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HISTOLOGICAL EVALUATION OF TWO EUTHANASIA METHODS IN A TOXICITY STUDY IN LABORATORY RATS

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Abstract

Toxicity tests are mandatory preclinical regulatory studies for the authorization of a medicinal product. The testing protocol include a complete analysis of the possible toxic action of the tested product. The most complex and defining analysis is post-mortem histological analysis. Depending on the place of action of the tested product and to avoid the interference of euthanasia methods with the results of analyzes, different euthanasia methods may be chosen. In a study of toxicity in rats for a substance with action on the nervous system, two methods of euthanasia were chosen, namely anesthetic overdose and euthanasia by decapitation with deep sedation of animals. Histological evaluation of the main organs revealed congestion in the analyzed organs regardless of the euthanasia method used in most animals. Diffuse hemorrhage, perivascular edema and pulmonary edema have also been observed. Lesions were identified in both test and control groups, male and female. Statistical analysis showed significant differences between the two methods, euthanasia by overdose of anesthetic producing more lesions than decapitation, the latter being considered more appropriate for this type of study.

Key words*: euthanasia, anesthetic overdose, decapitation, histological evaluation.*

INTRODUCTION

For the market authorization of medicinal products and medical devices, preclinical and clinical studies are mandatory (Steinmetz & Spack, 2009). Preclinical studies refer to *in vitro* and *in vivo* on laboratory animal studies (Henderson et al., 2013). Laboratory animal studies can target biocompatibility and toxicity regardless of its type (acute, chronic toxicity, genotoxicity, cytotoxicity, etc.). Animal toxicity studies are usually performed on 2 species (rodents and non-rodents), but it is also approved to perform them on a single species, in the case of target drugs (Prior et al., 2018). In order to avoid erroneous results in toxicity studies, the elimination of all factors that may interfere with the obtained values is essential. In addition to microclimate factors (temperature, relative humidity, noise, light, etc.) and those related to the animal (gender, age, body weight, type of barrier in which they were breeding) an important influence on the results may be the study methods used, methods related to the administration of test substances and the collection of samples for analysis. In a toxicity study, all the parameters that can determine the possible toxic actions of the test substances (weight gain, food consumption, immunological, ocular, hematological and biochemical parameters) are analyzed (Vandivort & Eaton, 2014).

The most important analysis remains the histological analysis which can detect in detail the possible toxic effects of the test substance (Crismann et al., 2004).

However, histological analysis can also be affected by certain factors that need to be considered, the most important of which is how euthanasia is performed.

Euthanasia, which is the act of ending life with good methods, is strictly regulated by national legislation, there are methods on species, ages and weights, and the person performing the euthanasia must be trained for this purpose.

The Guide for the Care and Use of Laboratory Animals indicates the appropriate euthanasia method depends on many criteria, including compatibility with research objectives. It further states, "The selection of specific agents and methods for euthanasia will depend on the species involved and the objectives of the protocol. Euthanasia, as a process, separates the presentation of new variables, treatments or environmental changes to the living system from the terminal collection of tissues and blood for additional study or analysis" (National Research Council, 2011). In itself, the euthanasia method can alter physiologic parameters and responses (Close et al., 1997).

There are numerous studies that have analyzed the effects of different euthanasia methods on the value of the different parameters studied (hematological, hormonal, and histological) (Brooks et al., 1999, Schoell et al., 2009, Artwohl et al., 2006, Shomer et al., 2020). Regardless of the study, there were no euthanasia methods that did not interfere with some of the test results (Pierozan et al., 2017).

In a chronic toxicity study for a substance with an effect on the central nervous system, a study performed on rats, two methods of euthanasia, anesthetic overdose and decapitation under deep sedation were used.

The option for two euthanasia methods was justified by the exclusion in the interpretation of the results of the changes generated by the euthanasia method. The results obtained at the necropsy and histopathological examination were analyzed.

MATERIALS AND METHODS

All procedures during the study were performed in "Cantacuzino" National Medico-Military Institute for Research and Development (CI), Department of Research and Development, Preclinical Testing Unit and in the pathological anatomy laboratory of the Faculty of Veterinary Medicine. Animal studies have been approved by the CI Ethics Committee and authorized by the competent authority, in accordance with the provisions of national and European regulation on protection of animals used for scientific purposes, respectively Law 43/2004. CI is an authorized unit under current legislation as a user of animals for scientific purposes.

Wistar rats, male and female, were used for the toxicity study, with an average body weight of 280-320 g at the beginning of the study. The animals were kept in identical conditions in open cages, with wood chips as bedding. Food and water were provided *ad libitum*. The microclimate conditions were temperature 20-24^{0}C, relative humidity 45-65%.

Two groups were created, each of 40 animals, of which 20 females and 20 males, a batch inoculated with the test substance and a control batch inoculated with saline solution. The test substance which is still under clinical study, effective on disorders of the nervous system has been inoculated intramuscularly, as well as saline solution (Hemofarm, Romania) that was use as control substance. Weekly administrations were given, and the study lasted 90 days.

Rats were daily inspected and food consumption was recorded for each group once a week. Weight measurements were performed for each rat every 14 days during the entire study period. Blood collection from the retro-orbital sinus was performed on days 0, 30, and 60, under general anaesthesia, using a cocktail of Acepromazine 1% (5 mg/kg; Sedam, Farmavet, Romania) and Ketamine 10% (100 mg/kg; Vetased, Farmavet, Romania). At the end of the study, the animals were euthanized; blood and samples were taken for histological examination.

As an anesthetic we chose a combination of the injectable dissociative agent, α2-adrenergic receptor agonists and phenothiazine tranquilizer as sedative to potentiate the effects of anesthetic.

Ten animals in each groups and for each gender were euthanized with an overdose of Ketamine 10% (Vetased, Farmavet, Romania)/Xylazine 2% (Xylazin Bio, Bioveta, Czech Republic)/Acepromazine 1% (Sedam, Farmavet, Romania) "cocktail" - mix 300 mg/ml Ketamine, 50 mg/ml Xylazine, and 30 mg/ml Acepromazine in a 3:3:1 ratio and injected 1.5 - 2.1 ml/kg intraperitoneally.

Ten animals were euthanized by guillotine decapitation after sedation with 3% isoflurane (Anesteran, Rompharm, Romania). The

procedure was undertaken skillfully and rapidly by a trained operator.

After euthanasia, necropsy was performed and organs with vital functions were collected: brain, heart, lungs, kidney, and liver. The organs were paraffin embedded and stained with hematoxylin-eosin. Histological examinations were performed on 5 µm sections.

All data are shown as positive samples at the number of samples analyzed. Statistical comparisons were performed using the Microsoft Excel T-test for independent groups and one-way analysis of variance for comparison of means of parameters within the same group. P-values < 0.001 were considered statistically significant, and < 0.05 less significant.

RESULTS AND DISCUSSIONS

No mortality was recorded in any of the groups during the study. All animals gained weight and the clinical and blood parameters were normal. The results obtained at necropsy and histopathological examinations are highlighted in Tables 1 to 4. As there were no differences between the groups of test substance versus saline, the results were combined to better highlight the differences between the 2 euthanasia methods.

Table 1. The result of necropsy in females depending on the method of euthanasia

Euthanasia methods	Organ	Female	
		Congestion	Hemorrhage
Anesthetic overdose	Heart	9/20	0/20
	Lung	12/20	2/20
	Liver	12/20	4/20
	Kidney	7/20	4/20
	Brain	11/20	1/20
Decapitation	Heart	1/20	0/20
	Lung	4/20	0/20
	Liver	2/20	0/20
	Kidney	3/20	0/20
	Brain	1/20	0/20

Table 2. The result of necropsy in males depending on the method of euthanasia

Euthanasia methods	Organ	Males	
		Congestion	Hemorrhage
Anesthetic overdose	Heart	11/20	3/20
	Lung	13/20	2/20
	Liver	10/20	3/20
	Kidney	9/20	4/20
	Brain	12/20	1/20
Decapitation	Heart	3/20	0/20
	Lung	2/20	0/20
	Liver	2/20	0/20
	Kidney	2/20	0/20
	Brain	2/20	0/20

Table 3. The result of histological analyses in females depending on the method of euthanasia

Euthanasia methods	Organ	Females			
		Congestion	Diffuse hemorrhage	Petechiae	Edema
Anesthetic overdose	Heart	11/20	3/20	11/20	3/20
	Lung	13/20	2/20	13/20	2/20
	Liver	10/20	3/20	10/20	3/20
	Kidney	9/20	4/20	9/20	4/20
	Brain	12/20	1/20	12/20	1/20
Decapitation	Heart	3/20	0/20	3/20	0/20
	Lung	2/20	0/20	2/20	0/20
	Liver	2/20	0/20	2/20	0/20
	Kidney	2/20	0/20	2/20	0/20
	Brain	2/20	0/20	2/20	0/20

Table 4. The result of histological analyses in females depending on the method of euthanasia

Euthanasia methods	Organ	Males			
		Congestion	Diffuse hemorrhage	pointed hemorrhage	Edema
Anesthetic overdose	Heart	15/20	4/20	3/20	0/20
	Lung	16/20	4/20	2/20	3/20
	Liver	13/20	5/20	1/20	0/20
	Kidney	11/20	6/20	1/20	1/20
	Brain	15/20	2/20	3/20	1/20
Decapitation	Heart	4/20	0/20	0/20	0/20
	Lung	6/20	1/20	0/20	0/20
	Liver	6/20	1/20	0/20	1/20
	Kidney	5/20	2/20	1/20	0/20
	Brain	4/20	0/20	0/20	0/20

The analysis of the results highlights differences between the two euthanasia methods. Congestion and hemorrhages were observed on the necropsy examination. The differences between the two methods of euthanasia were significant regardless of organ in congestion ($t < 0.001$), and less significant ($t < 0.05$) in the case of hemorrhagic lesions, being more obvious and more numerous in groups euthanized with anesthetic overdose. Regarding the results from the histopathological examinations, there were congestive lesions, diffuse hemorrhages, edema and pointed hemorrhages lesions being the consequence of the action of euthanasia methods. The differences between the method of euthanasia by an overdose of anesthetic were more numerous and more obvious on histopathological examination than by the method by decapitation. Regardless of the organ, the differences were significant ($t < 0.001$) in congestive lesions and less significant in diffuse hemorrhage lesions, even insignificant in the case of edema and pointed hemorrhage. The analysis of the results between the genders did not show significant differences.

The definition of euthanasia is a good death, and if we are trying to provide a good death for an animal, we should do that at all costs (Person et al, 2020). The two methods of euthanasia have different actions, one being of chemical origin (anesthetic overdose) and one physical (decapitation).

Ketamine is a short acting anesthetic agent being widely used, but has emerged as an abused drug in recent years. Ketamine is a dissociative anesthetic developed in 1963 to replace phencyclidine and is being currently used for human anesthesia and veterinary medicine (Dinis-Oliveira, 2017). Ketamine is not acceptable for euthanasia when used alone but can be humane when used in conjunction with sedatives and tranquilizers. However, it is not very efficient as it requires very high doses. Xylazine hydrochloride is a thiazine derivative that acts by activation of central presynaptic α2 receptors, producing sedation and muscle relaxation. As an anesthetic, it is typically used in conjunction with ketamine.

Acepromazine is a phenothiazine tranquilizer that blocks dopamine receptors in the CNS and depresses the reticular-activating system, resulting in sedation. Acepromazine also blocks alpha-adrenergic receptors. Acepromazine is a sedative that potentiates the effects of other anesthetic agents. The combination of the 3 substances works very well for anesthesia, and in overdose, as we used it, it quickly induces euthanasia in animals.

Physical methods of euthanasia have a high potential for being inhumane and are only acceptable when scientifically necessary and must be performed by carefully trained personnel. Physical methods are acceptable for fully sedated animals (Kongara et al., 2014). It is very important that we make sure that the animal is dead as a result of the euthanasia action. Very deeply anesthetized animals may appear dead; yet, they may recover from the anesthesia at a later time. Rapid euthanasia of laboratory rodents without the use of anesthesia is a necessary research technique whenever there is the likelihood of anesthesia or stress

interfering with the chemistry of the tissues under investigation. Decapitation has long been the procedure of choice under such circumstances (Holson, 1992). Recently, the American Veterinary Medical Association (AVMA) panel on euthanasia recommended that decapitation to be avoided on the grounds that the decapitated head may be conscious and suffering for as much as 15 seconds (AVMA, 2020).

The histopathologic changes caused by various methods of euthanasia were studied in rats, mice, guinea pigs, and rabbits. Lesions resulting from particular methods of euthanasia were consistent from species to species. Each method studied affected the organs to some degree, ranging from mild congestion to edema and alteration of vascular permeability. Euthanasia of experimental animals by overexposure to CO_2, or intraperitoneal injection of concentrated sodium pentobarbital seemed most suitable for pulmonary studies. Decapitation (mice, rats, guinea pigs), cervical dislocation (mice), CO_2, and intracardial injection of sodium pentobarbital were more suitable for examination of abdominal viscera (Feldman & Gupta, 1976).

However, it is clear that the applicability of these euthanasia methods may change with the model of study, experimental treatments and other factors.

Consequently, euthanasia should be assigned cautiously and preferably after preliminary studies to prevent aberrant research results. Equipped with the basic principles of euthanasia, investigators can make informed decisions that meet current standards of animal care while still achieving the scientific goals of their research studies.

CONCLUSIONS

The choice of euthanasia methods to generate as few side effects as possible is important in preclinical toxicity studies. The methods chosen in the presented study, respectively overdose of anesthetic and decapitation with deep sedation generated gross and histopathological changes such as congestion and hemorrhage in most organs examined. They were significantly more common with an anesthetic overdose. The method of decapitation with prior sedation can be considered an acceptable method of euthanasia from this point of view.

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