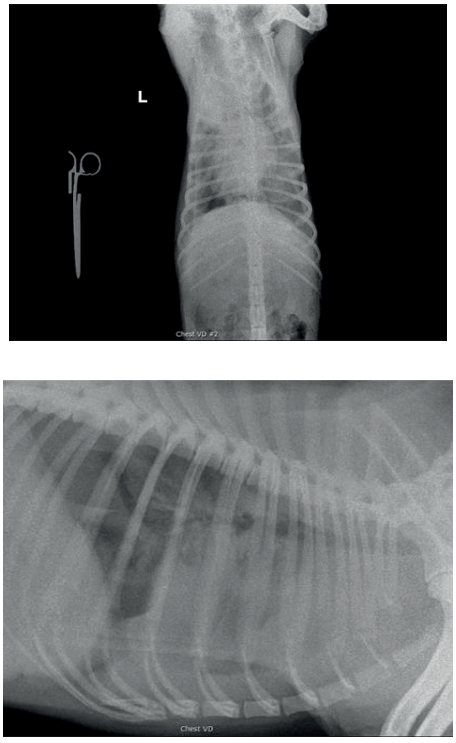

Figure 1. Pleural effusions in a 11-months Beagle dog (dorsal and lateral view) - radiography

Subsequently, in order to collect and examine the fluid, a puncture was performed. A total of

60 ml of bloody liquid (Figure 3) was collected, which was subjected for examination. An active bleeding in the thoracic cavity, was confirmed.

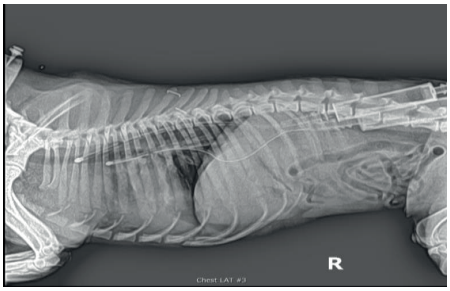

Figure 2. Dog with free fluid in the thoracic cavity

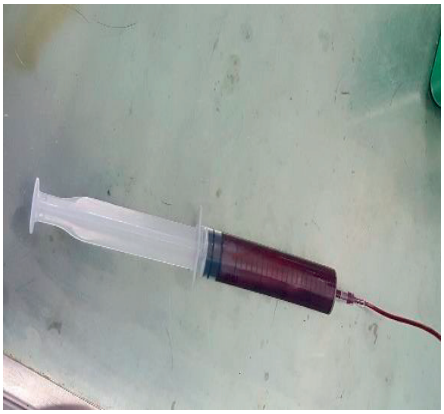

Figure 3. Bloody fluid drained from the thoracic cavity

Additionally, a drop of venous blood was collected to determine the coagulation time, which was longer than 10 minutes. Therefore, by corroborating the anamnesis and the clinical and pathological changes registered, an intoxication with anticoagulant rodenticides was suspected, and accordingly a therapy with vitamin K1 (phytomenadione) at a dose rate of 2.5 mg/kg body weight, s.c. was administered.
As it was considered an emergency, the dog was transferred to a specialized clinic for hemo-transfusion. Thereafter, the dog returned to the vet clinic where it was monitored post-transfusion, for a week. The haematological investigations performed during this period showed a stabilised condition (PCV improved from 22.2% to 32.0%); subsequently, the dog was released.

Over other several days, the dog returned to the clinic with a febrile syndrome, showing apathy, and accelerated breathing. The dogs displayed clinical signs compatible for babesiosis, such as: hyperthermia (40.6ºC), pale mucous membranes, tachypnea. Therefore, blood sample was collected and subjected for hematological, biochemical, and parasitological investigations.
The results of hematological and biochemical investigations revealed moderate non-regenerative anemia (PCV: 28.8%) and trombocytopaenia (Table 1).
Additional, a rapid, in-clinic, immunochromatographic (Snap 4DX, Plus, Idexx Laboratories) for detection of arthropod-borne pathogens (*Dirofilaria immitis* antigen and antibodies for *Anaplasma* spp., *Ehrlichia* spp., and *Borrelia burgdorferi* sensu lato), which was negative.
At the miscroscopic examination of thin blood smears Diff-quick stained, intraerythrocytic large *Babesia* piroplasms were identified.

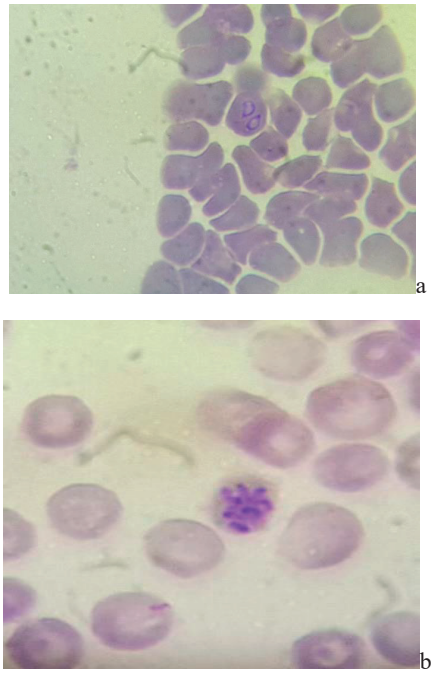

Figure 4. Dog peripheral blood smear showing intra-erythrocytic large *Babesia* piroplasms [paired (a) and multiple (b) intra-erythrocytic pyriform piroplasms] (Diff-quick staining; x1000)

Diagnostic, treatment, and follow-up
The owner was informed about the diagnostic, the potential severity of disease' evolution, and therapeutical protocol. Imidocarb dipropionate therapy was refused by the owner, who

requested for other therapeutic alternative. Therefore, a clindamycin-based therapy (Baneth, 2018) was initiated, at a dose rate of 15 mg/kg/day, *per-os*. Initially, an improvement in the dog's clinical status was observed, but the treatment has not been fully effective (Table 1, Figure 5). Due to the fact that a worsening of clinical condition and pathological changes were registered (PCV decreased at 33.7%; day 9 p.t.), finally the owner accepted the babesiicid treatment protocol, and imidocarb dipropionate was administered (6.0 mg/kg, body weight). At 48 hours p.t., the haematological parameters (PCV: 39.9%) and clinical status improved substantially.

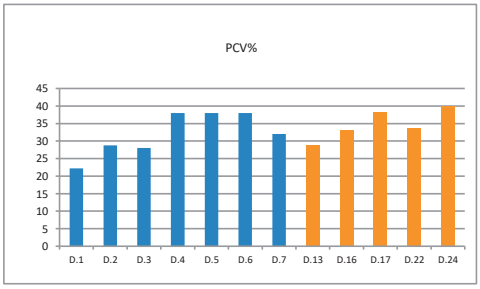

Figure 5. Dynamic of the packed cell volume (PCV%) in dog with coagulopathy and diagnosed with babesiosis (from the day of presentation-D.1 and the follow-up 24 days)

The second injection of imidocarb dipropionate was administered at 14 days, as manufacturer recommendations.

Table 1. Dynamics of haematological parameters on the dog after diagnostic of babesiosis (day 0), during of the treatment (with different drugs) follow-up

Parameter	**Drug administered/Day post-treatment** (D.pt.)					Limits and units of measurement
	clindamycin				imidocarb dipropionate	
	D.0* (18.10)	D.3pt.* (21.10)	D.4pt.* (23.10)	D.9pt.* (27.10)	D.2pt.** (29.10)	
WBC (white blood cells)	7.9	17.85	**17.8**↑	**4.09**↓	13.69	6-17 *10^9/L
LYM (lymphocytes)	1.92	30.7	2.86	**0.87**↓	**4.9**↑	1.0-4.80 *10^9/L
MON (monocytes)	0.33	5.9	0.86	0.38	0.65	0.20-1.5 *10^9/L
NEU (neutrophils)	5.63	ND	**14.5**↑	**2.83**↓	8.09	3.0-12.0*10^9/L
EOS (eosinophiles)	**0.02**↓	ND	**0.02**↓	**0.02**↓	**0.06**↓	0.1-1.0*10^9/L
BAS (basophiles)	0	ND	0.01	0	0	0.00-0.50*10^9/L
LYM %	24.3	ND	16.1	21.2	**35.8**↑	12.0-30.0
MON %	**4.2**↑	ND	4.8↑	9.2	**4.7**↑	2.0-4.0
NEU %	71.2	ND	79	69.2	**59.1**↓	62.0-87.0
EOS %	**0.3**↓	ND	0.1	0.4↓	**0.4**↓	1.0-8.0
BAS %	0	ND	0	0	0	0.0-3.0
RBC (red blood cells\0	**3.66**↓	**4.69**↓	**4.85**↓	**4.24**↓	**5.15**↓	5.50-8.50*10^{12}/L
HGB (hemoglobine)	**8.4**↓	**11**↓	12.4	**10.3**↓	12.3	12.0-18.0 g/dL
HCT (haematocrite) = PCV (packed cell volume)	**28.88**↓	**33.2**↓	38.16	**33.77**↓	39.93	37.0-55.0
MCV	**79**↑	70.9	79	80	77	60-77 fL
MCH	**23.1**↓	23.4	25.6	24.3	23.9	19.5-24.5 pg
MCHC	29.2	33.1	32.5	**30.5**↓	**30.8**↓	31-34.0 g/dL
RDWc	14	12.1	15.9	14.8	15.4	
RDWs	45.3	ND	51.6	48.4	49.2	
PLT (platelets)	**95**↓	ND	240	58↓	**35**↓	200-500*10^9/L
MPV	**12.4**↑	ND	**12.8**↑	9.9	**11.9**↑	3.9-11.1
PCT	0.12	ND	0.31	0.06	0.04	
PDWc	41.5	ND	43.4	39.5	41.9	
PDWs	22.5	ND	26.9	18.6	23.4	

D.0: day of treatment; *: post-treatment with clindamycin; **: post-treatment with imidocarb dipropionate

During the whole period of antiparasitic therapy the patient received supportive therapy, including liver protectors.
One month later, at the check-up, the clinical condition of the dog was very good and at the microscopic examination of blood smears was negative for piroplasms.
The ultrasound examination did not revealed any thoracic or abdominal changes, and the coagulation time returned to normal.

Discussions
Overall, this case supports the statement that babesiosis can lead to systemic disorders (Jacobson, 2006). Some authors hypothesized that multiple organ dysfunction syndrome (MODS) in canine babesiosis appears as a consequence of the dysfunction of pro-inflammatory and anti-inflammatory mechanisms (Goris et al., 1985; Welzl et al., 2001). The circulatory complications of canine babesiosis arising from systemic inflammatory response syndrome and disseminated intravascular coagulation, may lead to multiple organ dysfunction syndrome in babesiosis.
In the last decades, a high prevalence of canine babesiosis is reported in Romania, with values ranging from 10.7% in western Romania, Banat area (Imre et al., 2013), to up 27.8% in Dobrogea (Leica et al., 2017; 2019), or from 26.10 to 30.5% in the metropolitan area of Bucharest (Anghel, 2016; 2017), even with severe, complicated, life-threatening clinical cases (Leica et al., 2019). Moreover, molecular testing of ticks infesting dogs revealed the presence of *Babesia canis* in 21% of the investigated *Dermacentor reticulatus* tick (Ionita et al., 2016). Additionally, an increasing frequency and abundance of the *D. reticulatus* tick populations in the last decades, particularly in southe-southeastern Romania suggests increasing risks as well for canine babesiosis (Ionita and Mitrea, 2017).
Moreover, apart of dogs, recently, in Romania, outbreaks of clinical babesiosis have been also reported for horses (Ionita et al., 2018).
By this case report, the challenges for diagnostic of babesiosis in the acute phase and its impact on the mechanisms of hematopoiesis, hemostasis and vascular integrity are emphasized.
Moreover, the importance of the monitoring ante- and post-hemotransfusion, as well as the administration of the babesiicide medication it is highlighted.

CONCLUSIONS

This case report emphasizes the importance of the early diagnostic and specific treatment in due time in canine babesiosis, in order to avoid occurrence of complications that may lead to multiple organ dysfunctions.
Despite of various ways for diagnosis and treatment, canine babesiosis still remain a disease with major potential risks and challenges.

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A COMPARATIVE CLINICAL AND PARACLINICAL EVALUATION OF THE EFFECTS OF POLYSPECIFIC AVIAN IMMUNOGLOBULIN Y IN FIV+/- CATS

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Abstract

Feline Immunodeficiency Virus (FIV) infection represents a viral immunosuppressive condition that predominantly affects the immune system, having no curative therapeutic options. The present research focused on the biological and physical effect of the use of poly-specific avian immunoglobulin Y (IgY) on some immune molecules of FIV infected cats, especially on gamma-globulins and white blood cells, as well as on how this therapy can amend or improve the quality of life of infected cats. The polyspecific avian immunoglobulin Y was used to treat two groups of feline patients: 10 patients infected with FIV and 10 patients free of the disease. The patients were monitored via blood samples collected on days 0, 20 and 40. The samples were used for testing the biochemical profile, CBC, WBC and the plasma concentration of 10 cytokines: Fas, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p40 and RANTES. The study also attempted to establish the eventual side effects associated with the suppressed immune system of FIV infected cats.

Key words*: Feline Immunodeficiency Virus (FIV), Immunomodulators, IgY, Cytokines.*

INTRODUCTION

FIV induces a chronic and latent infection in domestic cats (Erol & Pasa, 2013; Kahn, 2014) characterized by a polymorphous clinical manifestation (Bendinelli et al., 1995; Najafi et al., 2014), which can develop into a severe immunodeficiency in its final stage of evolution (Sykes, 2014; Najafi et al., 2014). The highest infection rate is in old, male stray cats that also manifest an aggressive behavior (Kahn, 2014; Gil et al., 2013). The main transmission route is through biting (Day, 2008; Mosallanejad et al., 2010a). FIV induces a disease similar to AIDS, characterized by the progressive depletion of CD4+ T helper cells (Shimojima et al., 2004; Hohdatsu et al., 2000). Additionally, it infects CD8+ and B cells, macrophages and dendritic cells, microglia and astrocytes (Day, 2008; Collado et al., 2012). Moreover, it is characterized by dysfunctionalities in the production of cytokines. Three distinct phases of disease evolution have been described: the primary or acute phase, the subclinical phase and the terminal phase (Sykes, 2014; Day, 2008). Of these, the terminal phase is frequently associated with overlapping infections (Sykes, 2014; Hartmann, 2015). The immune abnormalities secondary to FIV infection include: 1) the progressive reduction of the CD4:CD8 ratio; 2) the suppressed reaction of mononuclear blood cells to mitogens or specific antigens, with a reduced IL-2 production consequent the mitogen activation and an increased production of IL-1, IL-6 and TNF-α; 3) modified activity of macrophages; 4) modified lymphoid tissue; 5) polyclonal gammopathy (Day, 2008). The major clinical signs of FIV account for loss in weight, fever, muscular atrophy (Sykes, 2014; Shimojima et al., 2004), periodontal disease, respiratory infections, otitis (Sykes, 2014), enteritis, enlarged lymph nodes, opportunistic infections and neoplasms (Mosallanejad et al., 2010b; Kahn, 2014). Palliative therapy includes antimicrobials, pain management, nutritional

support (Greggs et al., 2011), fluids and teeth extraction (Gil et al., 2013). Immunomodulators are frequently used (Hartmann, 2015) and include interferon (IFN), staphylococcal protein A, *Serratia marcescens*, inactivated Parapoxovirus, Acemannan (de Mari et al., 2004). Antiviral chemotherapy is rarely indicated due to the lack of evidence for benefits of numerous antivirals, as well as due to the significant toxicity they inflict (Hartmann, 2015).

Avian Immunoglobulin Y (IgY) represents the main antibody obtained from the domestic hen (Gallus gallus domesticus) (Bentes et al., 2015; Gao et al., 2016) and is continously synthetised and transfered into the egg yolk (Dias da Silva & Tambourgi, 2010; Alustiza et al., 2016; Thomsen et al., 2016). IgY is the functional equivalent of mammalian IgG (Murai et al., 2016; Jiang et al., 2016b; Aranda-Uribea et al., 2017). Due to the fact that modern technologies allow IgY production at high and long lasting titers with relatively low costs of production (Jiang et al., 2016a; He et al., 2016), its use has been introduced in various medical fields: xenotransplant, diagnosis tools, passive immunization and alternatives to antibiotics (Ko & Ahn, 2007; Alustiza et al., 2016). IgY therapy has proven to be a successful alternative to traditional treatments for viral or bacterial pathologies (Alustiza et al., 2016; da Rocha et al., 2017), offering a passive protection against a broad spectrum of infections, as *Salmonella* spp., *Campylobacter jejuni* (Nasiri et al., 2016; Müller et al., 2015), *Escherichia coli* (Suartini et al., 2014; Kalantar et al., 2015), *Helicobacter pylori* (Müller et al., 2015), *Staphylococcus aureus* (Al-Edany, 2011; Nasiri et al., 2016), *Listeria monocytogenes, Pseudomonas* spp., Newcastle Disease virus, *Yersenia ruckeri, Edwardsiella tarda, Aeromonas salmonicida, Pseudomonas fluorescens* (Zorriehzahra et al., 2016), *and toward* Rotavirus strains or bovine coronavirus in mice, pigs and cattle (Dias da Silva & Tambourgi, 2010). The most important advantages of IgY use: 1) it is a natural compound; 2) no side effects reported; 3) not toxic for the environment; 4) it does not induce specific resistance by microorganisms; 5) it is highly specific and 6) it allows for targeted action against specific pathogens (Thu et al., 2017).

Objectives of the research. The present study continued the previous research in studying the effects of IgY in cats (Supeanu et al., 2015; Supeanu et al., 2016). The two previous studies targeted the clinical, haematological and biochemical evolution of feline patients after 10 days of IgY administration. The results of the two studies showed that chicken egg IgY administration in FIV infected felines had a positive impact on the general status of the organism and was free of adverse reactions. The positive results suggested the need for a larger study. Thus, a research protocol was initiated by using classic testing techniques as well as innovative ones, respectively the quantitative determination of 10 pro/anti-inflammatory interleukins by using the microarray method. The main objective was to identify the innermost effects avian IgY has on the feline organism. The side objectives were to identify clinical changes, variations in the haematological and biochemical parameters as well as establishing the way in which the cytokines concentration changes during and after IgY administration.

MATERIALS AND METHODS

Overview of study. A clinical-based, case-control study was conducted for non-stray cats that were brought for veterinary assistance in the Veterinary Medical Clinic of the Faculty of Veterinary Medicine in Bucharest and in private veterinary clinics, during august 2017-august 2018. Inclusion in the study was based on the written consent by the owners, a detailed anamnesis, the clinical inspection and a series of laboratory assays.

Potential risk factors assessed. All patients included in the study were of common breed (European short haired). A single patient was adopted from a different owner, the rest of the cats having a stray background. For this particular reason, no medical history of genetically transmissible pathologies existed. Moreover, the age of the patients was estimated at the time of adoption, allowing for significant errors especially in the case where they were adopted as adults. The body condition score was calculated at the time of the clinical

inspection, similar to the procedure applied in the study by Gil et al., 2013.

Patients included in the study. Inclusion in the study for the 20 cats was based on the evaluation of the FIV/FeLV status and on that for coronavirus. Quick diagnosis assays were used, and when the test results raised suspicions, further exploratory assays were performed in an authorised veterinary laboratory.

The following categories were not included in the study: FeLV positive cats, cats positive for coronavirus antigens, cats with severe clinical signs unable of self-sustaining basic functions, cats that had outside access due to the risk of not complying with the protocol, cats extremely reluctant to veterinary actions (the risk of self-injury and that for the owners and veterinarians, as well as the stress induced possibility of altering the final results).

Case and control recruitment. The triage of the patients was in accordance with their FIV status. Thus, 2 groups of patients were created:

- a case group: 10 cats infected with the FIV virus (FIV+);
- a control group: 10 cats not infected with the FIV virus (FIV-).

The product. The product used was highly purified polyvalent avian immunoglobulin Y in sterile water solution, in a concentration of 200 mg/100 mL of solution. The eggs from which the IgY was extracted were obtained from the hyperimmunization of laying hens, by using an immunogenic intramuscular administration of a mixture of several inactivated bacterial and fungal strains in accordance with a previously established vaccination protocol. The eggs and their derived products were periodically tested by the producer in order to verify the compliance with the expected IgY concentration.

For the clinical trial, a daily single dose of 10 mg IgY was used, respectively 4 mL oral solution, for 20 days. The administration was done directly in the oral cavity with the help of pipette or ad libitum by mixing it with a small quantity of the daily diet. It was expected that the time of the administration of the solution was the same in every day. Also, it was recommended that food should not be provided to the patients for at least one hour following the treatment.

Work protocol. The study spread over a period of 40 days, of which the first 20 days involved treating each patient with IgY. Three days were established for evaluating the patients:

- Day 0 (prior to the first IgY dose) - a detailed anamnesis was performed for each patient, alongside the first clinical assessment and the first blood sampling;
- Day 20 (last day of treatment) - the second clinical inspection and the second blood sampling;
- Day 40 - the third clinical evaluation and the last blood sampling.

The detailed anamnesis performed for all 20 cats included in the study is summarised in Table 1.

Table 1. Demographic and clinical data of cats

		Group				Total	
		FIV+		FIV-			
		Count	%	Count	%	Count	%
Gender	**Male**	9	90.0%	5	50.0%	14	70.0%
	Female	1	10.0%	5	50.0%	6	30.0%
Neutering	**No**	0	0.0%	1	10.0%	1	5.0%
	Yes	10	100.0%	9	90.0%	19	95.0%
Origin	**Stray**	10	100.0%	9	90.0%	19	95.0%
	Owner	0	0.0%	1	10.0%	1	5.0%
Deworming	**Occasionally**	5	50.0%	2	20.0%	7	35.0%
	Up-to-date	5	50.0%	8	80.0%	13	65.0%
Vaccinations	**No**	0	0.0%	2	20.0%	2	10.0%
	Occasionally	5	50.0%	1	10.0%	6	30.0%
	Up-to-date	5	50.0%	7	70.0%	12	60.0%

The clinical evaluation of the patients included in the study. The clinical evaluation was performed in days D0, D20 and D40 and was followed, during the same days, by sampling blood necessary for laboratory assays. D0 was considered as the standard for all

patients considering the following evaluations of the symptoms and paraclinical aspects (D20 and D40). It was taken into consideration that the study included both clinically health cats as well as those that had diverse symptomatology. A clinical scoring system was used for the evaluation of all included cats (Table 2). The system was adapted after one used in a similar study (Gil et al., 2013). 14 clinical parameters were scored using values from 0 to 2, where 0 was considered the absence of the respective symptom and 2 was noted as severe symptomatology. A total clinical score was calculated by adding individual scores for each clinical parameter previously multiplied by a factor attributed according to the relevance for the FIV clinical diagnosis.

Table 2. Clinical score used to evaluate the feline patients
(adapted from Gil el al., 2013)

Clinical parameter	Classification	Multiplication factor
Oral ulcers/ Gingivitis	0 - No evidence of oral lesions 1 - Mild to moderate oral lesions 2 - Severe oral gingivitis	1.9
Stomatitis/ Palatitis	0 - No evidence of stomatitis 1 - Mild to moderate hyperemia and stomatitis 2 - Severe hyperemia and caudal stomatitis	1.6
Ophthalmology abnormalities	0 - No evidence of ophthalmology changes 1 - Mild conjunctival hyperemia (mainly unilateral), mild keratitis 2 - Severe conjunctival hyperemia (mainly bilateral), active keratitis	0.7
Ocular discharge	0 - No evidence of ocular discharge 1 - Serous ocular discharge +2 – Muco-purulent ocular discharge	0.7
Lymphadenopathy	0 - No evidence of lymphadenopathy 1 - Mild localized lymphadenopathy 2 - Generalized lymphadenopathy	1.6
Nasal discharge	0 - No evidence of nasal discharge 1 - Serous nasal discharge 2 – Muco-purulent nasal discharge	1.1
Mucous membranes	0 - No evidence of pale mucous membranes 1 - Mild pale mucous membranes 2 – Severe pale mucous membranes	1.4
Dry coat/ Seborrhea	0 - Normal coat condition 1 - Dry coat and/or seborrhea	1.3
Body condition score	0 - Normal or fat: body condition score 4/6 to 6/6 1 – Mildly reduced body condition score 3/6 2 - Underweight animal with a body score of 1/6-2/6	1.8
Faecal appearence	0 - No evidence of diarrhea 1 - Clinical evidence of diarrhea	0.7
Concurrent diseases or Comorbidities	0 - No evidence of concurrent diseases 1 - Clinical evidence of concurrent disease 2 - Severe prostration/global weakness	2
Appetite for food and water	0 - Physiological appetite for food and water 1 - Low appetite for food and water	0.5
Social status	0 - The animal si socially active 1 - The animals tend to isolate itself	1.4
Body temperature	0 - Normal 1 - Fever	1.5

Laboratory testing. In order to establish the possible effects of poly-specific immunoglobulin Y on the organisms of feline patients, 3 blood samples from each patient were taken, as follows:

- the first blood sample was collected by using a Li-heparin recipient for the testing of the biochemical parameters;
- the second blood sample was collected by using an EDTA recipient for the testing of the haematologic parameters;

- the third blood sample was collected by using an EDTA recipient for the testing of the cytokines.

The following classes of paraclinical indicators were assessed: 5 biochemical parameters, 24 haematological parameters, included in the full blood-WBC count and 10 feline cytokines, determined through a microarray quantitative method; all the parameters are shown in Table 3.

Table 3. The parameters analyzed in the study

Parameter type	Analyzed biochemical parameter	Physiological interval (according to IDEXX, 2019a and IDEXX, 2019b)
Biochemical	ALT	12-130 U/L
	AST	0-48 U/L
	ALB	23-39 g/L
	UREE	5.7-12.9 mmol/L
	CREA	71-212 µmol/L
Haematological	RBC	5.0-10.0 *10^12/L
	HCT	30.0-45.0%
	HGB	9.0-15.1 g/dL
	MCV	41.0-58.0 fL
	MCH	12.0-20.0 pg
	MCHC	29.0-37.5 g/dL
	RDW	17.3-22.0%
	# RETIC	3.0-50.0 K/µL
	% RETIC	-
	WBC	5.50-19.50 *10^9/L
	# NEU	2.50-12.50 *10^9/L
	% NEU	-
	# LYM	0.40-6.80 *10^9/L
	% LYM	-
	# MONO	0.15-1.70 *10^9/L
	% MONO	-
	# EOS	0.10-0.79 *10^9/L
	% EOS	-
	# BASO	0.00-0.10 *10^9/L
	% BASO	-
	PLT	175-600 K/µL
	MPV	-
	PDW	-
	PCT	-
Cytokines	Fas (TNF RSF6 /Apo-1)	-
	IFNγ	-
	IL-1β (IL-1 F2)	-
	IL-2	-
	IL-4	-
	IL-5	-
	IL-8 (CXCL8)	-
	IL-10	-
	IL-12p40	-
	RANTES (CCL5)	-

The biochemical and haematological parameters were assessed in an authorized veterinary laboratory by using IDEXX equipment, shortly after the blood was collected.

Preparation and storage of the plasma samples for cytokines testing The blood samples that has been collected from the patients were processed in the same day for the extraction of 1 mL of plasma that was stored for future testing, using the following protocol: centrifugation of the blood sample (2500 rcf) for 10 minutes, extraction of 1 mL of plasma in the sterile laminar flow hood and the preservation in a sterile cryotube at -20°C until processing.

Plasma samples processing. After collecting 3 plasma samples from each patient, the samples were processed. For the quantitative determination of the 10 cytokines, the Abcam Feline Cytokine Antibody Array kit was used (10 targets, ab197414), in the immunology laboratory of the "Stefan S. Nicolau" Institute of Virology of the Romanian Academy.

Statistical analysis. The Linear Mixed model was used to analyse clinical scores and blood parameters. In these models, animals and days were included as subjects and repeated factors, respectively. The models evaluated the main effects of group (2 levels: FIV+ and FIV-), time (3 levels: 0, 20 and 40 days), and their interaction. Sidak adjustment was used for carrying out multiple comparisons. Diagnostic graphics were used to check assumptions and outliers. Log10 transformation was used to analyse AST but raw data are reported in the table. When the transformation did not improve the model, a nonparametric approach was chosen. Mann-Whitney tests were used to compare the groups at each time, while Friedman followed by Dunn's multiple comparisons tests were used to compare the time effect for each group. Data were presented as mean and standard error (SE) or median (Mdn) and interquartile range (IQR). Moreover, the coefficient of variation (CV) was calculated.

Statistical analyses were performed with SPSS Statistics version 25 (IBM, SPSS Inc., Chicago, IL, USA) and GraphPad Prism, version 7.0 (GraphPad Software, San Diego, California USA). Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSIONS

Clinical score. A significant group effect was found in Total clinical score ($P < 0.001$). The estimated marginal mean of the score was higher in the FIV+ group (7.9 ± 1.1 and 2.5 ± 1.1 in FIV+ and FIV-, respectively; $P < 0.001$) and multiple comparisons showed significant differences at each observation day ($P < 0.01$; Figure 1). The total clinical scores did not change over time ($P = 0.070$ and $P = 0.745$ for Time and interaction effects, respectively).

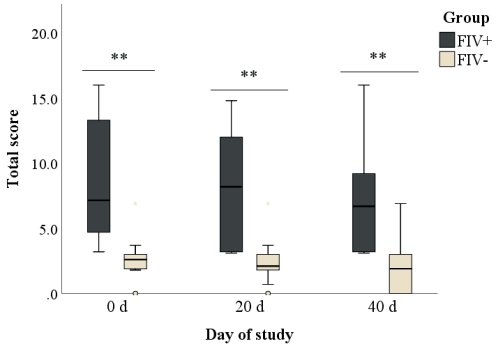

Figure 1. Changes in the total clinical score during the observation period in FIV+ and FIV- groups. **P < 0.01 (FIV+ vs FIV- in each day; Sidak correction)

Biochemical parameters (Table 4), blood count (Table 5), leukocyte formula and reticulocytes (Table 6). A significant effect of Group or Group x Time interaction was found for most of the parameters assessed. Multiple comparisons showed that RBC ($P < 0.05$), HCT ($P < 0.01$), HGB ($P < 0.01$) were higher in FIV- at days D0 and D20 while, in the same days, RDW ($P < 0.01$), and PLT ($P < 0.05$) were higher in FIV+ group. These parameters had similar values on day D40.

PCT had lower values in FIV- than FIV+ at D0 but no difference was found in the following days. Differences between FIV+ and FIV- were found only at D20 for ALT ($P < 0.05$), AST (log transformed data; $P < 0.05$), and Urea ($P < 0.05$): ALT and AST were higher in FIV- while Urea in FIV+ group.

Conversely, MCV and Eos% were higher in FIV- than FIV+ only at D40 ($P < 0.05$).

Lower values in FIV- than in FIV+ were found at all observation times for WBC ($P < 0.05$) while the opposite was always found for ALB ($P < 0.01$) and Lym% ($P < 0.05$).

As regard the changes over time, in FIV- group were found a significant increase at D40 compared to D0 and D20 for MPV ($P < 0.01$) as well as an increase at D40 compared to D20 for Baso% ($P < 0.05$). In both groups, a reduction was found at D40 compared to D20 for ALT ($P < 0.05$) while Ret% decreased at D20 compared to D0 only in FIV+ ($P<0.01$).

Finally, Crea, MCH, MCHC, PDW, Neut%, and Mono%, were not influenced neither by the group nor by time.

The coefficient of variation ranged from 0.075 for RDW to 1.333 for untransformed AST.

Table 4. Statistical variation of the serum concentration of the biochemical parameters

	Day of study	Group				*P value*		
		FIV+		FIV-		*Group*	*Time*	*Group x Time*
		Mean	SE	Mean	SE			
ALT (U/L)	**D0**	48.0a	7.3	64.0a	5.4	***0.035***	***0.048***	*0.593*
	D20	52.8a	8.0	80.6b	13.1			
	D40	42.2a	8.1	59.2a	3.9			
AST* (U/L)	**D0**	65.4a	25.2	162.2a	63.9	***0.002***	*0.457*	*0.469*
	D20	37.6a	8.1	174.4b	48.4			
	D40	35.6a	12.7	104.0a	38.7			
ALB (g/L)	**D0**	22.5a	2.4	33.3b	1.0	***0.005***	*0.341*	*0.082*
	D20	26.3a	3.1	32.4b	0.7			
	D40	24.4a	2.4	33.7b	1.9			
UREA (mmol/L)	**D0**	13.3a	3.9a	9.4a	0.6	*0.150*	*0.141*	***0.001***
	D20	16.3a	4.2	7.8b	0.5			
	D40	14.2a	4.5a	7.7a	0.3			
CREA (µmol/L)	**D0**	146.0a	24.3	136.0a	8.5	*0.695*	*0.564*	*0.140*
	D20	145.2a	25.8	147.2a	9.2			
	D40	151.5a	25.3	128.9a	7.6			

*analysis on log transformed data

Bold P values denote statistical significance at the 0.05 level.

Values followed by the same letter in each row do not differ significantly ($P \leq 0.05$; multiple comparisons with Sidak correction)

Table 5. Statistical variation of the haematological parameters

Parameter	Day of study	Group				*P value*		
		FIV+		FIV-		*Group*	*Time*	*Group x Time*
		Mean	SE	Mean	SE			
RBC	**D0**	7.05a	0.56	9.52b	0.73	***0.030***	*0.445*	*0.135*
	D20	7.08a	0.48	9.08b	0.36			
	D40	7.36a	0.66	8.13a	0.87			
HCT	**D0**	30.0a	2.1	41.8b	2.8	***0.005***	*0.558*	*0.247*
	D20	29.9a	1.4	40.1b	1.6			
	D40	31.0a	2.7	36.9a	3.8			
HGB	**D0**	11.8a	0.7	16.2b	0.9	***0.007***	*0.140*	*0.120*
	D20	11.6a	0.6	14.3b	0.4			
	D40	11.9a	0.7	13.4a	1.5			
MCV	**D0**	42.9a	1.4	44.4a	0.8	*0.188*	*0.709*	*0.086*
	D20	43.0a	1.6	44.3a	0.9			
	D40	42.2a	1.3	46.1b	1.4			
MCH	**D0**	17.2a	1.2	17.6a	1.3	*0.754*	*0.583*	*0.776*
	D20	16.9a	1.4	15.9a	0.7			
	D40	17.0a	1.4	16.4a	1.3			
MCHC	**D0**	36.9a	2.3	34.1a	0.3	*0.351*	*0.813*	*0.333*
	D20	35.5a	1.1	34.9a	0.8			
	D40	34.6a	1.0	34.9a	1.9			
RDW	**D0**	20.8a	0.3	19.5b	0.2	***0.001***	*0.502*	*0.370*
	D20	20.8a	0.3	18.7b	0.5			
	D40	20.6a	0.6	19.5a	0.4			
WBC	**D0**	14.96a	2.77	8.45b	1.11	***0.011***	*0.358*	*0.489*
	D20	16.19a	2.68	7.25b	1.03			
	D40	13.24a	2.32	7.05b	1.81			
PLT	**D0**	481a	75	296b	49	***0.017***	*0.093*	*0.265*
	D20	401a	52	180b	38			
	D40	370a	64	289a	55			
MPV	**D0**	9.8a	1.1	9.1a	1.0	*0.885*	***0.003****	*0.630*
	D20	9.6a	0.7	9.1a	0.8			
	D40	11.8a	1.6	12.5a	1.3			
PDW	**D0**	24.3a	1.0	24.2a	0.7	*0.302*	*0.527*	*0.338*
	D20	23.5a	0.7	24.3a	0.7			
	D40	23.7a	0.8	26.0a	1.5			
PCT	**D0**	0.48a	0.08	0.28b	0.06	*0.072*	*0.126*	*0.275*
	D20	0.38a	0.04	0.20a	0.05			
	D40	0.40a	0.07	0.35a	0.07			

*in FIV- group, significant increase at day 40 compared to days 0 and 20.
Bold P values denote statistical significance at the 0.05 level.
Values followed by the same letter in each row do not differ significantly ($P \leq 0.05$; multiple comparisons with Sidak correction)

Table 6. Statistical variation of the leukocyte formula and reticulocytes

Parameter	Day of study	Group				*P value*		
		FIV+		FIV-		*Group*	*Time*	*Group x Time*
		Mean	SE	Mean	SE			
% NEU	**D0**	48.7a	5.9	39.2a	3.4	*0.069*	*0.715*	*0.845*
	D20	49.8a	7.3	36.2a	3.3			
	D40	45.7a	5.9	35.9a	3.7			
% LYM	**D0**	27.29a	5.35	40.35b	4.46	***0.019***	*0.075*	***0.029***
	D20	23.32a	4.35	44.82b	4.09			
	D40	33.01a	4.35	39.11b	3.26			
% MONO	**D0**	15.76a	4.83	8.72a	1.60	*0.246*	*0.640*	*0.528*
	D20	11.77a	3.16	8.99a	2.70			
	D40	13.22a	3.54	9.46a	1.52			
% EOS	**D0**	7.76a	1.35	11.14a	1.09	*0.076*	*0.431*	*0.301*
	D20	7.95a	1.37	9.55a	1.11			
	D40	7.46a	1.44	14.78b	4.45			
% BASO	**D0**	0.44a	0.08	0.62a	0.06	*0.230*	***0.041****	*0.348*
	D20	0.47a	0.09	0.44a	0.08			
	D40	0.58a	0.11	0.75a	0.12			
% RETIC	**D0**	1.09a	0.21	0.68b	0.13	***0.038***	***0.005**#*	*0.613*
	D20	0.68a	0.10	0.47a	0.09			
	D40	0.68a	0.11	0.43a	0.07			

*in FIV- group, significant increase at day 40 compared to day 20.
#in FIV+ group, significant decrease at day 20 compared to day 0.
Bold P values denote statistical significance at the 0.05 level.
Values followed by the same letter in each row do not differ significantly ($P \leq 0.05$; multiple comparisons with Sidak correction)

Cytokines (Table 7). The coefficients of variation of the cytokines were all >1.000, ranging from 1.077 for IL-1β to 2.463 for IL-8. No cytokines were influenced by the group while a significant time effect was found for IL-8 ($P<0.01$) in FIV+ and for IL-12 both in both groups ($P<0.05$). However, multiple comparisons only highlighted the reduction from D0 to D20 of IL-8 in FIV+.

Table 7. Statistical variation of the serum concentration of the 10 cytokines

Parameter	Day of study	Group FIV+/-				*P value for group effect*
		FIV+		FIV-		
		Mdn	IQR	Mdn	IQR	
Fas	**D0**	205.65a	(0.00, 1895.77)	175.88a	(0.00, 503.62)	*0.631*
	D20	100.41a	(0.00, 1039.59)	29.64a	(0.00, 217.27)	*0.529*
	D40	129.78°	(0.00, 1122.40)	173.43a	(43.51, 655.36)	*0.739*
P value for time effect		*0.192*		*0.514*		
IFN-ɣ	**D0**	0.00a	(0.00, 201.33)	0.00a	(0.00, 0.00)	*0.739*
	D20	0.00a	(0.00, 54.28)	0.00a	(0.00, 0.00)	*0.529*
	D40	0.00a	(0.00, 0.00)	0.00a	(0.00, 0.00)	*0.739*
P value for time effect		*0.562*		*0.444*		
IL-1β	**D0**	103.77a	(22.71, 173.31)	56.89a	(37.74, 104.38)	*0.393*
	D20	57.37a	(12.63, 93.58)	42.11a	(17.63, 83.95)	*0.796*
	D40	39.33a	(10.74, 103.27)	35.48a	(9.68, 51.25)	*0.529*
P value for time effect		*0.316*		*0.316*		
IL-2	**D0**	1047.60a	(0.00, 2493.43)	429.75a	(0.00, 1317.35)	*0.529*
	D20	722.90a	(0.00, 1863.55)	405.02a	(32.73, 562.62)	*0.739*
	D40	345.66a	(2.05, 2054.97)	141.54a	(14.82, 615.56)	*0.579*
P value for time effect		*1.000*		*0.830*		
IL-4	**D0**	7.49a	(0.00, 173.29)	0.00a	(0.00, 86.00)	*0.739*
	D20	0.00a	(0.00, 88.34)	11.29a	(0.00, 52.74)	*0.436*
	D40	0.00a	(0.00, 112.53)	33.06a	(14.63, 61.87)	*0.529*
P value for time effect		*0.901*		*0.616*		
IL-5	**D0**	141.48a	(90.37, 331.55)	142.60a	(83.63, 215.03)	*1.000*
	D20	88.27a	(55.27, 292.53)	128.65a	(120.98, 346.25)	*0.123*
	D40	92.34a	(49.66, 553.08)	159.44a	(98.28, 277.09)	*0.853*
P value for time effect		*0.316*		*0.223*		
IL-8	**D0**	15.92a	(10.73, 69.06)	13.74a	(8.38, 70.14)	*0.912*
	D20	8.38b	(1.09, 21.84)	12.08a	(3.19, 14.64)	*0.481*
	D40	10.20a	(4.02, 49.54)	24.24a	(10.43, 37.15)	*0.971*
P value for time effect		***0.008***		*0.710*		

	D0	3.19a	(0.00, 26.09)	5.93a	(0.00, 19.89)	*1.000*
IL-10	**D20**	0.00a	(0.00, 13.63)	0.84a	(0.00, 21.36)	*0.853*
	D40	1.04a	(0.00, 33.29)	0.00a	(0.00, 15.96)	*0.529*
P value for time effect		*0.071*		*1.000*		
	D0	53.83a	(0.00, 149.17)	17.20a	(3.66, 86.22)	*0.613*
IL-12p40	**D20**	2.54a	(0.00, 90.83)	6.27a	(0.00, 36.20)	*1.000*
	D40	3.04a	(0.00, 79.09)	8.87a	(0.00, 75.63)	*0.631*
P value for time effect		***0.025***		***0.023***		

Bold P values denote statistical significance at the 0.05 level.
For each parameter and group, values followed by the same letter in each column do not differ significantly ($P \leq 0.05$; Dunn's multiple comparisons test)

CONCLUSIONS

All feline patients tolerated well the IgY treatment. No case of rejecting the treatment was recorded, regardless if it was administered directly in the oral cavity or if it was mixed with the usual food.

No patient recorded a negative clinical picture in D40 comparative to D0. Several FIV+ patients showed improvement in the symptomatology during the IgY administration (D20). However, these clinical signs relapsed within 20 days from the end of the treatment and were consistent with the observations made in D0. In the case of the FIV- patients the same amelioration was noted for the clinical symptomatology, with the remark that the relapse did not occur after the treatment period ended, as was the case for the FIV+ patients.

When discussing the biochemical parameters, the IgY administration determined an increase of the serum concentration for most of them (ALT, AST, UREA), with the tendency of going over the upper physiological limit values. Twenty days after the completion of the IgY treatment, the values for the biochemical parameters' serum concentrations returned to the initial levels. No other observations could be made concerning the biochemical parameters, as they tend to follow the disease specific patterns for each case.

The haematological parameters also seemed to follow the disease's specific patterns, as there was only a slight improvement for most patients that were more notable close to D20, but not enough to claim that the IgY therapy had a decisive impact on their evolution. It is also very likely that they are influenced in a more significant manner by secondary infections and by other concurrent pathologies.

The 10 analyzed cytokines manifest either a pro-inflammatory influence (IFN-γ, IL-1β, IL-2, IL-5, IL-8, IL-12p40, RANTES) or an anti-inflammatory one (Fas, IL-4, IL-10). The observed IgY effect on the anti-inflammatory cytokines was a relatively inconsistent one. However, the IgY therapy induced a decrease of the pro-inflammatory cytokines levels during the time of the treatment, especially in the case of IL-8 IL-12p40. This effect was noticed strictly during the product use.

The present study recorded no side effects recorded to the administration of IgY.

The authors consider that it can reasonably conclude that IgY administration in FIV+ and FIV-feline patients has a positive impact on the general functions of the feline organism, without side effects, thus confirming the observations recorded in the two studies aforementioned. Additionally, the study proves that the IgY administration has an anti-inflammatory effect that was highlighted by the drop in the values of pro-inflammatory cytokines, a particularly important effect in relation with the immune impairment registered in this patients.

The final conclusion of the study is that avian immunoglobulin therapies could be usually used as adjuvant to support the immune functions in FIV infected patients that have no allergies for eggs.

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Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Compliance with ethical standards

All of the procedures used in the clinical trial were compliant with the provisions of the European regulations for the welfare and

protection of animals used for scientific purposes.

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A CASE REPORT ON A RESCUED RED FOX IN AN URBAN AREA (BUCHAREST, ROMANIA) SUGGESTS POTENTIAL RISKS FOR PARASITIC DISEASES IN PETS

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Abstract

Red foxes (Vulpes vulpes) have been increasingly detected as carriers of multiple pathogens throughout Europe, being considered to be asymptomatic to the presence of most. Currently, a rapid urbanization of red foxes has been reported in many European countries, posing high risks for the both human and animal health. Here we describe a clinical case on a young male red fox which was rescued in March 2020 from an urban area of Bucharest (Romania). The fox was found collapsed and unresponsive on the street and immediately was referred to a wildlife rehabilitation center. On the clinical examination, the animal showed underweight (5 kg), hypothermia (T = 35.6°C), severe dehydration, pale mucosae, nystagmus, tremors, and hemoglobinuria. Whole body, cranial and thoracic radiographs revealed no traumatic injuries. Subsequently, a specific therapy for stabilizing the body temperature, oxygen and supportive therapy was administered. In the following two days, the general status of animal was improved, however, anorexia, slight fever (39.1°C-39.3°C), hemoglobinuria, glucosuria, proteinuria and apathy, were registered. Hepatic and renal parameters determined by biochemical analyses showed increased values. Based on this pathology, babesiosis was suspected and subsequently a blood sample was collected and analyzed by molecular qPCR technique which confirmed the Babesia DNA in the fox blood. The animal showed good response to the symptomatic treatment, therefore, no babesiicid treatment was considered. During the monitoring period, the fox displayed a clinical status significantly improved and at 13 days after its admission, it was released in a natural wild habitat. This case clearly shows that foxes invading urban areas pose potential risks for pathogens of medical and veterinary interest.

Key words*: fox, urban area, pathogen-risks, Romania.*

INTRODUCTION

Red foxes (*Vulpes vulpes*) are increasingly adapting to urban environments all around the world, as their opportunistic feeding habits are allowing them to establish stable populations close to human settlements (Handler, 2020). This can favor increased contact rates within different wildlife species and between foxes and domestic animals or humans that can lead to a higher transmission rate of infectious and parasitic diseases (Couper, 2016).

Currently, a rapid urbanization of red foxes has been reported in many European countries, posing high risks for the both human and animal health (Plumer et al., 2014).

Due to the expansion of their geographic range, increase in population density and probably a more present scientific interest, foxes have been reported to harbor a variety of parasitic agents, such as *Babesia/Theileria*., *Hepatozoon, Leishmania*), but also bacterial diseases transmitted by hematophagous vectors (Hodžić et al., 2017).

This report describes a clinical case of a red fox rescued from an urban area (Bucharest, Romania) and discusses the associated risks for emerging wild-life borne diseases for domestic animals.

MATERIALS AND METHODS

Case presentation

In March 2020, a young male fox (approx. 1 year old) was referred to a wildlife center by the local police. The fox was found collapsed and unresponsive on a closed boulevard in the urban area of Bucharest (Romania).

Immediately, the animal was subjected for a routine general clinical examination followed by laboratory and imagistic investigations. Accordingly, a supportive therapy was administered to the fox under a permanent clinical follow-up.

RESULTS AND DISCUSSIONS

On clinical examination, the animal, with an underweight (5 kg) aspect, was unresponsive to external stimuli, hypothermic (T = 35.6°C), severely dehydrated (> 10%), displaying pale and dry mucosae, nystagmus, and tremors.
The capillary refill time was over 2 seconds. No lesions of the musculoskeletal system or any other evidence of trauma were identified.
Ophthalmological examination has been performed and excluded any hemorrhages or retinal detachment.
In order to stabilize the body temperature, a specific, shock and analgesic therapy, and oxygen have been administered. An injectable protocol, of dexamethasone (1.6 mg/kg) and Butorphanol (0.15 mg/kg) were administered intramuscularly as shock therapy and analgesia. Supportive treatment, including fluids (Ringer Braun - 50 ml as bolus in the first hour and 10 ml/h for the next 24 h), vitamin cocktails (Duphalite), glucose (10%) and antihemorrhagic (250mg etamsylate) drugs were iv administered.
The next day, whole body, cranial and thoracic radiographs were performed and no traumatic injuries were present (Figures 1 and 2).

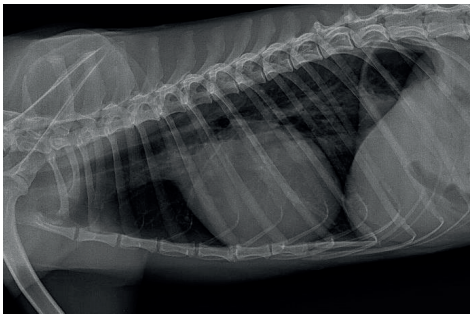

Figure 1. Latero-lateral thoracic radiograph of a rescued fox showing no abnormalities

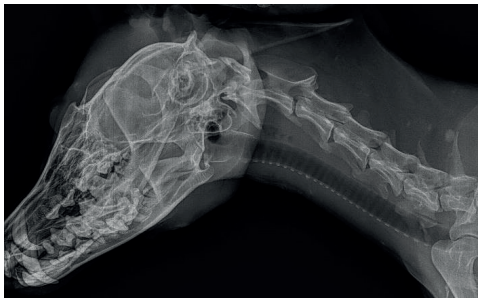

Figure 2. Latero-lateral cranial radiograph of a rescued fox showing no abnormalities

After 12 hours of its admission, the fox became awake but not responsive to external stimuli and appeared to be blind (Figure 3). The rectal temperature increased at 39.2°C.

Figure 3. The rescued fox at 24 hours after

Urine was sampled and analyzed using a multiparameter strip (Combur^{5} test® HC, Roche Diagnostics) that showed: the presence of glucose and proteins in moderate levels; hemoglobin in high levels; hematuria was not indicated (Figure 4).
Capillary refill time was around 2 seconds and mucosae were still pale.

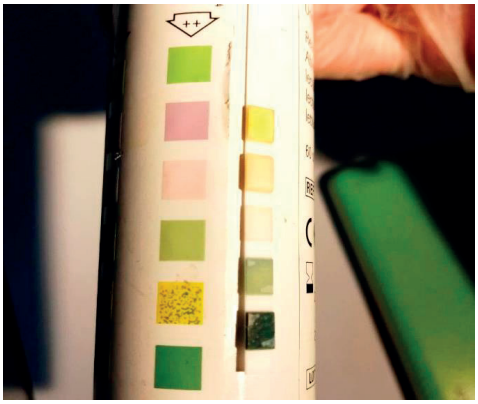

Figure 4. Urine analysis of a rescued fox showing: hemoglobinuria, proteinuria and glucosuria

The medical team decided to administer antibiotic (Amoxicillin trihydrate, dosed at 15 mg/kg (0.5 ml q 48 h, subcutaneous injection, for eight days) and antipyretic (metamizole sodium, Novasul®; 1 ml, every 24h) therapy. The body temperature was monitored daily (at 12 pm), before and after (p.t.) the administration of the antipyretic drug. The body temperature daily dynamics is depicted in Figure 5.

For the next 3 days p.t., the body temperature was characterized as slight hyperthermia (39.1°C-39.3°C), according to the normal range of body temperature in red foxes (*Vulpes vulpes*) of 37.8-39°C (Stocker, 2013).

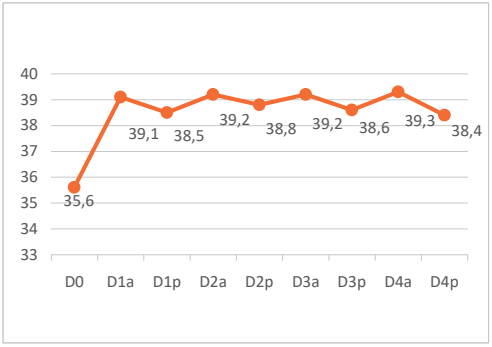

Figure 5. Dynamics of the body temperature in a rescued fox, ante (a)- and post (p)- antipyretic treatment
(D= day; D0: admission day; D1-D4: day 1-day 4)

Three days after admission, the fox regained its appetite and the clinical signs improved. However, the hemoglobinuria was still present (during of the next five days). Therefore, a blood sample was collected and subjected for laboratory investigations. The biochemical examination of the blood revealed higher levels of glucose, total cholesterol, total bilirubin, total protein, and uric acid, and lower levels of blood urea, creatinine and albumin, indicating slight alterations of the hepatic and renal functions (Tabel 1).

Table 1. Blood biochemistry parameters in the rescued fox

Parameter*	**Value registered**	**Reference value****
Glucose	141 mg/dL ↑	65-120 mg/dL
T-Cho	13.37 mmol/L ↑	2.23-12.64 mmol/L
BUN	1.6 mmol/L ↓	3.57-21.78 mmol/L
T-Bil	30.78 µmol/L ↑	7-26 µmol/L
AST	54 IU/L	8-160 IU/L
ALT	502 IU/L	39-607 IU/L
T-Pro	81 g/L ↑	35-76 g/L
Albumine	23 g/L ↓	23-44 g/L
UA	0.099 mmol/L↑	0.006-0.03 mmol/L
Creatinine	27.36 µmol/L ↓	35-194 µmol/L

*T-cho: total cholesterol; BUN: blood urea; T-BIL: total bilirubin AST: Aspartate Aminotransferase; ALT: Alanine transaminase; Pro: total protein; UA: uric acid.
**Reference values according to the BSAVA Manual of Wildlife Casualties, which adapted them from the International Species Information System reference ranges.

Blood sample was sent also to the diagnostic laboratory of molecular biology and analyzed by using a Real Time- qPCR (polymerase chain reaction) technique for the presence of *Babesia* DNA, which was found positive (Ct = 34). However, piroplasms were not detected by light microscopy, probably due to the low parasitemia (Ct = 34).
Considering the different approach used in wildlife medicine (Couper, 2016) and the suspected natural resistance of foxes to piroplasms (no clinical cases have been described), instead of the babesiisicid treatment, to continue the symptomatic therapy, under permanent clinical monitoring, was the treatment protocol of choice.
In the following four days, the animal responded well to the symptomatic treatment, its clinical status improved significantly, and the body temperature became normal (38.4°C), it began eating normally and gaining weight (BW = 5.4 kg). Therefore, at 10 days post-admission, the fox was moved in an outside pen, where showed to continue its recovering during the following days, expressing normal behavior (hiding, digging, jumping on fences) (Figure 6). Therefore, on the 13th day after its admission, the fox was released in a wild habitat, outside of the metropolitan area.

Figure 6. The rescued fox, displaying normal behavior (hiding), 13 days post-admission

Discussion

Rapid urbanization of wildlife forced many species to adapt to anthropic habitats, including foxes, hedgehogs, bats, hares and different bird species. As most species of wildlife are carriers for various pathogens, the distribution of those diseases is changing at the same pace (Hassel et al., 2017). Moreover, it is stated that at least 70% of the current emerging zoonotic infection

diseases have a wildlife origin, with cross-species spread and transmission (Jones et al., 2008). Red foxes are nowadays invading many cities worldwide, and they have colonized urban areas in numerous European countries (Plumer et al., 2014),

Recently, foxes have been found positive for different tick-borne pathogens, such as *Babesia canis, Babesia vulpes,* or *Hepatozoon canis* in studies performed in Europe (Hodžić et al., 2015). All of these species have been reported to cause clinical disease in domestic dogs (Simões et al., 2011; Ionita et al, 2012; Solano-Gallego et al., 2016).

Several recent molecular studies have reported the presence of diverse bacterial and protozoan tick-borne pathogens at the tick-host interface in Romania, highlighting potential risks for serious diseases of veterinary and medical interest (Ionita et al., 2013; 2016).

Wild animals, including foxes, represent a readily available host for hematophagous tick-vectors and subsequently reservoir for the vectored-pathogens. Therefore, urban foxes may represent a source of infections, especially for domestic carnivores, but also for other zoonotic pathogens.

Thus, recent studies in Romania report foxes as carriers of various tick-borne pathogens, such as *Anaplasma phagocytophilum* (2.55%) and *Borrelia burgdorferi* (1.42%) (Dumitrache et al., 2015). Other pathogens, *Toxoplasma gondii* (Șuteu et al., 2014), *Angiostrongylus vasorum* (Deak et al., 2017), have been also reported.

Additionaly, seven different species of fleas and five species of ticks (*Ixodes hexagonus*, *I. ricinus, I. crenulatus, Dermacentor marginatus* and *Haemaphysalis punctata)* infesting foxes in Romania have been reported, all potential vectors of pathogens of medical and veterinary interest (Dumitrache et al. 2014; Foley et al., 2017). Of these, *I. hexagonus* was reported as the most common tick infesting foxes in Romania (Mihalca, 2012; Dumitrache, 2014; Sandor et al., 2017).

I. hexagonus is recognized as main vector of the newly named piroplasm, *Babesia vulpes* n. sp., infecting dogs (Baneth et al., 2015; 2019).

Therefore, risk associated with the migration of foxes towards anthropic habitats is posed especially for domestic dogs, as many pathogens, including piroplasms can cause severe clinical diseases.

Of these, an increasing interest is emphasized on *B. vulpes* n. sp. (Baneth et al., 2015). This is a small piroplasm (for which several synonyms have been used such as, *Babesia* Spanish dog isolate, *Babesia cf. microti, Babesia microti*-like, *Theileria annae*), highly pathogenic for dogs, causing anemia, thrombocytopenia, and azotemia, but are mostly subclinical in red foxes (Criado-Fornelio et al., 2003; Baneth et al., 2019).

Infection of red foxes by *T. annae* was molecularly detected, by using PCR-based methods and subsequent sequencing, in many European countries, such as Croatia (5.0%) (Dežđek et al., 2010), Hungary (20.0%) (Farkas et al., 2015), Italy (22.88%) (Ebani et al., 2017), Austria (50.7%) (Hodžić et al., 2017), Spain (72.0%) (Checa et al., 2018).

Also, a recent study in Romania reports a prevalence of 20.17% (70/347) of the examined foxes (hunted and found dead as road kills) positive for *T. annae* (Daskalaki et al., 2018).

Therefore, as *B. vulpes* n. sp. is spreading from its thought-to-be endemic area (North-western Spain, Galicia), and is being harboured by wild canid species, all reports about its presence are important for the assessment and control of the clinical disease in domestic dogs (Falkenö et al., 2013).

Clinical disease associated with the fox-related piroplasm species, *T. annae*, has been recently described in a domestic dog (a 12 weeks old puppy), in Sweden, characterized by severe regenerative anaemia; a vertically transmission it was assumed for the infection route (Falkenö et al., 2013).

Despite of the fact foxes are believed to be asymptomatic carriers of *Babesia* infections, the clinical panel described in the present case indicate that some animals might suffer from subclinical forms of the disease, probably associated with immunosuppression or other concurrent pathologies.

However, more data and further investigations are planned in order to asses the real prevalence and molecular epidemiology of piroplasm infections in Romanian foxes and the risks they may represent for domestic animals and humans.

CONCLUSIONS

This case clearly shows that foxes adapting to urban habitats may represent potential risks for the spread and transmission of pathogens of veterinary interest.

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COMPARATIVE STUDY REGARDING THE QUALITY OF DIFFERENT TYPES OF UNPASTEURIZED BEER

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Abstract

The main purpose of this study was to compare the quality of the different types of unpasteurized beer from a profile unit in Bucharest area and samples coming from the market. The material was represented by 12 unpasteurized blonde beer samples divided in 4 batches. The samples were submitted to physico-chemical and microbiological analysis determining: alcohol concentration, original, apparent and real extract, density, limpidity, colour, pH, RDF (real fermentation degree) and microbial loading in order to verify the efficiency of pasteurization using microfiltration. pH is one of the most important parameters regarding the taste and beer stability, the values obtained were between 4.24-4.63. Another important parameter was the microbial loading, which was negative both in aerobic and anaerobic media. After analyzing all the results obtained, it is concluded that these products comply with the quality standards imposed by the legislation in force.

Key words*: unpasteurized beer, physico-chemical analyse, microbial loading, food safety.*

INTRODUCTION

Over time, beer has been defined as a low-alcohol beverage that can be obtained from malted and unmalted cereals, water, hop and yeast (Muste S. et al., 2005; Masschelein C.A. et al., 2008; Banu C., 2009; Branyik T., 2012; Hlatky M., 2013; Ghimpețeanu, M., 2017; Petcu C.D. et al., 2019).

Nowadays, on the Romanian market, there are countless types of beer obtained from different types of malt and hop, through ingenious technological processes, followed, tested and verified constantly, to make a finished product as tasty as possible, which aims to satisfy as many consumers as possible by taste, aroma, flavour and colour (SR 13355-1:1997; Cercel C., 2008; Mihaiu M. et al., 2013).

The beer contains the nutritional components of the cereals from which it is obtained and new products resulting from the alcoholic fermentation: organic acids, aldehydes, higher alcohols, water-soluble vitamins (B_1, B_2, B_6, B_{12}, PP, H), but also mineral substances (potassium, magnesium, calcium, phosphorus, iodine) (Berzescu., 1981; Banu, 2001; 2009; Banu et al., 2010; Tăpăloagă., 2013; Petcu et al., 2019).

Consumed in moderation, this drink can be beneficial to health, due to the natural ingredients from which it is obtained. Barley is an important source of proteins, fibers and vitamins, and hop is a source of antioxidants (Banu et al., 2001; 2009).

All raw materials and ingredients used to obtain beer assortments must comply with the applicable legal requirements regarding the possible presence of contaminants (Goran et al., 2012a; 2012b; Murariu O.C. et al., 2019; Murariu F. et al., 2019).

Due to the development of micro-breweries and the mass marketing of "craft" beers, all the major brands from Romania, but also from all over the world, have developed a technological system that can manufacture a product able to compete with "craft" beer, in industrial quantities, and so in the years of 2016 and 2017, the production and distribution of unpasteurized beer began in Romania.

MATERIALS AND METHODS

The study took place in a brewery, located in the Bucharest area. The general purpose of the paper is to evaluate the different types of unpasteurized beer in correlation with food safety, in order to find the physico-chemical differences between the brands.

In order to identify the different particularities regarding the beer obtaining technology, the technological flow of beer manufacturing was followed within the processing unit.

The factory is equipped with the latest technology, in terms of machinery, which are periodically checked, so that the results would reach the required level of market demands.

The technological stages take place in a completely automated stainless steel closed system. The employees at the control points can follow a series of essential parameters for the safety of the finished product (Petcu C.D., 2006). Monitoring can be done throughout the technological process of obtaining beer through several display monitors, and in case of an error, the operators are alerted visually, but also by sound to be able to solve the problem as soon as possible.

To increase the shelf life, the beer goes through a preservation process made with the help of pasteurizers or pasteurization tunnel, but for unpasteurized beer, preservation is done by cold pasteurization, meaning microfiltration of all microorganisms and enzymes using filter cartridges and avoiding contamination of beer at bottling by creating a sterile, tightly sealed environment around the bottling machine (Petcu C.D., 2014a; Petcu C.D., 2014b).

Microfiltration will be performed using a composite filtration system (BSF Alfa Laval) consisting of two sets of filter cartridges:

- 0.65 µm cartridge - used for retaining yeasts and various coarse particles;
- 0.45 µm cartridge - used to retain 1,000,000 bacteria/cm^2.

The factory has two microfiltration lines that can continuously filter 200 hl/h of beer each, and their sanitization is made with the help of a special internal cleaning system, which uses a NaOH-based sanitizer.

In the sterile sealed room in which the bottling of unpasteurized beer takes place, the temperature and humidity level are permanently controlled. An air purification system is installed to prevent the development of other microorganisms. In addition to these verification systems, the sterile chamber is equipped with a microparticle sensor that can stop the bottling if the microparticle limit in the air exceeds the set value.

During the study, 12 samples were examined. Beers come from 4 different brands, including beer produced by the study unit.

The 12 samples were divided into 4 batches by brand, so batch number 1 consists of unpasteurized beer produced in the study unit, and the other beer brands make up batches 2, 3 and 4.

In order to find out the potential differences between the beer brands studied, in the laboratory of physico-chemical analysis of the study unit, using the Alcolyzer Beer Anton Paar Analyzing System (Figure 1), the following determinations were made: percentage of alcohol, original extract, apparent extract, real extract, RDF (actual fermentation degree) and density. With the Haze-meter, the degree of limpidity was determined (Figure 2), while the pH was determined using the pH-meter (Figure 2), and the colour, using the spectrophotometer (Figure 3).

Figure 1. Alcolzyer Beer Anton Paar Analyzing System

Figure 2. Haze-meter and pH-meter with electrode

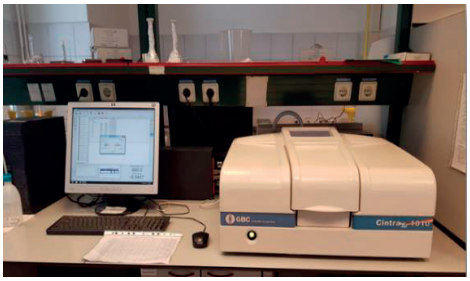


Figure 3. Spectrophotometer

In addition to to these, microbiological analyses carried out in the microbiology laboratory, were also performed, through which the microbiological load of the beer samples analysed, was determined.

Given that beer is a fermentative drink, in addition to beneficial microorganisms that transform carbohydrates into ethyl alcohol and carbon dioxide, due to minor accidents, like incorrect filtration, incorrect pasteurization or, in the case of unpasteurized beer, incorrect microfiltration, different microorganisms which could destroy the beer, can develop.

After the 10-day incubation period at 20-27°C, 100 ml of beer is filtered through a filter membrane, which is then placed in the Petri dish with the UBA or WLN culture medium. Next, the samples are incubated inside an aerobic enviroment for 3 days, respectively 7 days in anaerobic environment at 27°C, after which the counting of the colonies under the microscope is carried out.

RESULTS AND DISCUSSIONS

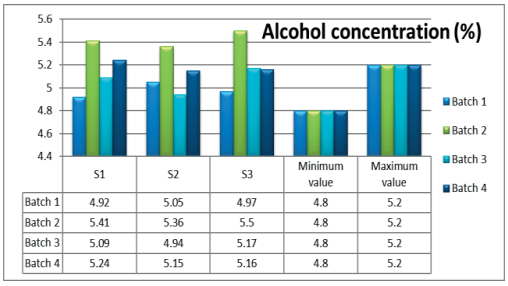
Results and discussions regarding the alcohol concentration determination

From the analysis of the obtained values it can be observed that in batch 1 all the results fall within the desired limits of 4.8-5.2%, these being the reference values.

In the case of batch number 2, all values exceed the reference values, but without distorting the product.

The alcoholic concentration of 5.3% is recorded on the label of the products that form batch 2, in correlation with the values of the determined parameters.

Batch number 3 did not exceed the limits imposed, forming a lot with compliant results. The last analysed batch registered a single minor deviation of 0.04% which does not endanger the integrity of the product (Figure 4).



	S1	S2	S3	Minimum value	Maximum value
Batch 1	4.92	5.05	4.97	4.8	5.2
Batch 2	5.41	5.36	5.5	4.8	5.2
Batch 3	5.09	4.94	5.17	4.8	5.2
Batch 4	5.24	5.15	5.16	4.8	5.2

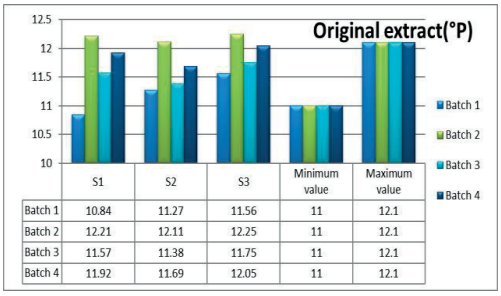
Figure 4. Evolution of the values obtained after determining the alcohol concentration (%)

Results and discussions regarding the original extract determination

The first batch analysed for the determination of the original extract started with a small deviation of the first beer sample of - 0.16°P. This deviation does not endanger the integrity of the final product.

All the results of batch 2 have exceeded the maximum limit of 12.10°P, but considering that the values are very close to this limit, the quality of the product is not jeopardized.

The values obtained from the determination of the original extract of the samples of batch 3 and batch 4 were in compliance, not exceeding the maximum and minimum values (Figure 5).



	S1	S2	S3	Minimum value	Maximum value
Batch 1	10.84	11.27	11.56	11	12.1
Batch 2	12.21	12.11	12.25	11	12.1
Batch 3	11.57	11.38	11.75	11	12.1
Batch 4	11.92	11.69	12.05	11	12.1

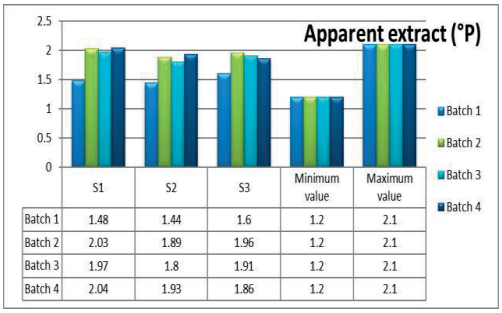
Figure 5. Evolution of the values obtained after determining the original extract (°P - Plato degrees)

The results obtained in the present study, regarding the original extract, were close to the ones obtained in the study conducted by Mudura E. et al. in 2006.

Results and discussions regarding the apparent extract determination

The results obtained from the determinations made for finding the apparent extract of all the

samples were within the quality limits of 1.20 - 2.10ºP (Figure 6).



	S1	S2	S3	Minimum value	Maximum value
Batch 1	1.48	1.44	1.6	1.2	2.1
Batch 2	2.03	1.89	1.96	1.2	2.1
Batch 3	1.97	1.8	1.91	1.2	2.1
Batch 4	2.04	1.93	1.86	1.2	2.1

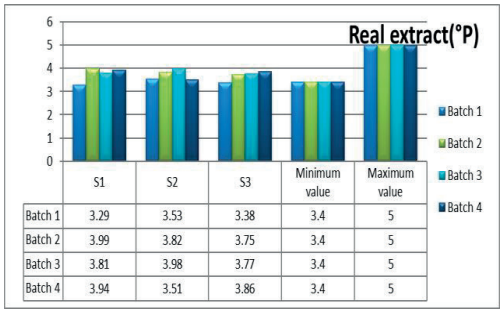
Figure 6. Evolution of the values obtained after determining the apparent extract (°P - Plato degrees)

Petcu C.D. et al., conducted in 2019 a study on a batch of 20 blond beer samples, the results of the apparent extract being close to the results obtained in the present study.

Results and discussions regarding the real extract determination

Following the determination of the real extract of the samples from batch number 1, it was found that two of the samples have values below the minimum allowed limit (sample 1, which has 0.11°P less, and sample 3 with 0.02°P less than the minimum allowed limit). Since these values are very close to the allowed limit, the quality of the beer is not considered to be altered.

Results obtained from the analysis of the samples from batches 2, 3 and 4 did not register real extract values that exceed the limits imposed, having obtained compliant values (Figure 7).

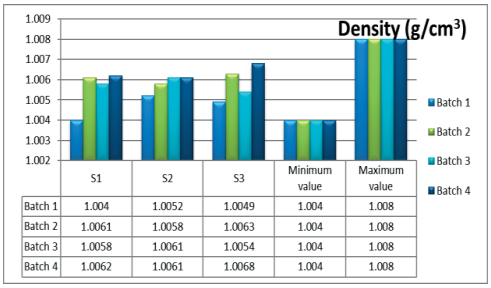


	S1	S2	S3	Minimum value	Maximum value
Batch 1	3.29	3.53	3.38	3.4	5
Batch 2	3.99	3.82	3.75	3.4	5
Batch 3	3.81	3.98	3.77	3.4	5
Batch 4	3.94	3.51	3.86	3.4	5

Figure 7. Evolution of the values obtained after determining the real extract (°P - Plato degrees)

Results and discussions regarding the density determination

The beer samples from all analysed batches obtained density results that do not exceed the limits imposed by 1.0040-1.0080 g/cm^3 (Figure 8).

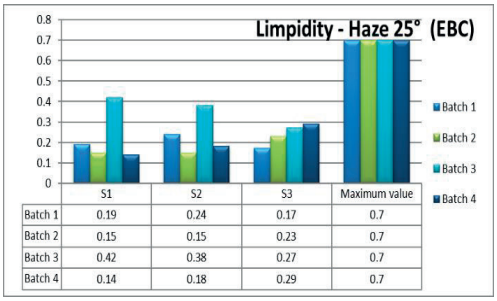


	S1	S2	S3	Minimum value	Maximum value
Batch 1	1.004	1.0052	1.0049	1.004	1.008
Batch 2	1.0061	1.0058	1.0063	1.004	1.008
Batch 3	1.0058	1.0061	1.0054	1.004	1.008
Batch 4	1.0062	1.0061	1.0068	1.004	1.008

Figure 8. Evolution of the values obtained after determining the density (g/cm^3)

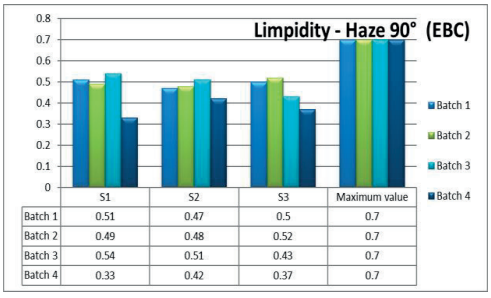
Results and discussions regarding the limpidity determination

The degree of limpidity of all the examined batches did not exceed the limit of 0.70 EBC units, neither in the case of the Haze at an angle of 25°, nor in the case of the Haze at an angle of 90° (Figures 9 and 10).



	S1	S2	S3	Maximum value
Batch 1	0.19	0.24	0.17	0.7
Batch 2	0.15	0.15	0.23	0.7
Batch 3	0.42	0.38	0.27	0.7
Batch 4	0.14	0.18	0.29	0.7

Figure 9. Evolution of the values obtained after determining the limpidity - Haze 25º (EBC)



	S1	S2	S3	Maximum value
Batch 1	0.51	0.47	0.5	0.7
Batch 2	0.49	0.48	0.52	0.7
Batch 3	0.54	0.51	0.43	0.7
Batch 4	0.33	0.42	0.37	0.7

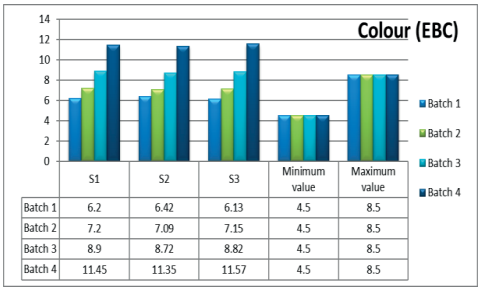
Figure 10. Evolution of the values obtained after determining the limpidity - Haze 90º (EBC)

Results and discussions regarding the colour determination

After determining the colour of the beer for the samples from batch 1 and batch 2, it was found that the results fall within the imposed limits (4.5-8.5 EBC).

The results of the samples from batch 3 registered the exceeding of the maximum limit, but these do not influence the quality of the beer.

All the samples of batch 4 have exceeded the maximum limit, but the result does not influence the quality of the beer, but the colour difference between the beer analysed in this batch and the samples analysed in the other batches is very visible, as the producers may have used a darker type of malt (Figure 11).



	S1	S2	S3	Minimum value	Maximum value
Batch 1	6.2	6.42	6.13	4.5	8.5
Batch 2	7.2	7.09	7.15	4.5	8.5
Batch 3	8.9	8.72	8.82	4.5	8.5
Batch 4	11.45	11.35	11.57	4.5	8.5

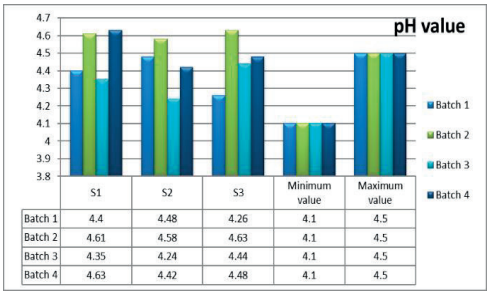
Figure 11. Evolution of the values obtained after determining the colour (EBC)

Results and discussions regarding the pH determination

The values recorded after the determination of the pH of the beer samples from batch 1 and batch 3 were within the limits of 4.10-4.50.

The values recorded from the analyses for determining the pH of the beer samples from batch 2 exceeded the maximum limit of 4.50. The pH value may be influenced by yeast that has not been removed by microfiltration or by high alcohol concentration.

In the case of beer samples comprising batch number 4, only one of them exceeded the limit imposed with 0.13 units on the pH scale. Since the values of the other samples were within the limits imposed, the integrity of the batch is not affected (Figure 12).



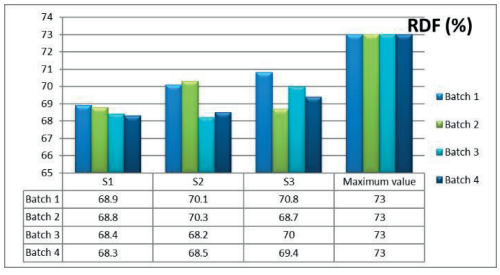
	S1	S2	S3	Minimum value	Maximum value
Batch 1	4.4	4.48	4.26	4.1	4.5
Batch 2	4.61	4.58	4.63	4.1	4.5
Batch 3	4.35	4.24	4.44	4.1	4.5
Batch 4	4.63	4.42	4.48	4.1	4.5

Figure 12. Evolution of the values obtained after determining the pH value

Comparing the results obtained in the present study regarding the pH value, with the results obtained by Petcu C.D. et al. in 2019, it is found that the accepted reference range is 4.35-4.8, a range in which the current study fits.

Results and discussions regarding the RDF (real fermentation degree) determination

The values recorded after the analysis of all beer samples for the determination of RDF do not exceed the maximum value of 73% (Figure 13).



	S1	S2	S3	Maximum value
Batch 1	68.9	70.1	70.8	73
Batch 2	68.8	70.3	68.7	73
Batch 3	68.4	68.2	70	73
Batch 4	68.3	68.5	69.4	73

Figure 13. Evolution of the values obtained after determining the RDF (real fermentation degree)

The results of these determinations recorded similar values of the RDF (real fermentation degree) with the results obtained in the study conducted by Mudura E. et al. in 2006.

Results and discussions regarding the determination of microorganisms using filter membranes

After the incubation period of 3 days in an aerobic environment, respectively 7 days in anaerobic environment at 27°C, the samples are removed from the incubator. It is noted that no microorganisms were developed on any culture medium used (UBA or WLN), so we can say

that microfiltration was effective for each type of beer (Figure 14).

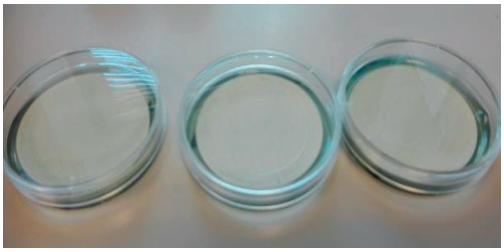


Figure 14. Representative samples of the batches of beer after incubation

CONCLUSIONS

The results were compared with the quality standards for pasteurized beer, and some samples show minor differences from the applicable standard.

Four of the analysed parameters namely: the apparent extract, the density, the limpidity and the RDF (real fermentation degree) recorded corresponding values for all the analysed samples.

On the other hand, after the determination of the real extract, deviations were recorded for 2 samples, the values obtained being with 0.11°P, respectively 0.02°P lower than the minimum allowed limit, although the quality of the beer is not considered to be altered.

In the case of the alcohol concentration determination, the exceedence of the reference values by 0.16%, 0.21% and 0.30% for the 3 samples of batch 2, is not considered a deviation from the beer quality standard.

Regarding the values obtained after the colour determination, half of the samples were within the reference range, and the results of two batches exceeded the maximum allowed limit for blonde beer. The colour difference from the other samples is clearly visible, which most likely correlates with the darker hue of the type of malt used by the processor unit.

With the help of the results obtained from the analyses carried out on each batch, we can say that most of the unpasteurized beer samples comply with the legal quality standards.

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IMPLICATIONS OF THE ONE HEALTH CONCEPT IN AGRICULTURE

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Abstract

The new conception of the sciences of life, One health, logically combines, in a unitary whole, the knowledge and practices of human medicine, veterinary medicine, agronomy, environment that can influence the public health, in one way or another. The first selection and integration of these seemingly disparate areas was made by military medicine, based on the experience of war medical protection and counterterrorism. Epidemics and pandemics can spread quickly, through travel and international trade, uncontrolled migration, military or terrorist attacks. Most biological warfare agents kill or incapacitate humans but also animals or plants and then contaminate the environment for a variable time forming a reservoir of pathogens.Disease of the live - stock and cultivated plants can lead to food crisis and can be used to defeat a country or unfair economic competition, and their effect, regardless of whether they are real or false attacks, can be very important. As a result, is required not only a close civilian-military (CIMIC) collaboration but also between the different departmental components of the state: Ministry of Health, Ministry of Agriculture, Ministry of Defense, Ministry of Finance etc..

Key words*: agriculture, biological agents, One health concept, medical protection, public health.*

INTRODUCTION

The new conception of the sciences of life, ***One health***, logically combines, in a unitary whole, the knowledges and practices of human medicine, veterinary medicine, agronomy, environment that can influence the public health, in one way or another. The first selection and integration of these seemingly disparate areas was made by military medicine, based on the experience of war and counterterrorism medical protection (Ordeanu et al., 2012; Ordeanu et al., 2015). Epidemics and pandemics can spread quickly, through travel and international trade, uncontrolled migration, military (directly, through biological attack or indirectly through precarious living conditions following the battles) or terrorist attacks (through bio-chem attacks on the opponent). Most biological warfare agents (BWA) both living: bacteria and viruses, or nonliving: toxins and bioregulators, kill or incapacitate humans but also animals or plants and then contaminate the environment (soil, water, air, objects and beings) for a variable time (days, months or years) forming a reservoir of pathogens.

Disease of the live - stock and cultivated plants can lead to food crisis and can be used to defeat a country or unfair economic competition, and their effect, regardless of whether they are real or false attacks, can be very important.

As a result, is required a close collaboration between the different departmental components of the state: the Ministry of Health, the Ministry of Agriculture, the Ministry of Internal Affairs, the Ministry of Defense, the Ministry of Finance (because without funding, none of them can act effectively), the secret services, the non-governmental organizations, the local communities and citizens as well as the civilian-military cooperation (CIMIC).

1. ANTHROPOZOONOSES

Anthropozoonoses are infectious diseases common to humans and animals, can affect humans and other animals, and those that are contagious can cause epidemics and pandemics, respectively epizootics and panzootics. The pathogenic microorganisms involved are from almost all known kingdoms of the living world: on cellular (prions, DNA and RNA viruses), Monera (aerobic and

anaerobic eubacteria), Protista (protozoa), Fungi (yeasts and molds), Vegetables and Animalia (helminths and arthropods). They act directly, through multiplication and parasitism, and/or indirectly, through their metabolism products (endotoxins and exotoxins).

Human-specific living pathogens can be controlled by specific medical counter-measures: diagnosis (clinical and laboratory), anti-infectious treatment (specific and non-specific) and prophylaxis (epidemiological surveillance, hygiene, isolation, quarantine, vaccination). In this way, for example, the eradication of human smallpox was achieved; in 1977, progress was made in the eradication of polio and other human-specific infectious diseases. Barriers are not only the poor health education of the population and the "anti-vaccination" current, but also the existence of the "animal reservoir".

In the case of most infectious diseases, which are anthropozoonotic, medical countermeasures are no longer sufficient, because the animal reservoir of microorganisms cannot be known and / or eradicated, but only controlled and diminished. This is how, in the 21st century we still have cases and outbreaks of plague, because the *Yersinia pestis* bacillus has a natural reservoir in rodents (wild and peridomestic) and a natural vector, the flea, which can transmit it to humans. And the cases occur not only in undeveloped countries, but also in the US, China, Russia, etc. An example of particular gravity is the endemic Ebola hemorrhagic fever, with repeated epidemics in West Africa, where the animal reservoir of viruses is multiple: monkeys, bats and other wild animals of hunting interest to the locals. Although both the disease and the virus have been known for a long time, we still do not have specific antiviral chemotherapy, specific reagents and no authorized vaccine, and the medical care and health education in the area are inadequate. As a result, the risk of anthropozoonosis is permanent, with epidemic outbreaks at local, regional or even pandemic level (Soulsby, et al., 2005).

We are currently facing another evolving anthropozoonosis, caused by a new COVID-19 coronavirus, with a multiple animal reservoir: bats, owls, pangolins and possibly snakes. After the species barrier is overcome, the virus spreads by interhuman transmissibility with respiratory febrile illness, which can be complicated by lethal pneumonia. This epidemic, with its epicenter in China, has been declared by WHO as a high risk international health emergency (PHEIC).

2. BIOLOGICAL AGENTS

It is no coincidence that, some of the diseases we exemplified here are also on the list of biological warfare agents (BWA). Ever since Neolithic Antiquity, when *Homo sapiens sapiens* started organizing conquering wars, he has used, besides various cold weapons and some poisons, in fact toxic and/or infectious substances, to facilitate the defeat of the adversary by disease. The Hittites used the bodies of dead sheep from anthrax, the corpses of dead soldiers from plague, etc. Only in the nineteenth century, after Pasteur laid the foundation for microbiology, was the concept of ***biological weapon*** outlined, with "militarized" pathogens for an optimal effect. It disperses living pathogens (bacteria, viruses, etc.) or nevi (toxins, regulators, etc.) in order to disrupt enemy fighting forces, the civilian population, domestic animals (for work or food), wild animals (as microbial reservoir pathogens), cultivated and uncultivated plants (for food shortages) and to contaminate the environment (water, air, soil, objects). For example, in Romania, during World War I, German sabotage agents contaminated with moray and anthrax bacilli the ports of Braila, Galati and Constanta, to stop the export of horses and sheep to the allies. Biological attacks, whether up front or masked, have diversified into different parts of the world. But unpredictable "accidents" also occurred. In Vietnam, US aviation launched defoliants in the jungle to see Vietnamese fighters, but many pilots became intoxicated or became ill with cancer. In Iraq, the "Gulf syndrome" has emerged as an unwanted consequence of the combined protection (anti-anthrax vaccine, cholinesterase antidote and insecticide). In the USSR, at the biological agent factory in Sverdlovsk, an accidental aerosolization of mycotoxins "yellow rain" (from other sources - were anthrax spores) led to many deaths. After the last cases of smallpox recorded in 1977, an

aerosolization of smallpox virus in the laboratory in London led to the death of the photographer (the last death caused by smallpox), which prompted the design of Secure (BSL3) and Highly Secured (BSL4a and BSL4b) Labs (Ordeanu et al. 2008; Ordeanu at al. 2012).
Currently, military regulations list eight bacterial species (Table 1), three groups of viruses (Table 2) and five toxins (Table 3) that could be used as biological weapons with BWA [STANAG 4632] and for which we must be prepared, at least at the conceptual level, for medical countermeasures, in order to protect the troops and the civilian population in the affected area (Ordeanu et al., 2015).

Table 1. Bacterial warfare agents (STANAG no. 4632)

No.	BWA	Disease	Sickness	Effect
1	*Bacillus anthracis*	Anthrax	Anthropozoonosis	lethal
2	*Yersinia pestis*	Plague	Anthropozoonosis	lethal
3	*Francisella tularensis*	Tularemia	Anthropozoonosis	lethal
4	*Vibrio cholerae*	Cholera	Anthropozoonosis	lethal
5	*Brucella melitensis*	Brucellosis	Anthropozoonosis	-
6	*Burkholderia mallei*	Glanders	Anthropozoonosis	lethal
7	*Burkholderia pseudomallei*	Melioidosis	Anthropozoonosis	lethal
8	*Coxiella burnetti*	Q Fever	Anthropozoonosis	-

Table 2. Viral Biological Warefare Agents (STANAG no. 4632)

No.	BWA	Disease	Sickness	Effect
1	Venezuelean equine encephalitis virus	Venezuelean equine encephalitis	Anthropo zoonosis	lethal
2	Orthopox virus	Smallpox	-	lethal
3	Yelow fever virus	Yelow fever	-	lethal

Table 3. Biological Warfare Agents toxins (STANAG no. 4632)

No.	BWA	Sickness	Effect
1	Ricin	ricinism	Lethal
2	Saxitoxin		Lethal
3	Botulinum toxin	botulism	Lethal
4	Staphylococcal enterotoxin B	-	Lethal
5	T2 mycotoxin	mycotoxicoses	Lethal

It seems that at present the risk of ***biological warfare*** is minimal, because the effect is not immediate, the consecutive epidemic is random because of extremely different factors, and international law categorically prohibits the production, storage and use of biological weapons (BTWC 1972) (Chevrier et al. 2004; Dando, et al., 2000).
But here is another risk, validated by multiple episodes in recent times. ***Terrorism***, both national and international, uses ***bioterrorist attacks*** and ***biocrime***, for different purposes - political, economic, religious etc.. The problem is the great diversity, because it is unlikely for BWA to be obtained from military stocks, but they can use any pathogens they can harvest from nature, from hospitals, laboratories, etc., so the list is open to any living agent (virus, bacteria, fungus or parasite), toxin or bioregulator, even if lethality is reduced. This results in difficulties of diagnosis, treatment, prophylaxis but also panic, capable of leading to disturbances of the economic-social life, which is exactly what the terrorists want (Eremia et al., 2019; Popescu et al. 2016).

3. IMPACT ON PUBLIC HEALTH IN THE BROADER SENSE

Factors that lead, directly or indirectly, to the sickening of people, animals and plants may be common, or even if they are different, the result will be affecting the public health and the economy, thus reducing the standard of living, work capacity and the struggle of the population of the attacked country, a biological crisis with multiple implications.
The purpose of the military biological attack can be tactical (on the military in the field sector where the attack will occur), operative (on troop concentrations and reserves) or strategically (on large cities with political, administrative, economic etc. importance), the latter being able to cause devastating epidemics among the civilian population ("USAMRIID's, 2011).
The purpose of the bioterrorist attack may be motivated by any of the terrorist claims. The attack can be local (tactical or biocrime) or disseminated (as were multiple attacks with letters with anthrax spores from the US and EU) to gain various advantages.

Any one of these attacks can cause an out of control epidemic, to make the military and civilians sick, from both camps, having *in extremis*, a pandemic potential. Also, when it comes to anthropozoonotic agents, the animals in the area, on which the quarantine measures are not very efficient, can be sickened, and the environment remains contaminated for a variable time, depending on the nature of the agent (Gal et al., 2019).

The medical countermeasures m ust be taken by the Ministry of Health, through the human, state and private medical network, the Ministry of National Defense, the Ministry of Internal Affairs, the NGOs (for example the Red Cross National Organization), the local authorities and each individual citizen, as well as by the Romanian Intelligence Service, if there is a suspicion of a terrorist attack. These must be broadcasted, in a correct and quick manner, by the press (not as it is usually done) in order to reach everyone.

4. BIOLOGICAL ATTACK ON ANIMALS

A particular feature is presented by the pathogens specific to certain species of animals, especially mammals. They will not make people sick, but they can cause serious economic losses by killing or incapacitating work animals, by reducing the livestock septum and creating microbial reservoirs in wild animals. The result may be insecurity in regards to food from animal products, stopping exports, bankruptcy of some producers and huge economic losses.

The countermeasures must be taken by the Ministry of Agriculture, by veterinary medicine and animal husbandry for domestic animals, and by the Ministry of the Environment for wild animals.

The deliberate spread of infectious agents for animals, in time of peace or immediately before the start of a war, can be concealed, to produce economic instability. There is also the potential risk that this type of attack will be committed for reasons of unfair economic competition, in order to eliminate another strong competitor from the market. In these situations, exotic agents such as the Ebola virus (which the Aum sect had tried to bring from West Africa to Japan) would not be used, but biological agents that are naturally found in the targeted area and cause either anthropozoonoses, either bacterial or viral diseases specific to domestic and wild animals. (Tables 4, 5)

Table 4. Diseases caused by bacterial agents targeting animals (Ordeanu V. and col., 2012)

No.	Disease	Sick animals
1	Anthrax	Man, cattle, sheep
2	Brucellosis	Man, cattle, sheep, goats,dogs
3	Tularemia	Man, cattle, sheep, rabbits
4	Q Fever	Man, cattle, sheep
5	Glanders	Man, horses
6	Melioidosis	Rodents, mammals, humans

Table 5. Diseases caused by viral agents targeting animals (Ordeanu V. and col., 2012)

No.	Disease	Sick animals
1	Rift Valley Fever	Human, cattle, sheep, goats
2	African swine fever	Swine
3	Foot and mouth disease	Cattle, sheep, pigs
4	Classical/African Swine fever	Swine
5	Vesicular stomatitis	Cattle, horses, pigs
6	Newcastle disease	Birds

A special mention deserves *Salmonella* sp, Gram-negative bacillus enterobacteria, but which is always pathogenic, causing ***salmonellosis***, with different degrees of pathogenicity and virulence, according to the species incriminated, from the most serious (typhoid fever with *Salmonella typhi*, paratyphoid fever with *Salmonella paratyphi* A and B), to medium and light ones, which spread through water (water epidemics). But other animals also contaminate humans: mammals, birds, reptiles, etc. Currently, ***salmonellosis is the most widespread disease transmitted through food***, although in the collective diet special measures are taken for the processing and storage of poultry meat, eggs, dairy products, etc. (Humphrey T. et al., 2005).

It is known that zoonoses can be natural or caused (bioterrorism or biocrime) to reduce livestock and exports through infectious diseases. Veterinary medicine describes the most important 26 bacterial diseases, 14 viral diseases and 30 parasitic zoonoses, most of which can infect the human being (Soulsby et al., 2005).

Some infectious diseases are specific to certain animal species and do not infect humans, but by reducing the livestock this causes significant economic damage and even starvation. Here are some examples of zoonoses specific to

domestic animals that could be used in biological attacks:

Horned: Infectious epididymitis of rams (*Brucella ovis*); Contagious agalactia of sheep and goats (*Mycoplasma agalactiae*); Chlamydial bronchopneumonia of the calves (*Chlamydophila pneumoniae*).

Swine: Pig's Pleuropneumonia- *Actinobacillus pleuropneumoniae*; Classical swine fever - (the virus of classical swine fever); African swine fever (African swine fever virus).

Birds: Infectious coryza of birds (*Haemophilus paragallinarum*); Egg drop syndrome (*Duck Adenovirus A type 1*); Infectious anemia of chickens (*Chichen anemia virus*)

Rabbits: Mucous enteritis of rabbits (*Haemophilus paracuniculus*)

Dogs: Canine parvovirus (*Canine parvovirus type 2, CPV-2*)

Cats: Parvovirus of cats (*Feline parvovirus, FPV)* (Mânzat et al., 2001)

5. BIOLOGICAL ATTACK ON PLANTS

Attacks with phytopathogenic biological agents on a country's agriculture will have serious economic consequences, and in the event of a blockade, it could lead to a decrease in food production and hunger. The quantitative or qualitative impact on the cultivated plants used in food, the technical ones, the forage and the non-cultivated plants would have multiple, direct and indirect, implications on public health.

In modern agriculture the increase of the crops is obtained using plants resistant to different diseases, selected or genetically modified. Diseases that attack plants can be caused by viruses, fungi, bacteria and animals.

In the case of ***viruses***, the transmission of diseases needs a vector to attack the plant (wheat, corn, rice, etc.). Because genetically identical plants are grown on large areas, a disease against them that they cannot resist could compromise all the vegetation. A classic example is the Tobacco Mosaic Virus, the first scientifically described virion.

Bacteria penetrate the epidermis through open spots or wounds caused by blows, and then multiply inside. They can produce toxins or destructive enzymes that affect plants in different ways. There are 160 known species of bacteria capable of generating diseases to over 150 species of plants. An example is the "wet potato rot" *Erwinia carotovora*, a bacterium that especially destroys vegetables stored for long periods: potatoes, carrots, but also affects the growth of plants, such as salad. The biological attack can cause heavy losses, both to crop plants and to stored plants. An example was the natural infection of potatoes in Germany, during World War I, which led to famine and contributed to the defeat of the German Empire.

The plant diseases caused by microorganisms are in great part caused by ***fungi***. They form resistant spores in a dry environment and easily spread by the wind. Upon reaching the spores on plants, under suitable conditions of humidity, they germinate and then penetrate from the outside of the epidermis and then can reproduce in the leaves, stem and root. After one or more weeks the plant is destroyed, new spores are formed which continue to disperse (Ordeanu et al., 2012).

The most important molds are those that affect the basic food of the population in the respective area: in the wheat civilization (Europe, North Africa, North and West Asia) is the "wheat rust" *Puccinia graminis* and in the secondary, the "rye horn" *Claviceps purpureea*, in the civilization of rice is the "rust of rice", in the civilization of corn it is the "corn stains" and in the potato culture it is the "hand of the potato" *Phytophora infestans.*

Many of these molds (filamentous fungi) produce ***mycotoxins***, which have a pathogenic, carcinogenic, teratogenic, abortive or lethal effect. An example was the toxicity given by the "rye horn" in Ireland, which was widespread, which depopulated the country and led to mass emigration to the USA. Also, during World War II, corn remained unused on the field, in Ukraine, it molded. To those who used it, as food appeared the epidemic Aleucia, and at the siege of Leningrad the moldy flour from which the bread was made led to deaths by aflatoxicosis. The mold of the feed, due to negligence, can lead to the death of animals, for example thousands of rabbits (for example at the Cantacuzino Institute, during the severe economies of the 1980s), to mycotoxin contamination of the meat, organs and milk consumed by humans.

Defoliants and herbicides are another means of attack, on agricultural and/or wild crops, including forests, to cause damage to agriculture, forestry or to counter the masking of tactical objectives.
The various insect *plagues* capable of living in/on the plants are also designed as biological weapons, for example the Colorado bug (harmful to potato crops) that arrived in Eastern Europe at the beginning of the Cold War, sent with weather balloons (including in Romania). Currently, the planet is facing invasions of locusts from Africa, for which China has used an "army" of 100,000 ducks. The protection against the biological attack on the plants is managed by the Ministry of Agriculture, through the Agricultural Directions for cultivated plants and food storage, as well as by the Ministry of the Environment for wild plants and forests, with the support of all the competent entities (Ordeanu et al., 2015).

6. PROTECTION AGAINST THE BIOLOGICAL ATTACK

The countermeasures will be integrated at national level through the Country's Supreme Council of Defense (CSAT) and at governmental level through the Commission for disasters: according to the modern concept one health, are also involved the Ministry of Public Health, the Ministry of National Defence, the Ministry of Internal Affairs and other departmental structures, including the secret services.
The protection in this area is complex, interdepartmental, but it is mainly addressed to the sector concerned (people, animals, plants, environment or mixed) with the purpose of defending public health. The measures are phased (BTWC 1972) before the attack: national and international legislation in the field, control, external and internal information, *scientific intelligence*, counter-attacks at the source, organization of defense, including anti-terrorist intervention;
- during the attack: alarm, means of individual and/or collective protection, primary CBRN detection and identification (Chemical, Biological, Radiological and Nuclear), antidotes, combating living and nonliving vectors, as the case may be, including counterterrorism intervention;
- after the attack: specific decontamination, including Disinfection, Disinsection, Deratization (DDD) identification and confirmation, sanitary treatment, specific treatment, case-tracking, integrated epidemiological measures (human, veterinary, phytosanitary and environmental), professional medical, veterinary and phytosanitary intervention for limiting the effects of the attack and normalizing the situation.
The more the measures will be faster and more appropriate to the situation, the more the consequences on the public health and the economy will be reduced. In the planet's Biosphere, the life and health of all are interdependent from the largest living being to the smallest.

The human being as an individual is a cenosis, a superorganism composed of tens of billions of "own" eukaryotic cells and hundreds of billions of other useful living organisms, microorganisms (prokaryotes, eukaryotes, cells) that are in saprophytic relationships with it. But sometimes the relationship is parasitic and infectious diseases occur. In order to obtain the vital energy, the human who is at the top of the "trophic chain" uses foods of animal, vegetable, fungal or microbial origin, and their quality is reflected in the human health: "tell me what you eat, and I will tell you who you are" (Ionescu, 2018; Ionescu, 2006).

CONCLUSIONS

The new ***One health*** medical concept encompasses the reality of human interconnection in the Biosphere, and shows the important role for the public health of animal and plant health, so direct and indirect links with veterinary medicine, agriculture and the environment. Good public health cannot be achieved without the good health of animals and plants, of all living things that constitute the biosphere.
Apart from the natural risks, which as professionals, we know and combat, there is the artificially created risk, with bad intent, in order to cause a damaging impact on the public health, to create economic damages (material and financial) that we must identify, at the conceptual and practical level, to have it counteracted in order to ensure the health of the nation.

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ECOLOGICAL DAIRY PRODUCTS: HEALTHY OR JUST A TREND?

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Abstract

With the development of the food industry, the products have undergone different phases of diversification, so today we meet products that did not exist yesterday or simply were not known, referring, for example, to "BIO", "ECOLOGICAL" or "ORGANIC" products. These can be defined as products of animal or vegetable origin, obtained without the use of chemicals or genetically modified components, which have not been exposed to irradiation, and as a result of their production, the environment has not suffered. In this study, we analyzed samples of dairy products represented by drinking milk, sour-batter milk, kefir, fermented cream, yogurt and fruit yogurt. A comparison was made between conventional products and ecological products of the same type, in terms of ingredients, and their nutritional value. To identify the different features of conventional or ecological dairy products, physico-chemical parameters such as fat content, carbohydrates, proteins, salt or other added substances were analyzed. Regarding the verification of the ecological products labeling, in all the analyzed samples it was found that the ingredients used come from the ecological agriculture, therefore respecting the labeling requirements. At the same time, these products were analyzed organoleptically, observing the appearance, colour, consistency, smell and taste. Analyzing the obtained results, it was found that the ecological products show some changes in the chemical composition and nutritional values. The salt level of the dairy products analyzed, although it is described on the label as part of the natural salt of raw milk, in the case of ecological products, the value obtained is lower, compared to that of conventional products. Ecological fruit yoghurts have a higher content of piece of fruit compared to conventional yogurts. In conclusion, some ecological products have a higher nutritional value compared to conventional dairy products, without registering statistically significant changes.

Key words: ecological agriculture, bio-eco-organic, milk, nutritional value.

INTRODUCTION

Milk and dairy products meet the body's needs in energy and in substances with a plastic and biostimulatory role, positively influencing the health of consumers (Worsley et al., 2003; Usturoi M.G., 2007; Sala C.C., 2008; Claeys W.L. et al., 2013; Claeys W.L. et al., 2014; Ladokun O. et al., 2014; Visoescu I.D. et al., 2015; Nistor C.E. et al., 2019; Oprea O.D. et al., 2019).

Currently, nutrition puts its mark on the pathology of contemporary humans, as a result of the imbalance between the intake and the need for biologically active substances.

Nutrition is a factor with permanent action, which determines the development of metabolic processes, because food represents their source and their moderator (Tăpăloagă D. et al., 2017). Also, maintaining the body's homeostasis depends on the nature of the diet, which influences the functions of the system, especially the enzymatic and hormonal factors

Until recently, the provision of sufficient quantities of food was the primary requirement, but today, special attention is paid to ensuring the integrity of foods and their nutritional value (Savu C. et al., 2002).

It has reached a stage where the concept of "food safety" is increasingly difficult to control and audit (Petcu C.D., 2006; Petcu C.D. et al., 2014), due to the increasing pollution of the atmosphere and due to the development of the industry that generates toxic gas emissions, which is affecting products in general, and food in particular.

The number of harmful elements in the environment has increased greatly and so did the number of preservatives or substitutes of taste or aroma used in the food industry.

Ecological agriculture is a production method that preserves soil structure and fertility, promotes a high standard of animal welfare and prohibits the use of substances such as: synthetic pesticides, herbicides, chemical fertilizers, genetically modified organisms or growth enhancers, such as antibiotics. Farmers use techniques that help maintain ecosystems and reduce pollution. Only a limited number of additives and technological aids can be used in the ecological processing of food products (European Regulation no. 848/2018; Gonciarov M. et al., 2014; Gonciarov M. et al., 2015; Gonciarov M., 2017; Tapaloaga D. et al., 2018).

Presently, in Romania, the trend of ecological products is expanding. In well developed countries, this is a concept already rooted in the lifestyle of the population. Despite the fact that the natural ingredients based products, without preservatives and dyes, are more expensive, the interest and the degree of information of Romanians has increased recently. Thus, in supermarkets or specialized stores, we can find a diverse range of ecological products.

ECOLOGICAL, ORGANIC, BIO are terms that have the same meaning (Figure 1), each being specific to another geographical area.

The term **"organic"** is used for food products in the Anglo-Saxon space (organic food, organic milk).

The term **"bio"** is used especially in the Franco-German space (agriculture biologique).

The term **"eco"** or **"ecological"** is used in Romania, being the term accepted by the Ministry of Agriculture and Rural Development (www.madr.ro).

Figure 1. The relationship between the terms bio, ecological, organic and natural

The term **"natural"** or **"natural 100%"** applied on the label of some products is only a marketing strategy, which does not necessarily offer the guarantee of a quality product and certainly does not indicate an ecological product. The legislation does not refer to the labeling and classification of products using the term "natural" (www.agrointel.ro).

Labeling of ecological products

In recent years, major advances have been made in terms of healthy food. The world has begun to get rid of unhealthy habits and place greater emphasis on the food quality and safety. This can be observed from the increased number of consumers interested to read the label and to check the packaging, this being the consequence of the ascertainment that most of the additives and chemicals used in the treatment of products can trigger pathological conditions (Petcu C., 2015).

The provisions regarding the labeling of products obtained from ecological agriculture, (Figure 2), established in Regulation (EC) no. 848/2018 regarding ecological production and labeling of ecological products are very precise and are intended to offer consumers complete confidence in ecological products, as products obtained and certified according to strict rules of production, processing, inspection and certification (Regulation (EC) No. 848/2018).

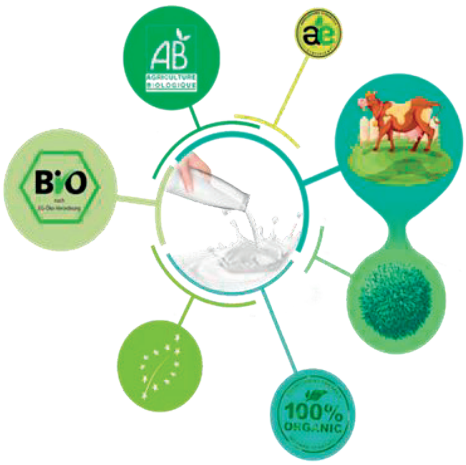

Figure 2. Logo used on the labels of ecological products

The Romanian ecological food products can be easily identified by the buyers because they have the "ae" logo on the label or packaging (Figure 3), which means product obtained in Romania from the ecological agriculture (www.madr.ro).

Figure 3. The logo of ecological agriculture (www.madr.ro)

The use of "ae" on the label is mandatory in the case of local products. However, to facilitate the identification of ecological products on the store shelves, the logo "ae" can also be applied to imported products, if they are also certified in Romania by an inspection and certification body accredited to us (www.tradiţiisibiu.ro - Guide "Ecological products", 2012).

The logo "ae" (Ecological Agriculture), owned by M.A.D.R. (Ministry of Agriculture and Rural Development), guarantees that the product so labeled comes from ecological agriculture and is certified by an approved inspection and certification body. The rules for the use of the "ae" logo are included in Annex No. 1 at the Common Order for the modification and completion, at the Annex to the Order of the Minister of Agriculture, Forests and Rural Development no. 317/2006 and at the President of the National Consumer Protection Authority no. 190/2006 for the approval of the specific rules regarding the labeling of ecological food products (www.madr.ro).

The right to use the logo "ae" (Ecological Agriculture) on the products, labels and packaging of the ecological products belongs to the producers, processors and importers registered with the M.A.D.R. (www.madr.ro).

The Community logo offers the recognition of ecological certified products throughout the European Union (Figure 4).

Figure 4. EU ecological logo for the certification of ecological products (www.madr.ro)

Consumers who buy products bearing the national logo as well as the Community logo can be confident that:

- at least 95% of the ingredients of the product were obtained according to the ecological production method;
- the product complies with the ecological production rules;
- the product bears the name of the manufacturer, processor or seller, as well as the name or code of the inspection and certification body;
- the label "ecological" is granted only to the producers inspected and certified by the inspection body;
- the inspection and certification bodies authorized by the Ministry of Agriculture may grant producers the right to use the ecological logo, if the results of the inspection carried out are in accordance (Gonciarov M., 2017).

MATERIALS AND METHODS

In order to identify the differences between ecological and conventional dairy products, physico-chemical parameters were analyzed such as: fat content (butirometric method or by using the Funke Gerber®LactoStar dairy analyzer), carbohydrates, proteins, salt (by using the Funke Gerber®LactoStar dairy analyzer) and other added substances. At the same time, these products were analyzed organoleptically, following the appearance, colour, consistency, smell and taste.

A number of 20 types of conventional and ecological dairy products were analyzed, represented by 1.5% fat drinking milk, 3.5% fat drinking milk, 3.7% fat drinking milk, 2% fat sour-batter milk, 3.5% fat kefir, 3.5% fat yogurt, 25% fat fermented cream, banana yogurt, strawberry yogurt and peach yogurt.

RESULTS AND DISCUSSIONS

The results obtained from the comparative analysis of conventional and ecological products were examined, finding that the ecological products show some changes in chemical composition and nutritional values.

Although the salt level of the product is described on the label as being part of the natural salt of milk, in the case of the

ecological 1.5% fat drinking milk, this level is lower compared to conventional products (Table 1).

Table 1. Differences and similarities between conventional 1.5% fat drinking milk and ecological 1.5% fat drinking milk

CONVENTIONAL PRODUCT	ECOLOGICAL PRODUCT
Conventional 1.5% fat drinking milk	**Ecological 1.5% fat drinking milk**
Ingredients: semi-degreased, standardized, homogenized and pasteurized cow's milk.	Ingredients: semi-degreased, homogenized and pasteurized at high temperature cow's milk, from ecological production. Contains milk lactose.
Nutritional information/100 ml product	Nutritional information/100 ml product
Energetic value: 185kJ/44 kcal	Energetic value: 185kJ/44 kcal
Fats: 1.5 g	Fats: 1.5 g
of which saturated fatty acids: 1 g	of which saturated fatty acids: 0.9 g
Carbohydrates: 4.5 g	Carbohydrates: 4.5 g
of which sugars: 4.5 g	of which sugars: 4.5 g
Protein: 3.1 g	Protein: 3.1 g
Salt: 0.1 g*	Salt: 0,06 g*
Calcium: 118 mg (14.75%)	Calcium: 125 mg (15.6%)**
*the natural salt of milk	*the natural salt of milk **from the daily reference nutritional value
Storage temperature: +2...+4ºC	Storage temperature: +2...+4ºC
	

Products with a high fat content are perceived by consumers as "creamy". Thus, ecological products with a higher fat content have an increased sensory score in terms of pleasing taste (Worsley A., 2003; McCarthy K.S. et al., 2017). Ecological 3.7% fat drinking milk is also recommended in children's nutrition, benefiting from a high intake of vitamins and minerals (Table 2).

Table 2. Differences and similarities between conventional 3.5% fat drinking milk and ecological 3.7% fat drinking milk

CONVENTIONAL PRODUCT	ECOLOGICAL PRODUCT
Conventional 3.5% fat drinking milk	**Ecological 3.7% fat drinking milk**
Ingredients: semi-degreased, homogenized and pasteurized at high temperature cow's milk. Contains milk lactose.	Ingredients: 99.83% organic whole milk, minerals (iron, zinc, iodine), vitamins (A, D_3, C, E, B_1, B_6, K_1, niacin, folic acid, biotin, pantothenic acid), natural flavors.
Nutritional information/100 ml product	Nutritional information/100 ml product
Energetic value: 260kJ/62 kcal	Energetic value: 273kJ/65 kcal
Fats: 3.5 g	Fats: 3.7 g
of which saturated fatty acids: 2.1 g	of which saturated fatty acids: 2.0 g
Carbohydrates: 4.5 g	Carbohydrates: 4.7 g
of which sugars: 4.5 g	of which sugars: 4.7 g
Protein: 3.2 g	Protein: 3.3 g
Salt: 0.1 g*	Salt: 0.1 g*
Calcium: 125 mg (5.6%)**	
*the natural salt of milk **from the daily reference nutritional value	
	
	Vitamins si minerals
	Calcium: 120 mg
	*Phosphorus: 90 mg
	Iron: 1.4 mg
	Zinc: 1.5 mg
	Iodine: 8.5 µg
	*Magnesium: 12 mg
	*Potassium: 140 mg
	*Sodium chloride: 85 mg
	Vitamin A: 80 µg
	Vitamin D_3: 1.7 µg
	Vitamin C: 5 mg
	Vitamin E: 1 mg
	Vitamin B_1: 0.15 mg
	*Vitamin B_2: 0.14 mg
	Vitamin B_6: 0.2 mg
	*Vitamin B_{12}: 0.4 µg
	Vitamin K_1: 4.7 µg
	Niacin: 1.8 mg
	Folic acid: 20 µg
	Biotin: 15 µg
	Pantothenic acid: 0.6 mg
	*Vitamin and mineral content is due exclusively to their naturally occurring presence in cow's milk
	Storage temperature: +2...+6ºC

Following the salt level in the ecological 2% fat sour-batter milk, compared to a conventional dairy product, a lower salt level is observed, although in both cases the salt content is described on the label, as being part of the natural salt of milk.

In contrast to the conventional product, the calcium content is also written on the label of the ecological product (Table 3).

Table 3. Differences and similarities between conventional and ecological 2% fat sour-batter milk

CONVENTIONAL PRODUCT	ECOLOGICAL PRODUCT
Conventional 2% fat sour-batter milk	**Ecological 2% fat sour-batter**
Ingredients: pasteurized cow's milk, milk proteins, selected lactic acid cultures.	Ingredients: high temperature pasteurized cow's milk from ecological production, selected lactic acid cultures.
Nutritional information/100 g product	Nutritional information/100 g product
Energetic value: 190kJ/45 kcal	Energetic value: 195kJ/ 46kcal
Fats: 2 g	Fats: 2 g
of which saturated fatty acids: 1.2 g	of which saturated fatty acids: 1.2 g
Carbohydrates: 3.6 g	Carbohydrates: 3.8 g
of which sugars: 3.6 g	of which sugars: 3.8 g
Protein: 3.2 g	Protein: 3.3 g
Salt: 0.1 g	Salt: 0.06 g
	Fiber: 0 g
	Calcium: 125 mg (15.6%)*
	*from the daily reference nutritional value
Storage temperature: +2...+6°C	Storage temperature: +2...+6°C
	

Following the evaluation of some assortments of kefir, it was concluded that there is no difference in their nutritional values. The only difference identified is given by the origin of the raw material, in the case of the ecological kefir, the milk comes from the ecological agriculture (Table 4).

Table 4. Differences and similarities between conventional and ecological kefir

CONVENTIONAL PRODUCT	ECOLOGICAL PRODUCT
Conventional 3.5% fat kefir	**Ecological 3.5% fat kefir**
Ingredients: high temperature pasteurized cow's milk, selected lactic acid cultures. Contains milk lactose.	Ingredients: high temperature pasteurized cow's milk from ecological production, selected lactic acid cultures. Contains milk lactose.
Nutritional information/100 g product	Nutritional information/100 g product
Energetic value: 247kJ/59k cal	Energetic value: 245kJ/ 59kcal
Fats: 3.5 g	Fats: 3.5 g
of which saturated fatty acids: 2.1 g	of which saturated fatty acids: 2.1 g
Carbohydrates: 3.7 g	Carbohydrates: 3.7 g
of which sugars: 3.7 g	of which sugars: 3.7 g
Protein: 3.1 g	Protein: 3.1 g
Salt: 0.06 g*	Salt: 0.06 g*
Calcium: 125 mg (15.6%)**	Calcium: 125 mg (15.6%) **
*the natural salt of milk **from the daily reference nutritional value	*the natural salt of milk **from the daily reference nutritional value
Storage temperature: +2...+6°C	Storage temperature: +2...+6°C
	

By studying the differences between conventional and ecological 3.5% fat yogurt, it is found that in the case of ecological products a high level of carbohydrates and proteins is observed (Table 5).

Table 5. Differences and similarities between conventional and ecological 3.5% fat yogurt

CONVENTIONAL PRODUCT	ECOLOGICAL PRODUCT
Conventional 3.5% fat yogurt	**Ecological 3.5% fat yogurt**
Ingredients: pasteurized whole milk, yogurt starter cultures.	Ingredients: pasteurized cow's milk, selected yogurt starter cultures. Ingredients from ecological agriculture.
Nutritional information/100 g product	Nutritional information/100 g product
Energetic value: 249 kJ/60 kcal	Energetic value: 291 kJ/70 kcal
Fats: 3.5 g	Fats: 3.5 g
of which saturated fatty acids: 2.3 g	of which saturated fatty acids: 2.1 g
Carbohydrates: 3.9 g	Carbohydrates: 4.5 g
of which sugars: 3.9 g	of which sugars: 4.5 g
Protein: 3.1 g	Protein: 5 g
Salt: 0.1 g	Salt: 0.1 g
Calcium: 120 mg (15%)*	
*from the daily reference nutritional value	
Storage temperature: +2...+6ºC	Storage temperature: +2...+6ºC
	

Nutritionally, the carbohydrate level is lower in the case of conventional 25% fat fermented cream, and the protein level is lower in the case of the ecological 25% fat fermented cream (Table 6).

Table 6. Differences and similarities between conventional and ecological 25% fat fermented cream

CONVENTIONAL PRODUCT	ECOLOGICAL PRODUCT
Conventional 25% fat fermented cream	**Ecological 25% fat fermented cream**
Ingredients: pasteurized cream and selected lactic acid cultures.	Ingredients: high temperature pasteurized cream from cow's milk from ecological production, selected lactic acid cultures. Contains milk lactose.
Nutritional information/100 g product	Nutritional information/100 g product
Energetic value: 1016 kJ/246 kcal	Energetic value: 1020kJ/247 kcal
Fats: 25 g	Fats: 25 g
of which saturated fatty acids: 15 g	of which saturated fatty acids: 15 g
Carbohydrates: 2.5 g	Carbohydrates: 3.3 g
of which sugars: 2.5 g	of which sugars: 3.3 g
Protein: 2.9 g	Protein: 2.3 g
Salt: 0.1 g	Salt: 0.06 g*
	*the natural salt of milk
Storage temperature: +2...+6ºC	Storage temperature: +2...+6ºC
	

Yoghurts with added fruit were evaluated, comparing products with different fat content. The added fruit quantity was especially monitored, not performing the nutritional values comparison, as in the prior situations, because the results would not have been eloquent, with the samples being of different categories and with different declared fat content.

The amount of fruit added to the conventional product is 2%, while in the case of the ecological product, the banana content is 15%. In both situations, pectin (E440) is used as a stabilizer, and the results are presented in table 7.

Table 7. Differences and similarities between conventional and ecological banana yogurt

CONVENTIONAL PRODUCT	ECOLOGICAL PRODUCT
Conventional 2.6% fat banana yogurt	**Ecological 3.1% fat banana yogurt**
Ingredients: pasteurized whole milk, sugar, bananas 2% (with the addition of: sugar, juice and mashed banana concentrate, water, modified starch, flavour, stabilizer: pectin, acidifier: citric acid), milk proteins, selected yogurt starter cultures.	Ingredients: 85% organic yogurt - pasteurized cow's milk from ecological agriculture, selected yogurt starter cultures (*L. bulgaricus, S. thermophilus*); organic banana preparation 15% - organic sucrose, organic mashed banana 30%, organic Tapioca starch, concentrated organic lemon juice, natural flavours, stabilizer (pectin E440).
Nutritional information/100 g product	Nutritional information/100 g product
Energetic value: 397 kJ/94 kcal	Energetic value: 414 kJ/98 kcal
Fats: 2.6 g	Fats: 3.1 g
of which saturated fatty acids: 1.7 g	of which saturated fatty acids: 1.8 g
Carbohydrates: 14.7 g	Carbohydrates: 13.0 g
of which sugars: 14.5 g	of which sugars: 11.8 g
Protein: 3 g	Protein: 4.6 g
Salt: 0.09 g*	Fiber: 0.1 g
Calcium: 103 mg (13%)**	Salt: 0.1 g
*the natural salt of milk **from the daily reference nutritional value	
Storage temperature: +2...+8ºC	Storage temperature: +2...+6ºC
	

Table 8. Differences and similarities between conventional and ecological strawberries yogurt

CONVENTIONAL PRODUCT	ECOLOGICAL PRODUCT
Conventional 1.9% fat strawberries yogurt	**Ecological 3.1% fat strawberries yogurt**
Ingredients: pasteurized milk, partially skimmed milk, sugar, strawberries 2% (with the addition of: glucose-fructose syrup, dyes: carrot juice, red beet juice, beta-carotene, flavour), milk proteins, modified starch, thickening agent, pectin, selected yogurt starter cultures.	Ingredients: 85% ecological yogurt - pasteurized cow's milk from ecological agriculture, selected yogurt starter cultures (*L. bulgaricus, S. thermophilus*); organic strawberries preparation 15% - organic sucrose, organic mashed strawberries 30%, organic Tapioca starch, concentrated organic lemon juice, natural flavours, stabilizer (pectin E440).
Nutritional information/100 g product	Nutritional information/100 g product
Energetic value: 342 kJ/81 kcal	Energetic value: 414 kJ/98 kcal
Fats: 1.9 g	Fats: 3.1 g
of which saturated fatty acids: 1.2 g	of which saturated fatty acids: 1.8 g
Carbohydrates: 13 g	Carbohydrates: 12.8 g
of which sugars: 12.1 g	of which sugars: 11.9 g
Protein: 3 g	Protein: 4.6 g
Salt: 0.09 g	Fiber: 0.1 g
Calcium: 120 mg (15%)*	Salt: 0.1 g
***from the daily reference nutritional value	
Storage temperature: +2...+6ºC	Storage temperature: +2...+6ºC
	

Analyzing conventional fruit yogurt, the amount of strawberries present in the product is 2%, while in the case of the ecological product, the strawberry content is 15% (Table 8).

In the case of conventional and ecological peach yogurt, there is not much difference in their fruit content, thus, the conventional product contains 23% fruit preparation and the ecological product contains 25% fruit preparation (Table 9).

Table 9. Differences and similarities between conventional Peach&Apricot yogurt and ecological Peach&Passion fruit yogurt

CONVENTIONAL PRODUCT	ECOLOGICAL PRODUCT
Conventional creamy yogurt with pieces of peaches and apricots (23% fruits)	**Ecological yogurt with pieces of peaches and passion fruit juice (25% fruits)**
Ingredients: yogurt, fruit preparation (36% peaches, sugar, 20% apricots, natural flavour), sugar.	Ingredients: yogurt, peach (12.5%), sugar, passion fruit juice (2.5%), corn starch, ingredients from ecological agriculture.
Nutritional information/100 g product	Nutritional information/100 g product
Energetic value: 435 kJ/103 kcal	Energetic value: 416 kJ/99 kcal
Fats: 3.2 g	Fats: 2.7 g
of which saturated fatty acids: 2.2 g	of which saturated fatty acids: 1.7 g
Carbohydrates: 14.4 g	Carbohydrates: 14 g
of which sugars: 13.6 g	of which sugars: 13 g
Protein: 3.3 g	Protein: 3.9 g
Salt: 0.16 g	Salt: 0.13 g
Storage temperature: +2...+6ºC	Storage temperature: +4...+8ºC
	

CONCLUSIONS

For the analyzed samples, regarding the ecological products labeling verification, it was found that the ingredients used come from the ecological agriculture, having met the requirements regarding their labeling.

In the case of the comparative study between conventional and ecological products, it was observed that, although the salt is described on the packaging as being part of the natural salt of milk, in the case of ecological products its level is lower, a fact that most probably correlates with the food that animals raised in ecological systems receive.

The organoleptic examination of the 20 types of products analyzed showed that all products have normal characteristics, without modification of an alterative nature or taste not specific to the assortment.

Analyzing fruit yogurt (banana yogurt and strawberry yogurt), it was found that ecological products have a higher percentage of fat, namely 3.1% fat, compared to conventional products, and the salt level is similar, registering insignificant variations. between product types.

Regarding the storage temperature, there were no major differences between the conventional and the ecological products, this being in the range +2 ... + 8ºC.

Some ecological dairy products, including drinking milk, 2% fat sour-batter milk and 3.5% fat yogurt have a higher nutritional value compared to conventional dairy products of the same type, without significant variation.

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***Ordin nr. 317/2006 privind modificarea și completarea anexei la Ordinul ministrului agriculturii, alimentației și pădurilor și al președintelui Autorității Naționale pentru Protecția Consumatorilor nr. 417/110/2002 pentru aprobarea Regulilor specifice privind etichetarea produselor agroalimentare ecologice.

***Ordinul nr. 190/2006 privind modificarea și completarea anexei la Ordinul ministrului agriculturii, alimentației și pădurilor și al președintelui Autorității Naționale pentru Protecția Consumatorilor nr. 417/110/2002 pentru aprobarea Regulilor specifice privind etichetarea produselor agroalimentare ecologice.

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EXPERIMENTAL MEDICINE

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EXPERIMENTAL TESTING OF A DEFIBRILLATION PROTOCOL ON PIGS

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Abstract

Testing of a defibrillation protocol is necessary in order to add additional security to the monitoring and support protocols during the experimental procedures developed on pigs. Pigs are placed under general anaesthesia and continuous monitoring (capnography, pulse-oximetry, blood pressure measurement). The experimental testing consists in creating the conditions needed for the heart to enter the states of ventricular tachycardia (VT) and ventricular fibrillation (VF) followed by the pre-chosen defibrillation protocol. By every defibrillation shock added, the normal sinus rhythm was sustained for a longer period, in relation with the number of shocks applied. Survivability could not be sustained only with defibrillation, the use of CPR and cardio tonic medication being recommended.

Key words*: defibrillation, protocol, pig.*

INTRODUCTION

The aim of this experimental study is to test the efficiency of defibrillation protocols in pigs, used during experimental procedures, in order to develop further cardio pulmonary resuscitation guidelines.

MATERIALS AND METHODS

All 5 subjects were selected respecting the same principles for housing, feeding, anaesthesia and surgical principles (Mitrănescu, 2008), in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes (E.C., 2010) and manipulated in regard to ensure a high level of animal welfare as a moral duty of human being regarding the animals (Mitranescu, 2009) and approved by the Ethic Commission of the Faculty of Veterinary Medicine of Bucharest, as part of a bigger research project regarding the use of pigs for experimental procedures.

Subject selection- five female domestic pigs (25-35 kg) were used for this study. Animals were acclimatised in the same conditions for 48h before the experiment was conducted and were fasted overnight but had access to water. For experimental surgery, there is a need for anaesthesia protocols depending on the complexity of the procedures. Subjects were evaluated trough a complete pre anaesthetic examination (clinical examination, body condition, weighing) 24 hours before the procedure and assigned to an anaesthetic risk group- ASA adapted for veterinary medicine (Table 1).

Table 1. **ASA risk classification** adapted for veterinary medicine (*American Society of Anaesthesiologists*) and subjects distribution

ASA 1	A normal healthy patient, with no organic disease	All 5 subjects
ASA 2	A patient with mild systemic disease	
ASA 3	A patient with severe systemic disease that limits activity but it`s not incapacitating	
ASA 4	A patient with sever systemic disease that is a constant threat to life	
ASA 5	A moribund patient who is not expected to survive 24 hours without intervention	

Pigs were anaesthetized and surgical prepared for an experimental laparoscopy surgery. Anaesthetic protocol- premedication by intramuscular injection with Xylazine 2 mg/kg and Ketamine 20 mg/kg, followed after 10 minutes by induction- with Propofol 5 mg/kg and maintenance with Isoflurane and oxygen for adequate anaesthetic depth (Bîrtoiu, 2009). The animals were intubated with 5.0 to 6.0 size tracheal tube.

All 5 subjects were monitored with: electrocardiography (ECG) (Figure 1) using standard bipolar limb leads with self-adhesive electrodes, capnography, pulse-oximetry (SpO2) with sensors placed on the tongue of the pigs and non-invasive blood pressure, by pressure cuff placed on one of the front limbs.
A continuous infusion of 10 ml/h of NaCl (0.9%) was infused via a peripheral catheter placed on the lateral auricular vein (Costea, 2019). Respiration was sustained by ventilation delivered by volume-controlled ventilator with a tidal volume of 10 ml/kg (IPPV), respiratory rate 10 breaths/min.).
The defibrillator used for this study was a human paediatric device (Biphasic Multifunctional Defibrillator/Monitor from South Korean Manufacturers Mediana), capable of delivering shocks from a large interval (1 J to 360 J) (Figure 1).
The defibrillator is used to apply an electric current to the heart, also known as a shock. Shocks are applied with pads that were cleaned before the experiment and lubed with gel (Savage, 2013).
The pads are applied on the chest of the animal, one to the sternum and one on the fifth to seventh intercostal space, on the left side, corresponding to the heart apex.

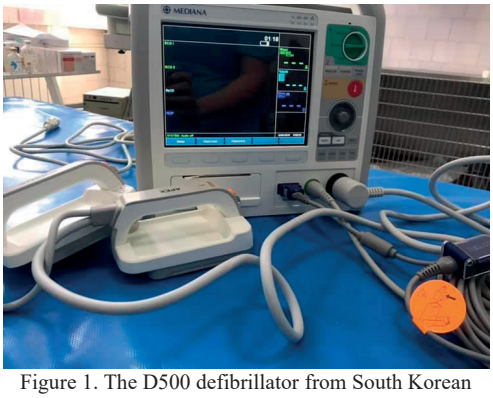

Figure 1. The D500 defibrillator from South Korean Manufacturers Mediana used for the experiment

The experiment began with a laparotomy procedure, performed via the medial abdominal line from the sternum to the umbilical scar, as part of a bigger research experimental procedure.
Pigs were under anaesthesia already for a period of 3 to 4 hours, for experimental laparoscopy procedure, when diaphragm was perforated, by a vertical incision of 10 cm, in the medial plan, from the xiphoid process, using a surgical scalpel to simulate a trauma.
The defibrillation protocol chosen for the procedure was composed of three shocks with different intensities: first shock-2 J/kg, second shock- 3.5 J/kg, third shock-5 J/kg.

RESULTS AND DISCUSSIONS

All 5 subjects were healthy, with no organic diseases and included in ASA risk group 1 and received the same anaesthetic protocol during the experimental laparoscopic surgical procedure.
We measured the duration (seconds) from the moment when the diaphragm was punctured (T = O) to the instalment of ventricular tachycardia (VT) and after until the confirmation of ventricular fibrillation (VF) through ECG.
Ten to fifteen seconds (mean of 13 seconds) after the puncturing was performed, pigs hearts entered a state of ventricular tachycardia (VT) followed in maximum 120 seconds (mean of 103 seconds) by ventricular fibrillation (VF) and confirmed by the monitor EKG in lead II (Figure 2).

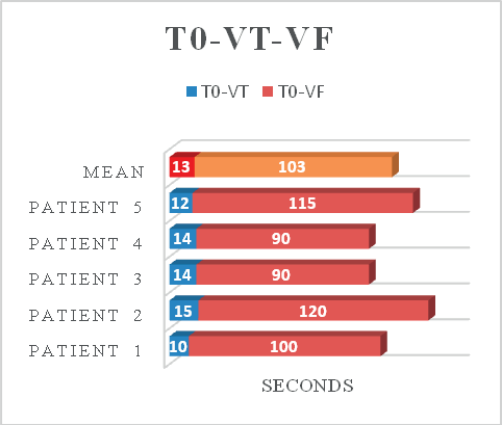

Figure 2. Mean time after diaphragmatic trauma (T = 0) to enter in ventricular tachycardia (VT) and time for each patient to enter ventricular fibrillation (VF)

Figure 3- Biphasic manual defibrillation with D500 defibrillator Mediana

Defibrillation protocol was applied after the VF was confirmed, starting with the first shock by biphasic manual defibrillation - with paddles (Figure 3). The first shock (2 J/kg) was applied and the response was a brief 2-6 seconds of sinus rhythm, in all 5 pigs, and after that reverting to ventricular fibrillation.

The second shock (3.5 J/kg) was applied and the response was a sinus rhythm sustained by itself for 270 to 360 seconds in three out of the five pigs (3/5). In the other two pigs (2/5) the response to the second shock determined a response similar to the first shock-5 seconds.

The third shock (5 J/kg) was applied and the response was a sinus rhythm sustained for by itself for 340 to 365 seconds in two out of the five pigs, followed by asystole. For pig number 5, after the third shock was performed, a brief (2-3 sec.) sinus rhythm was installed followed by a quick (1-2 sec.) instalment of VF. For this pig we decide to give a 4th shock, with the same intensity (5 J/kg), after the instalment of VF and the result was self-sustained sinus rhythm for 370 seconds (Table 2, Figure 4). Two pigs did not respond to the third shock and were removed from the protocol. By every defibrillation, shock added to the protocol the normal sinus rhythm was sustained for a longer period of time (Figure 5) for the entire group, but in different moments, respectively after a variable number of defibrillation shocks applied. After the normal cardiac rhythm was kept on its own, the heart went into fatigue that leads to asystole state and heart failure. No vasopressors and anticholinergic drugs were used during the resuscitation protocols and no chest compressions were to be given between shocks.

Table 2. Duration of sinus rhythm response, after 1st, 2nd, 3rd and 4th defibrillator shocks, for each of the 5 animals

Patient	1	2	3	4	5
1st shock 2 J/kg Sinus rhythm response	2 sec.	3 sec.	5 sec.	2 sec.	6 sec.
2nd shock 3.5 J/kg Sinus rhythm response	270 sec.	5 sec.	360 sec.	5 sec.	355 sec.
3rd shock 5 J/kg Sinus rhythm response	No response	340 sec.	No response	365 sec.	2 Sec.
4th shock 5 J/kg Sinus rhythm response	-	-	-	-	370 sec.

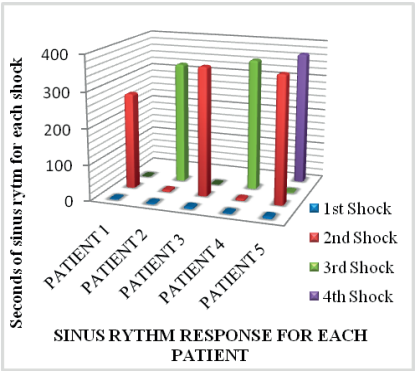

Figure 4. Evaluation of shock responses

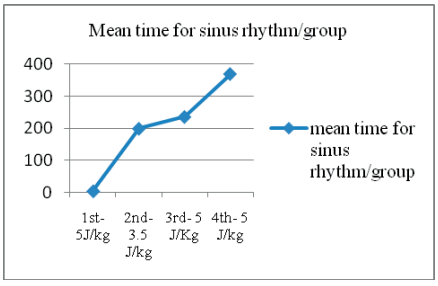

Figure 5. Mean time for sinus rhythm/group after each defibrillation shock

CONCLUSIONS

Traumatic diaphragmatic perforation induced ventricular tachycardia (VT) followed by ventricular fibrillation (VF) for pigs under anaesthesia for a period of 3 to 4 hours, for experimental laparoscopy procedures

Defibrillation protocols used on pigs have a good success rate at higher voltage since shocks of low voltage (1st shock-2 J/kg) did not produce reliable responses.

In this study, the results were promising with 3.5 to 5 J/kg shocks, respectively 2nd and 3rd shock applied.

Using a shock at higher intensity right after the first shock would increase the chances for a normal sinus rhythm instalment.

By every defibrillation, shock added to the protocol the normal sinus rhythm was sustained for a longer period of time for, in relation with the number of defibrillation shocks applied.

Long-term survivability cannot be obtained only with defibrillation, the use of CPR and cardio tonic medication should absolutely be used in cases like these.

These results open new possible hypothesis for further researches concerning the association between resuscitation medication and defibrillation protocols.

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EVALUATION OF THERAPEUTIC BENEFITS OF COLLAGEN BASED BIOMATERIAL FUNCTIONALISED WITH PLATELET RICH PLASMA

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Abstract

Platelet rich plasma (PRP) in combination with different type of biomaterials can promote tissue regeneration. Our study aimed to evaluate the adhesion, proliferation and chondrogenic differentiation potential of canine mesenchymal stem cells on functionalised collagen-based biomaterial. Characterized gingival mesenchymal stem cells ($1x10^5$) were seeded on PRP functionalized synthetic biomaterial and were cultured in DMEM/F12 culture medium supplemented with 10% fetal calf serum and 1% antibiotics-antimycotics. After 24, 72 h and 5 days cellular adhesion, proliferation was assessed using CCK8 assay. The absorbance was recorded using a a BioTek Synergy 2 microplate reader set at 450 nm. The bioactive potential of PRP functionalized biomaterial was evaluated after 21 days. Proteoglycans and sulfated glycosaminoglycan content were assessed. Statistical analysis was performed using GraphPad Prism 5.0 software and statistical significance was considered when $p < 0.05$. Our results indicated the potential of PRP on cells attachment, proliferation and due to the release of growth factors promote the cells differentiation.

Key words*: biomaterials, collagen, platelet rich plasma, mesenchymal stem cells.*

INTRODUCTION

Platelet-rich plasma (PRP), defined as an autologous leukocyte and PRF biomaterial (Tambella et al., 2018), contain various growth factors (platelet-derived growth factor (PDGF), transforming growth factor-b (TGF-b) 1, 2, vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor (IGF) I, IGF-II), extracellular matrix and cell adhesion molecules (fibrin, fibronectin, vitronectin and thrombospondin) (Ramaswamy et al., 2018), proteins, cytokines and other signalling molecules with important function in regulation of regeneration and tissue repair by supplying growth factors, cytokines, chemokines and other bioactive compounds (Boswell et al., 2012; Hsu et al., 2013; Jason et al., 2013; Albanese et al., 2013; Carr et al., 2016; Chang et al., 2018; Wenqiang et al., 2020). PRP are an autologous source of platelets (least 1,000,000/1 L in a small volume of plasma) (Marx, 1999; Saluja et al., 2011; Albanese et al., 2013) it is widely used in different areas of dentistry, orthopedics, reconstructive medicine, among which can be remembered mandibular reconstruction, periodontal defects and periodontal plastic surgery, head and neck surgery, surgical repair of the alveolar cleft, otolaryngology, cardiovascular surgery, maxillofacial surgery (Albanese et al., 2013), soft tissue injuries, osteoarthritis of the knee, ulnar collateral ligament tears, lateral epicondylitis (Mlynarek et al., 2016), hip dysplasia (Yun et al., 2016), hip osteoarthritis in dog (Malek et al., 2012) and muscle damage. Our hypothesis is that PRP in combination with different type of biomaterials can promote tissue regeneration. Therefore, the objective of the current study was to evaluate the adhesion, proliferation and chondrogenic differentiation potential of canine mesenchymal stem cells on PRP functionalised collagen-based biomaterial.

MATERIALS AND METHODS

Canine gingival tissue derived mesenchymal stem cells (MSCs) was used for this study. The gingival tissue and blood sample was harvested from a mixed breed dog, presented at a private

practice with dental disorders. The samples were collected after the informed consent from the owner. The MSCs were isolated using enzymatic dissociation method (Collagenase 0.075%, Sigma-Aldrich), after several passages, the cells were characterized according to the International Society for Cellular Therapy recommendations. For PRP the blood was collected from the external jugular vein under sterile conditions. The samples were centrifuged at 300x g for 7 min to separate the blood phases. The upper phase was harvested (approximately 1.5 ml) and freshly added on collagen-based biomaterial (Evolution, OsteoBiol^{R}) in three different concentrations (5%, 7%, and 10%). Prior the PRP was activated with calcium chloride (Sigma-Aldrich). The canine gingival stem cells at a density of 1 x 10^4 cells were seeded onto the functionalized biomaterial (0.7 cm x 0.7 cm) placed in culture plates. The cell-seeded biomaterials were transferred to a 12 well plates containing normal propagation medium containing DMEM/F12 (Gibco), 1% Antibiotic Antimycotic 100x (Sigma-Aldrich), 5% Horse serum (Gibco Life Technologies), at 37.5°C in a humidified 5% CO atmosphere for the attachment and proliferation tests. The medium was changed every day. Collagen based biomaterials without PRP was considered the negative control. The propagation medium in the control group was supplemented with 10% FCS (fetal calf serum, Sigma-Aldrich). After 24 h cellular adhesion was assessed using cytochemical stains, tetramethylrhodamine isothiocyanate (TRITC) phalloidin (Sigma-Aldrich) 1: 20 in phosphate-buffered saline (PBS; Sigma-Aldrich) for actin filaments and DAPI (4,6-diamidino2-phenylindole) for cell nuclei. The results were evaluated using Axiovision 3.0 image analysis software (Carl Zeiss, Hitech Instruments). Cell proliferation was assessed using CCK-8 assay after 24, 72h and 5 days of culture. CCK-8 solution was added in each well (100 µl/ml) followed by incubation at 37°C for 1 hour. The absorbance was recorded using a BioTek Synergy 2 microplate reader set at 450 nm. For chondrogenic differentiation, pellet cultures method was used. The cells (5 x 10^4/ml) were aggregated using hanging drops method. After 48h, the aggregates were washed and added onto the PRP treated collagen-based biomaterial in normal propagation medium. The control groups were treated with chondrogenic induction medium: DMEM/F-12 (Sigma-Aldrich) supplemented with 1% ITS (Insulin-Transferrin-Selenium, Sigma-Aldrich), 50 nM L ascorbic acid 2-phosphate (Sigma-Aldrich), 100 nM dexamethasone, 10 ng/ml of transforming growth factor (TGF-b; Sigma-Aldrich) and 1% antibiotic/antimycotic (Gibco).

The morphological characteristics of the cells from both cultures were monitored daily. Proteoglycans and sulfated glycosaminoglycan content were evaluated after 21 days. The proteoglycan accumulation was assessed by Alcian blue staining. The cultures were fixed with methanol for 20 minutes and were stained with Alcian blue in 0.1N HCl for 20 minutes, washed with distilled water and treated with 6 M guanidine-HCl (Sigma-Aldrich). The absorbance was measured at 620 nm. The glycolsaminoglycans were assessed after treatment of the paraformaldehyde fixed cultures with 0.02% Safranin-O (Sigma-Aldrich). The Safranin-O dye was extracted using 10% cetylpyridinium chloride (Sigma-Aldrich) for 20 min. The optical density was evaluated at 550 nm. Canine gingival cells cultured in normal propagation medium were represented the control group. The evaluations were performed in triplicate.

Statistical analysis was performed with GraphPad Prism 6 software. A value of $P < 0.05$ was considered to be statistically significant difference for all tests.

RESULTS AND DISCUSSIONS

In order to evaluate the potential of functionalized collagen-based biomaterial, characterized canine gingival stem cells were used. The biomaterial was functionalized with different concentration of PRP. Prior the biomaterial treatment the PRP was activated with calcium chloride.

This treatment is important for releasing the granule's contents (Cuervo et al., 2020). Cellular adhesion was assessed using cytochemical staining and quantitative morphometric analyses were performed.

The number of adherent cells was quantified in control group and in PFR functionalized

biomaterials. Cells were counted in 5 different microscopic fields (Figures 1, 2).

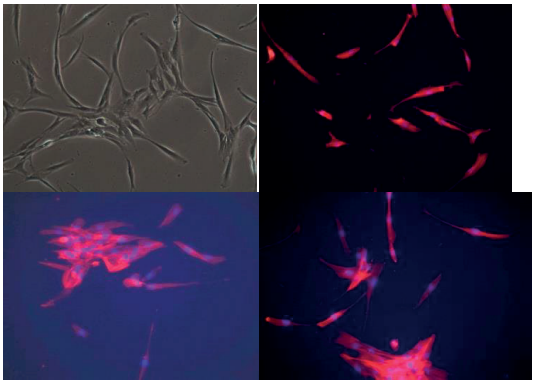

Figure 1. Gingival MSCS attached to the PRP treated biomaterial (1st images: cells cultivated on plastic surface, negative control, 2nd images: cells seeded on biomaterial functionalized with 5% PRP, 3rd images: cells seeded on biomaterial functionalized with 7% PRP, 4th images: cells seeded on biomaterial functionalized with 10% PRP), blue nuclei stained with DAPI, red actin filaments stained with TRITC-phalloidin

Microscopic fields were randomly selected. After 24 h of seeding on collagen-based biomaterial statistically significant results were identified especially for biomaterials treated with 5% PRP (Figure 1).

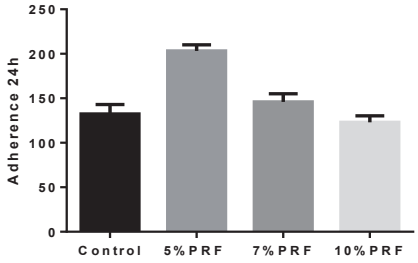

Figure 2. Cell number on functionalized collagen-based biomaterial 24 h after seeding (cells seeded on biomaterial functionalized with 5% PRP, 7% PRP and 10% PRP)

CCK-8 test was performed to examine the effect of different concentrations of PRP on canine gingival stem cells proliferation after 24 h, 72 h and 5 days of exposure (Figure 3). The results of the CCK-8 assay demonstrated that PRP-treated MSCs in different concentration showed improved cell growth compared to control groups.

After 24 hours of seeding the cells on 7% and 10% PRP treated biomaterial, a slight increase in cell proliferation rate can be observed.

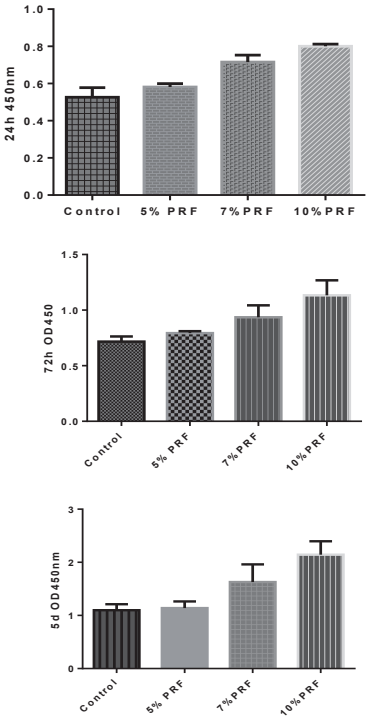

Figure 3. Canine gingival tissue derived MSCs proliferation on PRP functionalized collagen-based biomaterial (Evolution, OsteoBiol[R]). CCK-8 assay was performed to evaluate the effect of three different concentrations of PRP in three different period of time. The absorbance was read at 450 nm

After 5 days statistically significant results were identified in cultures treated with 10% PRP. Our results are in agreement with certain data from the literature. A similar behaviour was observed by Wang et al. (2019) in bone marrow aspirate derived mesenchymal stem cells treated with of the PRP. They study demonstrate that PRP in concentration of 1500 $\times 10^9$ pl/L. exerted different modulatory effects on cell proliferation and the osteogenic, adipogenic and chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells. The differentiation capacity is closely related to the increased content of cytokines and growth factors, a group of diffusible polypeptides that control cell growth, proliferation, differentiation, cellular

metabolism (Marx et al., 1999, Cuervo et al., 2020). PRP also promoted the production of the anti-inflammatory cytokines IL-10 by mesenchymal stem cells, but can suppress the IL-1β, IL-6 and TNF-α secretion (Wang et al., 2019).

To explore the bioactive potential of PRP, chondrogenic induction potential of functionnalized biomaterials were assessed. The canine gingival tissue derived stem cells were maintained on functionalized biomaterial for 21 days. The PRP supplementation was performed in every 3 days until the cells were fixed. The positive control group were treated with chondrogenic induction medium without PRP and for negative control the cells were treated with normal propagation medium. After 21 days Alcian Blue and Safranin O staining indicated the presence of glycosaminoglycans and proteoglycan deposition (Figures 4, 5).

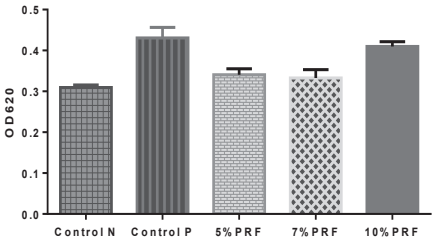

Figure 4. Chondrogenic differentiation - proteoglycan deposition
(The absorbance value of the solubilized Alcian blue at 620 nm. The results are shown as mean ± SD
Control N - negative control: canine gingival stem cells cultured in normal propagation medium; Control P - positive control: canine gingival stem cells treated with chondrogenic induction medium)

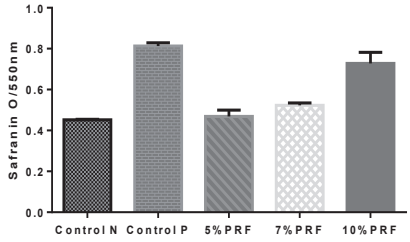

Figure 5. Chondrogenic differentiation-glycosaminoglycans evaluation with Safranin O staining
(Control N - negative control: canine gingival stem cells cultured in normal propagation medium; Control P - positive control: canine gingival stem cells treated with chondrogenic induction medium)

Compared with negative control (0.309 ± 0.006), the average of absorbance for proteoglycan deposition was 0.340 ± 0.01 on biomaterial functionalized with 5% PRP, 0.332 ± 0.02 on 7% PRP and 0.410 ± 0.01 on 10% PRP. The differentiation capacity of cells maintained on 10% PRP treated biomaterial was similar with positive control group where the average of absorbance was 0.430 ± 0.02. These results suggested that normal propagation medium supplemented with 10% PRP may be preferable for the accumulation of proteoglycans (Figure 4). Moreover, similar effects of PRP in glycosaminoglycans production were observed. The average of absorbance for positive control was 0.810 ± 0.01 compared with the negative control where the average of absorbance was 0.452 ± 0.002 (Figure 5).

The potential of the glycosaminoglycans synthesis increased exponentially with increasing PRP concentration. These data demonstrate that 10% PRP is effective and efficient for the chondrogenic differentiation of canine gingival stem cells. Numerous applications based on PRP have been tested for bone and cartilaginous disorders (Marmotti et al., 2015; Wang et al., 2019). All these applications suggested that the concentration of PRP is important for the restoration of the affected tissues. Our data showed that PRP in concentration of 10% would be effective. Our future concerns will be focused on evaluating the tested PRP concentrations on different biological models including organotypic cultures. Wang et al. (2019) data showed that PRP with platelet concentrations of 1000×10^9 pl/L to 3000×10^9 pl/L supported MSC adipogenesis, osteogenesis and chondrogenesis. They also suggest that for cartilage regeneration a concentration of 2000×10^9 pl/L PRP provides a strong proliferation and induce chondrogenesis (Wang et al., 2019). Platelet rich plasma in combination with stem cells may represent a promissory treatment for many diseases, but multiple clinical trials are required to establish the true efficiency of *in vitro* and *in vivo* treatments. PRP-based therapy could be valuable as an alternative therapy alone or in combination with other conventional treatments for different diseases (Ramaswamy et al., 2018).

CONCLUSIONS

Our results indicated the potential of PRP in combination with collagen-based biomaterial on cells attachment, proliferation and due to the release of growth factors promote the cells differentiation. These results may suggest promising clinical strategies for different diseases.

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