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Comparative study of antioxidants in fresh and frozen blueberries and cranberries fruits

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Abstract

The beneficial effect of blueberries and cranberries consumption is largely due to the high content of biomolecules with antioxidant properties, the most important are vitamins (especially vitamin C and provitamins A - carotenoids), anthocyanins and phenolic compounds. The purpose of this work is to determine how the blueberry and cranberry preservation at -80ºC influence the antioxidant content of these fruits. The biochemical parameters analyzed were as follows: anthocyanin pigments (total anthocyanin extraction and dosing, anthocyanin profile by TLC and HPLC chromatography); carotenoid pigments (total carotenoid extraction and dosing, carotenoid profile by HPLC); determination of ascorbic acid and total phenolic compounds. Antioxidants profile is different in blueberry and cranberry, both of quality and quantitative point of view. Preserving berries by freezing them for a period of time between 1 and 3 months induce different changes in the content of specific antioxidants: the concentration of vitamin C and anthocyanin pigments decreases, simultaneously with an increase in concentration of polyphenols and carotenoids.

Key words*: blueberries, cranberries, antioxidants, freezing*

Introduction

Blueberry (*Vaccinium myrtillus*) and cranberry (*Vaccinium vitis*) are part of the *Ericaceae* family and are spread in mountain areas in Asia, Europe and North America. The berries contain water, sucrose, proteins, pectin substances, vitamins (C, A, PP, B1, B2), and mineral salts. Anthocyanins, flavones, phenolic acids, and proanthocyanins are the main secondary metabolites [Bunea et al., 2012].

Epidemiological and *in vitro* studies suggest that blueberries help maintain the health and act as a barrier to the effects of aging, in particular neurodegeneration and cognitive defects. There is evidence of their action on the prevention of cardiovascular disease and certain types of cancer. Supplementing feed with blueberry extracts can be used to prevent or treat Alzheimer's disease and possibly other neurodegenerative disorders [Garcia da Rocha Concenço et al., 2014].

Anthocyanins from blueberries and cranberries act as cardio protectors by maintaining vascular permeability, reducing inflammatory responses and platelet aggregation, providing superior vascular protection compared to other cardiovascular drugs [Zafra-Stone et al., 2007].

In vitro studies have suggested that phenols, the class of compounds present in these fruits, can affect the pathogenesis of cardiovascular disease by increasing LDL resistance to oxidation, preventing platelet aggregation and thrombosis, reducing blood pressure and/or inhibiting the inflammatory processes [McKay and Bulmberg, 2007].

Another very important effect of these fruits is the neuroprotective effect. According to a study in which a stroke was simulated in rats, it was observed that after treatment with a blueberry extract, oxidative stress-induced necrosis was reduced by 43%, and ischemia-induced necrosis was reduced by 49% [McKay and Bulmberg, 2007].

Cranberries have been used since the earliest times as cataplasms for wounds and septicemia, and cranberry juice has been widely used as a popular remedy for treating women's urinary tract infections (UTIs) and other gastrointestinal disorders in infections with *E. coli* and

other pathogens. In many clinical trials, a positive relationship has been established between the consumption of cranberries and the prevention of UTIs, an effect due to the bacteriostatic activity of hippuric acid, which is formed by the metabolic conversion of p-hydroxybenzoic acid into the liver. Hippuric acid excreted in the kidney system produces urinary acidification and prevents E. coli growth in the urinary tract [Vattem et al., 2005].

Blueberries and cranberries are also used to treat diabetes, due to the presence of anthocyanins that prevent free radical production, lipid peroxidation, increased insulin secretion, and improved insulin resistance. Both *in vivo* and *in vitro* studies demonstrated a decrease in oxidative stress markers and an increase in insulin production in patients with type 2 diabetes [Andrei et al., 2014].

Age-related macular degeneration (AMD) is another condition that can be treated by eating blueberries, the anthocyanins present in them can cross the blood-retina barrier and the blood-brain barrier, which can accumulate in the eye and cause some biological effects, also acting indirectly by increasing blood flow [Andrei et al., 2014].

Blueberries and cranberries are frequently consumed fresh or frozen. The beneficial effect of these fruits is largely due to the high content of biomolecules with antioxidant properties, the most important being vitamins (especially vitamin C and provitamins A - carotenoids), anthocyanins and phenolic compounds. The purpose of this study was to determine how fruit preservation by freezing at -80 ° C influences the content of antioxidants. The biochemical parameters analyzed were as follows: anthocyanin pigments (total anthocyanin extraction and dosing, anthocyanin profile by TLC and HPLC chromatography); carotenoid pigments (total carotenoid extraction and dosing, carotenoid profile by HPLC); determination of ascorbic acid and total phenolic compounds.

Material and methods

Biological material:

The determinations were made on blueberries and cranberries, collected from the spontaneous flora (in the Băişoara Mountain region, Cluj county), during July - September. The determinations of the chemical parameters were performed immediately after harvesting. An aliquot of samples were subjected to freezing at -80ºC and is then analyzed at 1 month and 3 months after freezing. Thus, the analyzed samples were noted as follows: fresh blueberries = FB; fresh cranberries = FC; frozen blueberries 1 month = FrB1; frozen blueberries 3 months = FrB3; frozen cranberries 1 month = FrC1 and frozen cranberries 3 months = FrC3.

Extraction and determination of anthocyanins concentration:

The extraction of anthocyanins was carried out after homogenization with a mixture of acidified methanol (85:15 v/v, MeOH: HCl 0.03%). The total extract was evaporated to dryness at 40°C. The residues were taken up in 10 ml of methanol, centrifuged at 5000 rpm and filtered with a 0.45 μm Millipore filter [Bunea et al., 2011]. To determine the concentration of anthocyanins in the extracts was used the differential pH method proposed by Giusti and Wrolstad (2001).

Separation of anthocyanins by TLC and HPLC chromatography:

Extracts obtained from all types of fruit (fresh and frozen) were subjected to chromatographic separation, using two types of stationary phases, namely: paper chromatography and thin layer chromatography (TLC). Two different mobile phases were also tested. The methods were modified after Santos et al. (2013) for mobile phase 1 (ethyl acetate: acetic acid: formic acid: water - 100:11:11:26) and Halbwirth (2010) for mobile phase 2 (water: hydrochloric acid: acetic acid - 83:3: 5). The best results were achieved by TLC chromatography on silica gel and mobile phase 1.

In order to better characterize the profile of anthocyanin pigments in fresh and preserved fruits, on the total obtained extracts we performed the RP-HPLC chromatographic separation proposed by Bunea et al. (2011): Shimadzu chromatographic system equipped with LC-20 AT (Prominence) pumps, DGU-20 A3 (Prominence) degassing, photodetector SPDM20 A UV-VIS detector (DAD). For separation, column Luna Phenomenex C-18 column (5 μm, 25 cm x 4.6 mm) was used. The mobile phase consisted of two solvents: A - formic acid (4.5%) in bidistilled water and B - acetonitrile. The gradient separation system was as follows: 10% B, 0-9 min; 12% B, 9-17 min; 25% B 17-30 min; 90% B, 30-50 min; 10% B, 50-55 min. Separation was performed at a flow rate of 0.8 ml / min at 35°C. Chromatograms were monitored at 520 nm. Identification of the separated anthocyanins was based on retention time and UV-Vis spectra, by comparison with standard solutions and literature data.

Extraction and determination of total carotenoids:

The extraction of total carotenoids was performed using the method proposed by Breithaupt et al. (2000) and Bunea et al. (2012); with a mixture of methanol: ethyl acetate: petroleum ether (1: 1: 1). The partition of the extracts was carried out by the successive addition of distilled water, ethyl ether and saturated sodium chloride solution. The organic upper phase was separated, evaporated to dryness and the residue was dissolved in ethyl ether and saponified with a 30% methanolic KOH solution at room temperature for 12 hours. The saponified extract was then washed with large amounts of saturated sodium chloride solution and then water. The organic phase containing the extracted pigments was passed over anhydrous sodium sulfate and evaporated to dryness at 35°C. To determine the total carotenoid concentration, the formed residue was dissolved in 15 ml of petroleum ether and the absorption spectrum of the extracts was determined in the range 300-700 nm. The dosing was performed photometrically by reading the sample absorbance at 442 nm.

Separation of carotenoids by HPLC chromatography:

The separation of carotenoids was carried out using the method proposed by Bunea et al. (2012): Waters 990 chromatographic system with PDA detector, Kontron pumps and a reversed phase column C18 Zorbax ODS (250 mm × 4.6 mm, 3.5 μm). The mobile phase was a mixture of two solvents: acetonitrile: water (9: 1 with 0.25% triethylamine (solvent A) and ethyl acetate with 0.25% triethylamine (solvent B). The gradient program started at 15% B at 50% B from minute 0 to 16 minutes. The program was continued isocratic (16-30 minutes) with 50% solvent B.

Determination of ascorbic acid:

For vitamin C dosing the iodometric method was used [Moldovan et al., 2006], based on the oxidation of excess ascorbic acid with iodine.

Determination of total polyphenols concentration:

The amount of total polyphenol in the blueberry extracts was determined using modified Folin-Ciocalteu colorimetric method [Singleton et al., 1999]. The results were expressed as milligram of gallic acid (GAE) per 100 grams.

Results and discussion

The results obtained in determining the total anthocyanin concentration are detailed in Table 1 (mean and standard deviation). Concentration of anthocyanins is dependent on various factors, among which the most important is the species under consideration and its type (for example, whether it is cultured or spontaneous). The results obtained in this study are consistent with those presented by Bunea et al. (2011), according to which the concentration of the anthocyanins from wild blueberries harvested from Transylvania is between 250 and 300 mg /100g. In the case of fresh cranberries, the anthocyanin concentration is much lower compared to

blueberries, with an average value of 32.9 mg / 100g. These data are lower compared to those published by Celik et al. (2008).

Table 1: Concentration of total anthocyanins, total carotenoids, ascorbic acid and total polyphenol in fresh and frozen fruits (average and standard deviation; with different letters are significantly different at $P < 0.05$)

	Total anthocyanins mg/100g	**Total carotenoids µg/100 g**	**Ascorbic acid mg/100g**	**Total polyphenol mg GAE/100g**
FB	252.94±20.860	304.02±6.957	12.52±0.401	412.66±7.547
FrB1	211.78±8.533	353.12±19.786	8.73±0.224[a]	520.46±12.817[e]
FrB3	200.21±1.055	354.32±18.244	4.66±0.504[b]	537.40±10.541[f]
FC	30.17±2.110	189.77±8.892	15.67±0.851	342.45±20.066
FrC1	26.76±2.411	208.78±15.324	9.15±0.294[c]	407.88±3.790[g]
FrC3	26.21±2.648	209.34±7.273[i]	7.33±0.270[d]	416.98±10.395[h]

According to them, the concentration of anthocyanins is dependent on the species but also the degree of maturation of the fruits. In their study, the concentration of anthocyanins in immature (light red) and mature (dark red) fruits was followed. These concentrations varied from 52 to 111 mg /100g. However, the data obtained by us are consistent with those presented by Duthie et al. (2006), according to which cranberries have an average anthocyanin content of 28.19 mg / 100g. As can be seen from the table, freezing processes cause a decrease in anthocyanin concentration in both blueberries and cranberries, the decrease being more pronounced in the first month of freezing.

In the case of anthocyanin pigments, it was of interest to carry out a comparative study of the profile of these pigments in the two types of fruit. A first step consisted of a TLC separation on SilicaGel (in Figure 1). The identification of these pigments was made by comparing the values of the specific retention factors, for the chromatographic system used, with literature data [Halbwirth, 2010; Santos et al., 2013]. From the figure we can see the different profile of anthocyanins in the two types of fruit. Two different pigments were identified in cranberry fruit: cyanidin 3-glycoside (2) and peonidine 3-glycoside (4) respectively. In the cranberry fruits, in addition to the two pigments mentioned above, there were also identified: delphinidin 3-glycoside (1); malvidin 3-glycoside (3) and petunidin 3-glycoside (5). We can therefore say that these fruits differ in both the type and the concentration of anthocyanins.

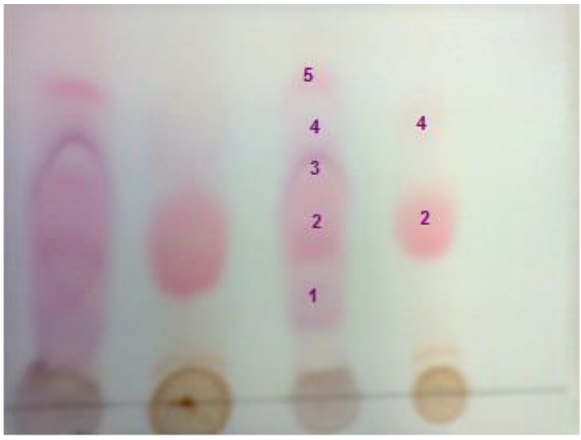

Figure 1: Separation of anthocyanins by TLC chromatography

A more accurate analysis of the qualitative profile of anthocyanins was performed by HPLC, the identification of separate peaks being performed by comparing retention times (Rt) with literature data for similar chromatographic systems [Bunea et al., 2011; Zheng and Wang, 2003; Prior et al., 2001]. Figure 2 shows chromatograms obtained in the separation of pigments from fresh fruit.

The anthocyanins identified in blueberries (whether fresh or frozen) were: (1) delphinidin-3-galactoside; (2) delphinidin-3-glucoside; (3) delphinidin-3-arabinoside; (4) petunidin-3-galactoside; (5) petunidin-3-glucoside; (6) petunidin-3-arabinoside; (7) peonidine-3-glucoside; (8) malvidin-3-galactoside and (9) malvidin-3-glucoside (Figure 2A). In the case of cranberry fruit, the number of pigments in the samples was lower compared to those in blueberries (Figure 2B), these being the following: (1) cyanidin-3-galactoside; (2) cyanidin-3-glucoside; (3) petunidin-3-glucoside; (4) peonidin-3-galactoside and (5) peonidin-3-glucoside.

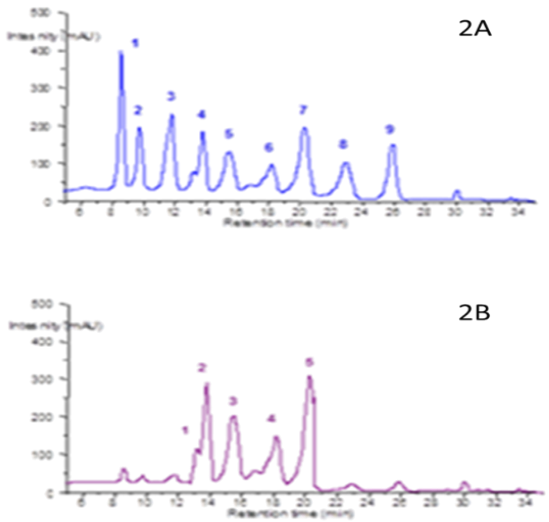

Figure 2: Separation of anthocyanins by HPLC chromatography

Carotenoid pigments are associated with a low risk of cardiovascular disease, muscle degeneration and cataracts, certain types of cancer, have immunostimulatory properties, and are involved in photo-protective mechanisms in the skin [Krinsky and Johnson, 2005; Andrei et al., 2014]. In the present study, it was of interest to determine the total concentration of these compounds in berries (Table 1) and the way in which freezing preservation influences these molecules. Concentration of carotenoids in blueberries was much higher compared to cranberries, both in the fruits analyzed immediately after harvesting and in those preserved by freezing. The results obtained in this study are consistent with those presented by Bunea et al. (2011), according to which the concentration of total carotenoid content of wild blueberries was in the range of 215–317 μg per 100 g of fruit. Fruit freezing induces an increase in carotenoid concentration, which can be explained by the fact that this freezing process causes a partial loss of water in the fruit, which facilitates the release and solubilization of these pigments.

The next step consisted in analyzing the carotenoid profile, in Figure 3 two of the chromatograms obtained were shown.

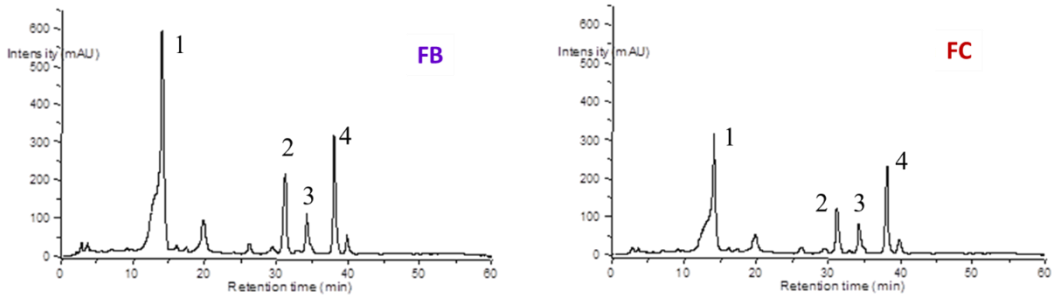

Figure 3: Separation of carotenoids by HPLC chromatography

Identification of separation peaks was performed by comparing retention times with literature data and based on absorption spectra [Bunea et al., 2012]. Thus, identified carotenoids are: lutein (pick 1); β-cryptoxanthin (pick 2); β-carotene (pick 3) and cis-β-carotene (pick 4). There is a very limited volume of data available on the composition of carotenoids in blueberries and cranberries. The data obtained for cranberry fruits are consistent with those presented by Bunea et al. (2012), according to which these fruits contain lutein, β-cryptoxanthin, and β-carotene. The profile obtained is different from that presented in the article published by Lashmanova et al. (2012). According to them, blueberry and cranberry fruits in the northern part of Europe contain neoxanthin; violaxanthin; anteraxanthin; lutein; zeaxanthin and β-carotene.

A water-soluble antioxidant present in berries is vitamin C (Table 1). The data presented are similar to those published by Borges et al. (2010), according to which the berries are characterized by a rather low concentration of vitamin C, averaging 1107 nmol / g for cranberries and 115 nmol / g for blueberries. It can be noticed that, regardless of the fruits, freezing causes a sharp decrease in the concentration of this vitamin. During storage of food, the vitamin C content decreases, because ascorbic acid is oxidized to dehydroascorbic acid, which in turn degrades by hydrolysis and the opening of the lactonic ring with the formation of 2,3-dicetogulonic acid, which has no biological activity. Vitamin C can also be reduced by exposure to oxidases present in plant tissues [Andrei et al., 2014].

Numerous epidemiological studies suggest that a correct diet is significantly associated with reduced risk of cardiovascular disease. From the category of natural compounds, polyphenols have been shown to be associated with a decrease in the incidence of cardiovascular disease. Polyphenols are the most abundant class of antioxidants in the human diet, being present in various food products of vegetable origin: fruits, vegetables, cereals, olive oil, vegetables, chocolate and various beverages [Andrei et al., 2014]. Blueberries are a rich source of polyphenols, with a mean concentration of 412.6 mg/100 g (table 1). The data obtained from wild blueberry fruit are lower compared to those presented by Bunea et al. (2011). According to the studies presented by these authors, wild blueberry fruits were characterized by average concentrations between 672.59 and 819.12 mg GAE /100g, while the fruits of culture ranged between 424.84 and 652.27 mg GAE /100g.

In this study, in the case of frozen fruits over a period of 1 to 3 months, an increase in the total polyphenol concentration was observed. The composition of phenolic compounds in fruits and vegetables is dependent on the product, the cultivar, the maturity stage and the post-harvest conditions. Because phenolic compounds are antioxidants, they are oxidized during storage and processing of food. The freezing preservative process inactivates the enzymes that cause the oxidation of phenols. [Rickman et al., 2006], which may explain the increase in the concentration observed in our study, in the frozen fruits for 1 to 3 months.

Conclusions

The antioxidant profile is different in fresh blueberry and cranberry fruits, both in qualitative and quantitative terms. Preserving the berries by freezing for a period of between 1 and 3 months induces different changes in the specific antioxidant content.

Freezing processes cause a decrease in the anthocyanin concentration in both blueberries and cranberries, the decrease being more pronounced in the first month of freezing.

The carotenoids identified in fresh and frozen fruits were: lutein; β-cryptoxanthin; β-carotene and cis-β-carotene. Fruit freezing induces an increase in carotenoid concentration, which can be explained by the fact that this freezing process causes a partial loss of water in the fruit, which facilitates the release and solubilization of these pigments.

Freezing causes a sharp decrease in vitamin C concentration, a variation that can be explained by two different mechanisms: ascorbic acid is oxidized to dehydroascorbic acid and vitamin C can also be reduced by exposure to oxidases present in plant tissues.

Blueberries are a rich source of polyphenols, while cranberry fruits are characterized by a lower concentration. In the case of frozen fruits over a period of 1 to 3 months, an increase in the total polyphenol concentration was observed.

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Influence of thermal preparation method on mineral composition of Pangasius fish

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Abstract

Determination of metallic/mineral elements in seafood, such as fish, is of great importance in assessing both their nutritional quality and also the risk of environmental contamination, and use of fish as a biomarker for aquatic environment pollution could represent a reliable approach. Cooking method changes the mineral concentrations and could contribute to loss or increment of some essential, non-essential or toxic elements concentration. This study aimed to evaluate the effects of three different cooking methods (boiling, roasting, and microwave cooking) on the mineral concentrations of Pangasius fish filets from the Bucharest (Romania) market. Mineral content in raw and cooked Pangasius fish samples was evaluated by ICP-OES, after microwave digestion, and the relative humidity of Pangasius fish samples was assessed by thermogravimetric method used. Ca, K, and Mg levels were higher in cooked samples compared to raw Pangasius fish, with the highest level in microwaved samples. Na levels were significantly higher in roasted and microwaved Pangasius fish, and significantly lower in boiled samples. The highest Fe concentration was found in roasted samples. Al and Zn levels registered the same pattern with the highest level in roasted samples, and Se level in roasted samples was insignificantly different compared to raw samples. Pb levels were significantly increased in boiled and roasted Pangasius fish meat samples and Cd levels registered the highest concentration in raw samples.

Keywords: *pangasius fish, mineral, heavy metal, thermal preparation*

Introduction

Overpopulation determined the need to increase the amount of food, and exploitation of seas and oceans for fish was one of the solutions. However, this has led to overfishing, and then to the development of aquaculture, a viable solution to these problems (Stankovic *et al.*, 2012), but not always a healthy one.

Among the aquaculture fisheries food supply, *Pangasius* sp. is one of the commonly farmed fish in the Mekong River fishery, one of the largest and most important inland fisheries in the world (www.fao.org). Pangasius fish fillets marketed in Romania are imported from Vietnam.

Metallic pollutants contamination of freshwater is a matter of concern because of their toxic potential ability to be accumulated in the food chain (Elnimr, 2011), particularly in some parts of the world, thus it is important to evaluate the aquatic environment. Fish are considered as one of the most susceptible aquatic organisms to pollutants (Alibalić *et al*., 2007). Fish that occupies the highest level of the aquatic food chain may concentrate an important level of hazardous chemicals, which could reach to humans. (Pourang, 1995; Adeyeye *et al*., 1996; Mansour and Sidky, 2002; Kah *et al*., 2016; Nor *et al*., 2017) Therefore, using the fish as a biomarker for aquatic environment pollution could represent a reliable approach (Rudneva *et al.*, 2011). The determination of metallic/mineral elements in food, such as fish, is of great importance in assessing both their nutritional quality and also the risk of environmental contamination (Conti *et al*., 2012).

Cooking method changes the mineral concentrations (Mesko *et al.*, 2016), and could contribute to loss or increment of some essential, non-essential or toxic elements concentration. In spite of knowledge about the toxicity of heavy metals and the great economic importance of the

Pangasius hypophthalmus, there is a lack of information available about the influence of different cooking methods on metallic/mineral elements as quality parameters which are considered quality indicators of fish. Most of the studies about *Pangasius hypophthalmus* refer individual chemical parameters such as mercury (Orban et al., 2008; Guimarães *et al*., 2016), or thermal preparation influence on lipid composition (Domiszewski et al., 2011).

This study aimed to evaluate the effects of three different cooking methods (boiling, roasting, and microwave cooking) on the mineral concentrations of Pangasius fish filets from the Bucharest (Romania) market. Mineral content in raw and cooked Pangasius fish samples was evaluated by ICP-OES, after microwave digestion, and the relative humidity of Pangasius fish samples was assessed by thermogravimetric method used.

Materials and methods

Samples preparation

The samples were represented by imported frozen fish fillets of *Pangasius hypophthalmus* without skin purchased from the supermarkets in Bucharest, Romania.

Before analysis the samples were thawed, weighed, labelled and packed in temperature resistant food plastic bags (samples of 100 g ± 5% each were placed in resistant plastic cooking bags). All Pangasius fish samples (n=30) were dived into four groups: raw samples, samples cooked by boiling (boiled in water with no contact between samples and water, for about 17 minutes, 100ºC), samples cooked by roasting (with no contact between meat samples and oven tray, 12 minutes, electric oven, 180ºC), and samples cooked by microwave irradiation (with no contact between meat samples and microwave plate, 5 minutes, consumer microwave oven, 850W).

For each cooking method, the time for cooking was estimated after several tests in order to achieve eatable samples. After cooking, samples were cooled, stored at 6ºC for 24 hours, and then raw and cooked samples were drained off before they were ground using the GRINDOMIX GM 200 knife mill. From each sample, 0.5 g (wet weight – ww) were digested using a Spedwave MWS-2 Berghof microwave oven as follows: Step 1: 120ºC, power 50%; Step 2: 180ºC, power 75%; Step 3: 100ºC, power 40%.

Spectrometric analysis

Digested samples were diluted to 25 mL with ultrapure water and analyzed by Thermo iCAP ICP–OES spectrometer (RF1100 W; reading time 30 s, washing time 30 s, nebulizer gas flow 0.5 $L \bullet min^{-1}$; auxiliary gas flow 0.5 $L \bullet min^{-1}$; sample injection pump flow 50 rpm). Calibration curves were developed using standard solutions of 0.001 ppm, 0.01 ppm, 0.1 ppm, 1 ppm, 5 ppm, 10 ppm, 50 ppm obtained by dilution from a multi-element ICP MERCK standard containing 1000 $mg \bullet L^{-1}$ of Al, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, Pb, Se, Sr, and Zn. Analyzed minerals for which no concentrations are reported in the present work were below method detection limit.

Relative humidity

The relative humidity of raw and cooked Pangasius fish samples was measured before digestion by thermogravimetry. The operating parameters of the thermogravimeter were: t = 9 minutes, T = 100°C.

Statistical analysis

Statistical analysis was performed using the software of VassarStats: Website for Statistical Computation (http://vassarstats.net/). One-Way ANOVA was performed for all samples' mineral concentrations, and when ANOVA generated $p \leq 0.05$, means comparison was carried out by all-pair Tukey HSD Test.

Results and discussions

In Table 1 are presented the mean heavy metal and mineral levels in Pangasius fish meat samples. Analyzed minerals for which no concentrations are reported in the present work were below method detection limit.

In general, mineral/heavy metal levels were significantly different between mussel meat samples independent of thermal preparation method.

Cooking method significantly influenced the level of Fe only in roasted and microwaved samples reported to raw ones, suggesting a high level of an insoluble fraction of this element in Pangasius fish. The highest Fe concentration was found in roasted samples, insignificantly different compared to microwaved samples.

Al and Zn levels registered the same pattern with the highest level in roasted samples. In the case of the other two types of cooking, Zn mean levels were insignificantly different compared to raw samples.

Se level in roasted samples was insignificantly different compared to raw samples, and it was significantly decreased in the case of the other 2 thermal preparation methods. Cu concentration in raw Pangasius fish meat samples was not significantly different compared to those in boiled and microwaved samples and significantly increased in roasted samples.

Ca, K, and Mg levels were higher in cooked samples compared to raw Pangasius fish, independent of cooking method, with the highest level in microwaved samples. Reported to raw samples, Na levels were significantly higher in roasted and microwaved Pangasius fish, and significantly lower in boiled samples.

Table 1. Mean heavy metal and mineral levels in Pangasius fish meat samples (ppm)

Element	Pangasius fish meat				*p*-value
	Raw	Boiled	Roasted	Microwaved	
Al	0.45[a]	0.43 [a]	0.56 [b]	0.265 [c]	<.0001
Ca	11.0 [a]	13.2 [b]	33.5 [c]	59.4 [d]	<.0001
Cu	0.016 [a]	0.017 [a]	0.025 [b]	0.018 [a]	<.0001
Cd	0.043 [a]	0.028 [b]	0.032 [c]	0.029 [b]	<.0001
Fe	0.4 [a]	0.4 [a]	1.4 [b]	1.3 [b]	<.0001
K	56.9 [a]	69.5 [b]	117 [c]	176.6 [d]	<.0001
Mg	8.8 [a]	9.5 [b]	18.6 [c]	22.4 [d]	<.0001
Na	1445 [a]	1297 [b]	2752 [c]	2839 [d]	<.0001
Ni	0.009 [a]	0.026 [b]	0.015 [c]	0.019 [d]	<.0001
Pb	0.004 [a]	0.006 [b]	0.007 [b]	0.005 [a]	0.0305
Se	0.022 [a]	0.003 [b]	0.02 [a]	0.001 [c]	0.0014
Zn	0.3 [a]	0.3 [a]	0.63 [b]	0.33 [a]	0.0011

*Levels not connected by the same letter are significantly different. The comparison can be made only between thermal preparation methods for the concentration of one element and not between different

Pb levels were insignificantly different in microwaved samples, but they were significantly increased in boiled and roasted Pangasius fish meat samples. Also, Ni levels in Pangasius fish meat samples were significantly increased in cooked samples, with the highest level in boiled samples.

Cd levels were significantly decreased in cooked samples reported to raw samples, in which registered the highest concentration.

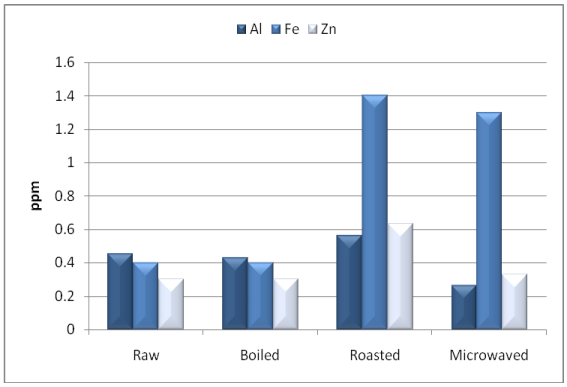

Fig. 1. Mean Al, Fe, and Zn levels in Pangasius fish meat samples (ppm)

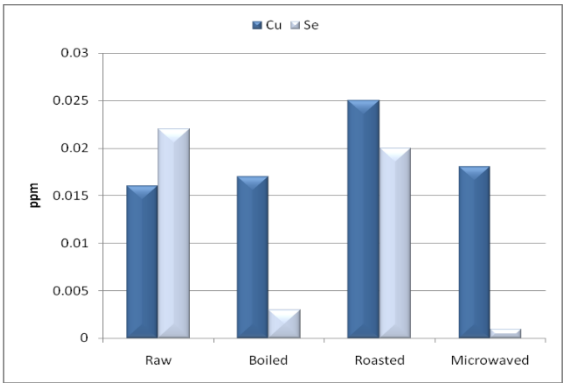

Fig. 2. Mean Cu and Se levels in Pangasius fish meat samples (ppm)

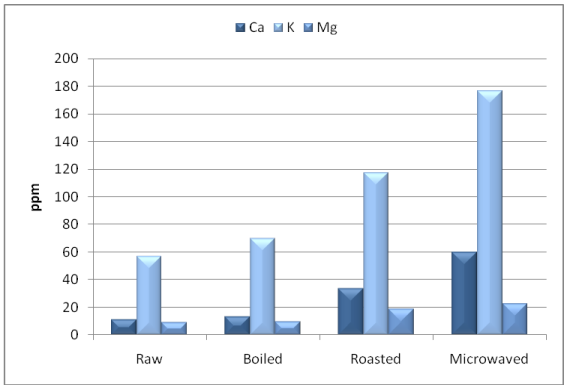

Fig. 3. Mean Ca, K, and Mg levels in Pangasius fish meat samples (ppm)

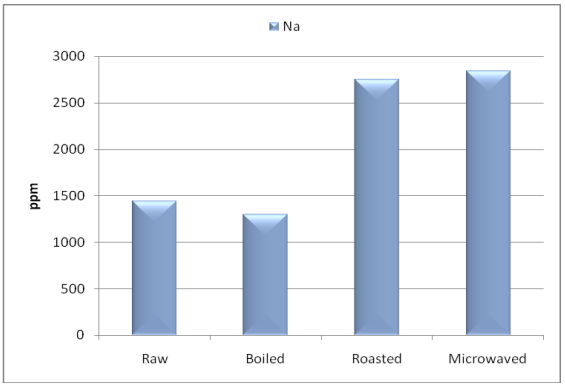

Fig. 4. Mean Na levels in Pangasius fish meat samples (ppm)

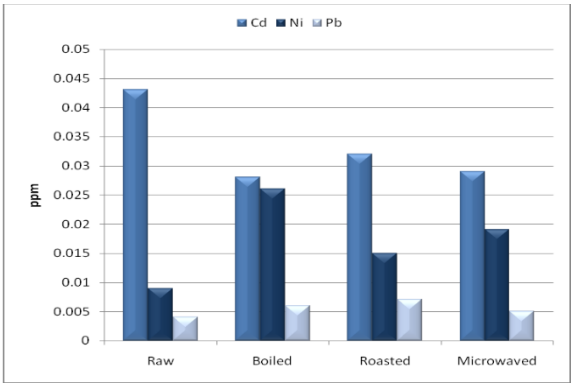

Fig. 5. Mean Cd, Ni, and Pb levels in Pangasius fish meat samples (ppm)

In cooked Pangasius fish samples, the highest mean relative humidity was registered in the case of boiling (84.49%), the lowest after microwave cooking (70.94%), while in the case of roasting, relative humidity was 75.64%. One-way ANOVA was performed for identifying significant differences between the relative humidity of cooked samples. The relative humidity of cooked samples was significantly different between cooking methods ($p<.0001$). The percentage of water loss during microwave cooking was higher than the other two thermal preparation methods.

Conclusion

Cooking influenced the mineral composition of Pangasius fish, with impact on the essential mineral nutrient intake.

In this research work, thermal preparation increased macromineral concentrations in cooked samples compared to raw Pangasius fish.

The highest mineral concentrations were identified in roasted samples.

Essential and non-essential minerals registered highest levels in roasted samples.

Cd registered significantly decreased levels in cooked samples.

The results obtained in this study can be a recommendation for consumers to choose the most effective method of cooking Pangasius fish in order to maintain or improve their nutritional qualities.

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The prophylaxis of major bacterial infections in the *Apis mellifera carpathica* bee through honey, pollen and bee bread control

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Abstract

For the purpose of controlling the evolution of major bacterial diseases in bees, which decimate bee colonies in Europe and Romania, respectively, we examined samples (honey, pollen and honeycombs) in the apicultural year 2016, from all over Romania. Sample collection and testing were done with the purpose to prevent the contamination of bee colonies with the etiological agents of major bacterial diseases, considering that worker bees and the food entering the hive (honey, pollen) represent the main contamination ways. The diagnosis method observed OIE regulations (2008) and was adapted in an original way in the Bee Pathology Laboratory in Bucharest. A total of 73 samples were examined, representing honey (51), honeycombs (6) and pollen/bee bread (16), from private apiaries all over the country, that presented depopulation without clinical evolution of contagious diseases in bees, and in which we diagnosed the presence of etiological agents of major bacterial bee diseases (36.98 %), while the rest of the samples were negative (63.02%). Of the 51 samples of honey that were examined, we identified 39.22% positive samples and 60.78% negative ones. Of the pollen samples that were examined, 31.25% were positive and 68.75% were negative, and the honeycombs samples showed 33.33% positive and 66.66% negative. Previous researches indicated that the positive samples (honey, pollen, bee bread), from apiaries in all the regions of the country, represented the basis for the prophylaxis of major bacterial diseases so that, by avoiding using them in bee nutrition, the evolution of major bee diseases did not confirm clinically or paraclinically in the following season (January-April 2017).

Keywords*: Apis mellifera carpathica, honey, pollen and bee bread control*

Introduction

Major bacterial diseases in bees, including the American foulbrood and the European foulbrood, represent a group of diseases with devastating action in bee hives, that also cause economic losses in apiculture. The American foulbrood and the European foulbrood affect young larvae, causing changes in smell and aspect and their death [1, 2, 4, 7], and adult bees carry the etiological agents of major bacterial diseases. Both diseases are declarable and quarantinable, quarantine measures being enforced to avoid spreading the disease, with emphasis on prophylaxis by natural nonaggressive means. According to legislation in effect, treatment by antibiotics are forbidden because of residues in hive products [1, 3, 7]. It is allowed in some countries but antibiotics only suppress the symptoms without eradicating the disease. Bacterial spores of the American foulbrood are not destroyed by treatment with antibiotics. Frequent use of treatment by antibiotics enables growth of resistant bacterial strains [3, 4].

Material and method

In the apicultural season 2016-2017 a total number of 73 samples were collected, from honey (51), pollen (16) and bee combs (6), in private apiaries all over Romania, to identify etiological agents of the American foulbrood and of the European foulbrood, as the apiaries presented depopulation without a clinical evolution of contagious diseases in bees. The diagnosis

method observed OIE regulations (2008) [4, 5] and was adapted in an original way in the Bee pathology Laboratory in Bucharest.

Results and discussions

The microscopic laboratory test permitted identification of etiological agents of major bacterial diseases in bees in a number of 27 samples (36.99%), while 46 samples were diagnosed negative (63.01%) (Fig. 1 and 2).

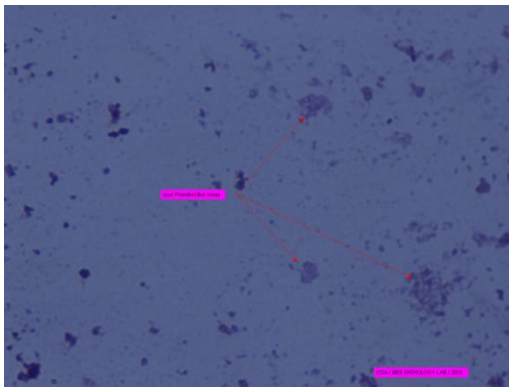

Fig. 1. Presence of the etiological agent of the American foulbrood (col. Gram x 1000)

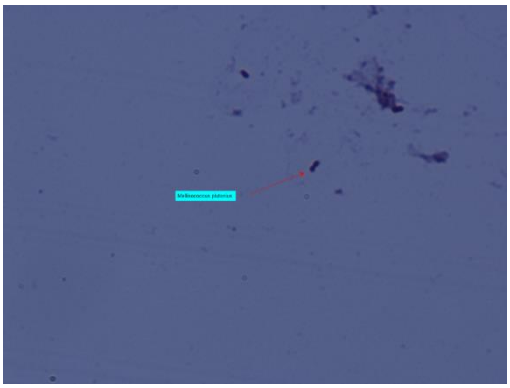

Fig. 2. Presence of the etiological agent of the European foulbrood (col. Gram x 1000)

As regards the presence of etiological agents of major bacterial diseases in bees in the 3 types of samples, laboratory tests showed the following results as in table 1.

Table 1. The presence of etiological agents of major bacterial diseases in samples examined microscopically

Type of sample	No. of examined samples	No. of positive samples			No. Of negative samples (%)
		Etiologic agent of the American foulbrood (LA)	Etiologic agent of the European foulbrood (LE)	Etiologic agents LA+LE	
1. Honey	51	4 (7.84%)	15 (29.41%)	1 (1.96%)	31 (60.79%)
2. Pollen/Bee bread	16	2 (12.50%)	3 (18.75%)	-	11 (68,75)
3. Honey combs	6	1 (16.67%)	1 (16.67%)	-	4 (66.66%)
TOTAL	73	7 (9.60%)	19 (26.02%)	1 (1.37%)	46 (63.01%)
		27 (36.99%)			

Table 1 shows that out of 51 honey samples examined (100%), 4 samples (7.84%) presented the etiological agent of the American foulbrood (spores of *Paenibacillus larvae*), 15 samples (29.41%) presented the agents of the European foulbrood (*Mellisococcus plutonius* and associated flora), while one sample (1.96%) presented a combined infection, both the etiological agents of the American foulbrood and of the European foulbrood. Of the total of honey samples examined, 31 samples (60.79%) were negative. Examination of the pollen/bee bread showed the

presence of the American foulbrood agent in 2 samples (12.5%), of the European foulbrood agents in 3 samples (18.75% showed the presence of the European foulbrood agents while 11 samples (68, 75%) were negative. Samples of honey combs presented in 7 samples (9.6%) spores of *Paenibacillus larvae*, 19 samples (26.02%) were diagnosed with agents of the European foulbrood and one sample (1.37%) presented a combined infection. The presence of the etiological agents of major bacterial diseases in the examined samples is showed in Figure 3.

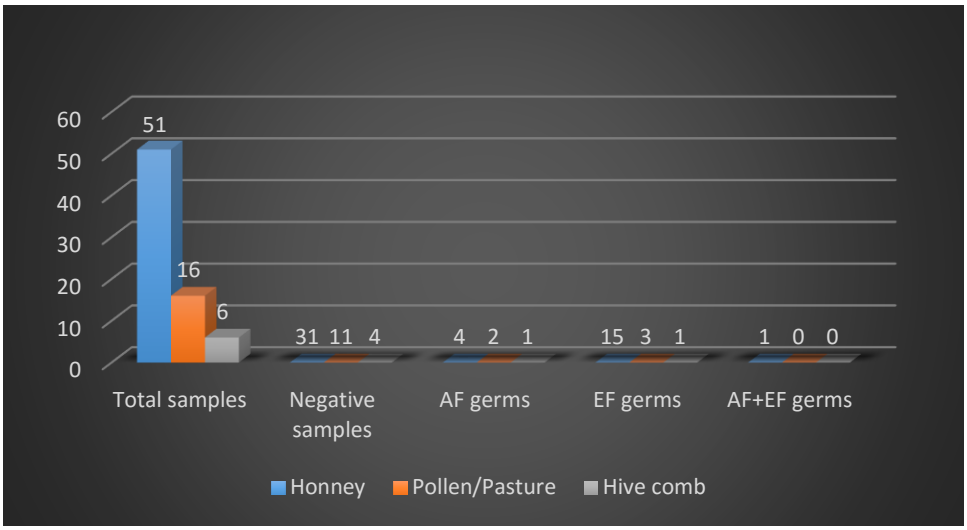

Fig. 3 The presence of germs of major bacterial diseases in examines samples (AF - American foulbrood; EF - European foulbrood)

Although examined sampled came from private apiaries all over the country that presented depopulation without clinical evolution of contagious diseases in bees, the diagnose of the presence of etiological agents of major bacterial diseases in bees in the examined samples imposed removing contaminated honey, pollen and combs from bees' food during the inactive season (winter), as these constitute sources of contamination in bees and a potential for serious bacterial diseases evolution in bees. Removing these sources from bees' food and feeding them in the winter with honey and pollen lacking in pathogens led to the absence of the clinical evolution of major bacterial diseases in bees in the following season (January-April 2017). Early identification of pathogens by bacterioscopic lab examination in the sample constituting food source for bees in the winter and removing them from bees' food was an efficient prophylaxis means for the major bacterial diseases that should be introduced as a mandatory examination prior to the inactive season of bees.

Conclusions

1. Of a total of 72 samples of honey, pollen/bee bread and combs examined by the bacterioscopic method, 27 samples (36.99%) were positive for etiological agents of major bacterial diseases in bees and 46 samples (63.01%) were negative.
2. The presence of the etiological agents of major bacterial diseases in bees per types of examined samples was the following: 7 samples of honey, pollen and combs (9.6%) were positive for the American foulbrood agent, 19 samples (26.02%) were positive for the etiological agent of the European foulbrood and one sample was diagnosed with combined infection (American foulbrood and European foulbrood), the rest of the samples being negative.

3. The fact that samples tested positive imposed removing contaminated food and feeding bees with honey, pollen/bee bread lacking in pathogens of major bacterial diseases in bees, being aware of the role of these sources in contaminating bees and the subsequent evolution of major bacterial diseases in the contaminated bee colonies.
4. Removing these sources from bees' food and feeding bees in the winter with honey and pollen lacking in pathogens led to the absence of clinical evolution of major bacterial diseases in bees in the following season.
5. Early identification of pathogens in the bacterioscopic lab examination of samples that constitute food source for bees in the winter should be introduced as a mandatory examination prior to bees' inactive season as a prophylaxis means in major bacterial diseases in bees.

Acknowledgments

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Canine behaviour type index in experimental Units trial

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Abstract

We ran the present research in canine behaviour over 18 months, on the premises of Experimental infrastructure of Horia Cernescu Research Unit, under behaviour study project of animal lodging Research contract no. 4833 / September, 4, 2014. The study considered a 360 dogs group, data being extracted from our (March, 31, 2015 to July, 31, 2017) pet databases. The research is structured based on Canine Behaviour Type Index (CTBI) 12 types canine behaviour, considering three psychological interactive factors further itemized into (1) Environmental (either Organized or Spontaneous); (2) Social (Alpha, Beta, or Gamma); (3) Motivation (either Medium or High), i.e. 12 possible outcomes. The breed type (χ^2=818.59, at p < 0.000), age (F=9.31, at p < 0.001) and period of staying (F=3.185, at p ≤ 0.001) appear to be associated with CBTI. The older dogs resulted more like Dreamer (SBM) and Aristocrat (SAM) behaviour types, while younger more like Adventurer (SBH) and Rebel (SAH). Our study results cannot sustain gender association hypothesis based on CBTI profiles (χ^2=17.31, at p = 0.099), suggesting, nevertheless, that CBTI is a useful tool in canine behaviour research, in matters of pets' owners – research financed by private funds, win-win case.

Keywords: *canine behaviour, Canine Behaviour Type Index (CTBI)*

Introduction

Our Experimental Units for canine and feline species have been operational in Banat University - *Horia Cernescu* Research Unit since 2012, starting March, 10, 2011 under Sanitary Veterinary and Food Safety Directorate's *Authorization no. 0317 - Pet lodging, temporary shelter, feeding and pet maintenance.* Our canine behaviour research project targeted development of a public – private research partnership. Practically, the project illustrates a win to win case of research vs. pet owners: behaviour research needs the animals to come from different environments, owners need animal facilities when they go away from home.

Specific target of present report was establishing correlations and association between *Canine Behaviour Type Indexes® (CBTI)* and a number of genetics and physiological factors *[1,2]*. One more (side) target was determining whether a pet management system can modify typical behavioural differences between males and females, as noticed in our experimental units.

Materials and methods

Animals and data collection: for each Owner, the Collaboration agreement of 4883 Contract was signed for the animal or animals included in research program; out of 668 cases, 360 dogs were sampled (206 male and 154 female). The animals used as our research samples are 47 breeds, including one crossbreed group. The behaviour pattern adopted is based on Pet Connect team, Australia, which developed CBTI, ranking companion dogs into distinct profiles.

Dagley & Perkins (2005) considered three psychological dimensions, which we based our research on, i.e.:

(1) Environmental Order (either Organized or Spontaneous);

The two variables of Environmental Dimension are Organised type (O) and Spontaneous type (S). The Organised type seeks an orderly controlled environment. It loves to herd things and is team focused. The Spontaneous type is more self-focused and interested in a particular facet of its environment at any time, rather than with the larger picture that the Organised type focuses on.

(2) Social Order (Alpha, Beta, or Gamma);

Such dimension refers to social position and willingness to comply with social rules. Such linear hierarchy manifests three types: Alpha, Beta, and Gamma, in that order. The Alpha (A) type is most dominant, confident and controlling, socially. The Beta type (B) is socially mobile and more challenging of the social order. The Gamma type (G) is a born follower and is highly rule bound, socially.

(3) Motivation (either Medium or High).

Motivation is a general term denoting how active the dog is. Dogs display either high or medium levels of motivation. High levels (H) will amplify other characteristics in the preceding two dimensions. Medium levels (M) will tone down the other behavioural dimensions.

The *Canine Behaviour Type Index* advances 12 type dog behaviour system, based on three dimensions of each interactive factor considered, as indicated in Table 1 which also indicates the number of dogs considered for each behaviour type.

Table 1. Twelve Canine Behaviour Type Index profiles

Behavioural type	No cases	Behavioural type	No cases	Behavioural type	No cases
Commando (OAH)	8	Director (OAM)	11	Defender (OBH)	11
Sentry (OBM)	9	Deputy (OGH)	11	Diplomat (OGM)	44
Rebel (SAH)	36	Aristocrat (SAM)	10	Adventurer (SBH)	89
Dreamer (SBM)	5	Investigator (SGH)	71	Companion (SGM)	55

Classes apud Dagley & Perkins, 2005.

During the entire hosting period, the veterinarian volunteer students registered behaviour aspects by filling in a questionnaire (see *www.petconnectgame.com*) together with owner, after the staying/care period. As per *CBTI*, the most frequent behaviour types were SBH (Adventurer – 89 dogs), SGH (Investigator – 71 dogs) and OGM (Diplomat – 44 dogs).

Except for the 82 cross breed dogs, the most common dog breeds in our experimental units were the 42 Bichon, and the 40 Labrador, probably the most popular in Timisoara area. The Poodles, Beagle and Cockers and are the next of the most common breeds – 15, 14 and 12 lodged animals. No animal from *Group 10: Sighthounds* was hosted in Experimental units during the trial period.

Table 2. Sample-groups of breeds involved

Names apud FCI[1] Standards Commission *[5]*	**No. of cases**
Group 1: Sheepdogs and Cattledogs	13
Group 2: Pinscher and Schnauzer - Molossoid and Swiss Mountain and Cattledogs	37
Group 3: Terriers	28
Group 4: Dachshunds	7
Group 5: Spitz and primitive types	19
Group 6: Scent hounds and related breeds	20
Group 7: Pointing Dogs	7
Group 8: Retrievers - Flushing Dogs - Water Dogs	61

[1] *Fédération Cynologique Internationale* (*World Canine Organisation*)

Group 9: Companion and Toy Dogs	85
Cross breed	83
Total	**360**

Housing and feeding. We kept the dogs in eight conventional dog pen rooms and an open air grassed paddock in Pet Experimental Unit. Experimental unit for pets was organized into 4x 6.0 m^2 pens, in 2 rows; pen minimal equipment: feeder, drinker, carpet on floor or on raised platform, and toys. In front of each pen there is a front stainless steel gutter, a corridor and a visual barrier *[3]*.

Fig. 1: Behaviou study grounds. *Horia Cernescu* Research Unit – Pet Sector *[3]*

Each dog was either single, or accompanied by other dogs, in the pen. Three times a day, each animal was walked into the paddock and/or near area of the Experimental Units. Dogs were fed as per individual preference, expressed in the owner's specifications: 1-3 teas or *ad libitum.* All pens and corridors were video monitored during entire lodging period. The owners had the possibility to see their pets in real time on smartphones, in the facilities during lodging, feeding and care activities.

Internal and external temperature and humidity were continuously monitored by multi-functional wireless digital device *Weather Station PCE-FWS 20*.

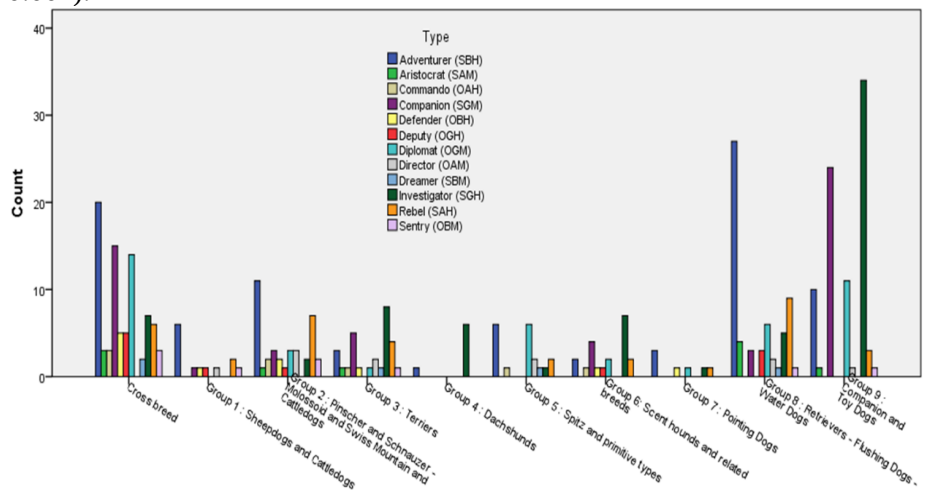
Statistical Analysis: Analysis of *CBTI* and association of *CBTI* with several factors or variables (age, days of staying) were performed based on *Variance Analysis (ANOVA)*. All data comparing male and female and nominal variables (group, breed, gender and feeding protocol) were analysed based on χ^2 tests.

Results

The Groups established by FCI Standards Commission (Graph no 1) including a number of 82 animals form hybrids group were associated with CBTI (χ^2=182.09, at $p < 0.000$). ***Breed*** appears to be associated with *CBTI* profiles (χ^2=818.59, at $p < 0.000$). Bichon breeds (26/42 animals, 61.90 dogs) were associated with Investigator (SGH) behaviour type, Labrador breed was associated (23/40 animals, 57.5% dogs) with Adventurer (SBH) and Poodle breed (10/15 animals, 66.6% dogs) was associated with Companion (SGM) behaviour type.

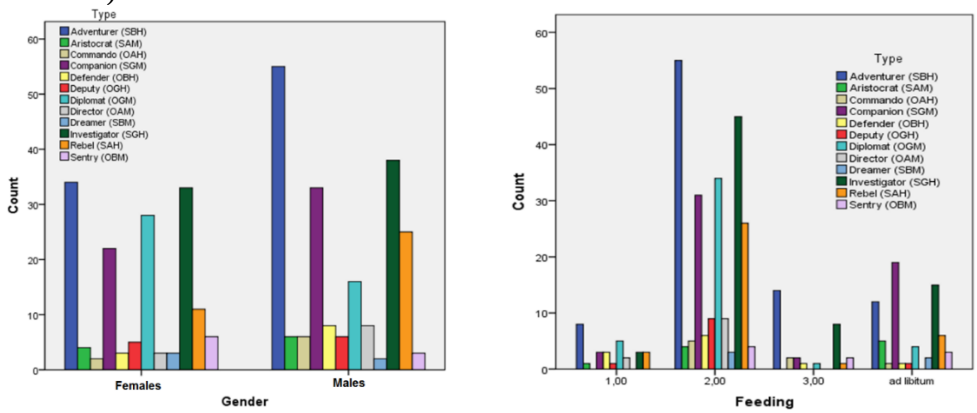
Age: *CBTI* is depending of the age; the Dreamer (SBM) and Aristocrat (SAM) behaviour types appear to be associated with older animals (6.25±1.76 years, respectively 6.20±2.35). The Adventurer (SBH), Commando (OAH), Investigator (SGH) and Rebel (SAH) behaviour types appear to be associated with younger animals (1.90±0.18 years, 2.13±0.82, 2.32±0.26 respectively,

2.55±0.50). There appears to be significant difference between behaviour types, based on age (F = 7.121, at $p < 0.001$).



Graph 1. CBTI Histograms, based on FCI groups

Gender: There was no statistical difference noted between males and females (Graph 2, *left*); our research cannot sustain the hypothesis of gender being associated with *CBTI* profiles (χ^2=17.31, at p = 0.099).



Graph 2. CBTI Histograms, based on gender and feeding protocol

Period of staying in the Experimental Units appear to be associated with *CBTI*; science does not explain how the animals staying longer (13.55±2.81 days) associate with Director (OAM) behaviour type, while the animal staying less (4.12±1.34 days) associate with Commando (OAH) behaviour type (F = 1.967, at p = 0.031). Care takers say that a longer stay permit the dog to better accommodate, which is expressed by medium activity level, in contrast with the first days' stay, when they can often act more restless, as a reaction to multiple stress factors – new environment, parting with owners, other animals around, and such like.

Feeding protocol in the Experimental Units appear to be associated with *CBTI* (Graph 2, *right*); science does not explain how come that the animals with two intake/day associate with Adventurer (SBH – 55/360 cases), Investigator (SGH – 44/360 cases) and Diplomat (OGM 34/360 cases) behaviour type (χ^2=55.44, at p = 0.009).

Discussion

CBTI helped us understand behaviors types displayed by dogs; increased the enjoyment that dogs produced; helped to improve dogs' lifestyles; and provided options for dog problems.

The *CBTI* tool was described as not breed-specific; however, behavior types may cluster around particular profiles. In present study we associated breeds and behavior types (χ^2=729.68, at $p < 0.000$); also, the FCI groups sustain the hypothesis of breed association with *Canine Behavior Type Index.* The authors of *CBTI [1]* suggested some precautions in following cases:

i) Dogs under 3 years old (or 5 in cases of late social maturity) may need to be profiled each 6 months, because their personality is still forming.

ii) Breeds tend to cluster around specific profiles, because they have been selectively bred for specific purposes. People often prefer a particular breed for their character, hence continuing to select the same breed with a similar personality profile.

iii) When a dog becomes depressed, such mood could be emphasized as an increase in irritability and anxious activity, unlike humans who typically become withdrawn and reduce activity levels. However, the neurochemical changes occurring in depressed humans and dogs are thought to be similar. If the dog changes from a Medium activity type to a High activity type, perhaps all is not well, and help from a local Veterinary Behaviorist should be sought.

iv) In cases of abnormal brain function, or a psychiatric condition, the test may need to be retaken at regular intervals, and after treatment.

v) Dogs' personality may change with senescence.

All precautions were taken over research time; however, considering the high number of cases, and the time needed for acceptance of hypothesis, the authors will continue the study for particular precautions, also considering extra variables.

Conclusions and implications

- Privately financed research projects could represent a solution, in context of generally scant research financing; for a win-win case, *Canine Behaviour Type Index* profiles will produce easy and useful results, to both researchers and owners.
- *Canine Behaviour Type Index* and several variables could be proved to associate: breed, age, and lodging time appear to associate with *Canine Behaviour Type Index*.
- Present study couldn`t sustain association of gender and *Canine Behaviour Type Index*.

Acknowledgments

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The importance of dietary control in skin and hair disorders in dogs

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Abstract

The frequency of hair and skin disorders in dogs has increased in recent years. Diet has a role to play in dealing with these disorders. Several companies produce commercial diets to help treat these disorders.These disorders include Atopic dermatitis, Zinc responsive dermatosis, food allergy dermatitis and dandruff. For this study two different foods were use.These were premium original chicken and brown rice and Super premium anallergenic.They were fed to four dogs of different breeds. One dog which had dandruff was fed with premium original chicken and brown rice. The other three, which included dogs with pruritus, dandruff and food allergy dermatitis, were fed with superpremium anallergenic dog food. The results of the trial were as follows: the dog with dandruff, which was fed with premium dog food, showed no modifications during the trial period. In fact its condition remained the same. The dog with pruritus worsened during the trial period when it was fed with super-premium anallergenic dog food. The dog with dandruff fed with super-premium analelergrnic did not show any modifications and its condition remained the same. The dog with food allergy dermatitis shows no modifactions or lesions when fed with super-premium anallergenic dog food. Three of the four dogs were reluctant to eat the foods initially. The conclusion of the trial was the fact that the diets used were unable to illustrate improvement in two of the four dogs. The condition of one dog worsened during the period while the condition of the other one was managed when eating the food.

Key words*: skin and hair disorders, commercial diets, food trial*

Introduction

Skin and hair disorders are an important part of small animal practice. Bacterial infections, ectoparasitism, allergies, fungal infections and neoplasia are common problems. The skin and coat can be affected by many nutritional factors. Therefore, it is important to investigate these factors in patients with skin disorders. Changes in the skin which occur due to nutritional abnormalities include a dry dull coat with brittle hairs, slow hair growth, abnormal production of scales, crusts and erythema in areas of stretch such as the distal extremities (Hand et al., 2010). Dogs are prone to a large range of inflammatory skin diseases. These include allergic disorders, parasitic infestations, bacterial infections and adverse reactions to food.

Skin disorders, which result in inflammation, are associated with Immunoglobin E (IgE) mediated type one hypersensitivity responses. These respond to changes in dietary fatty acid concentrations. They manifest in the form of atopic dermatitis, flea bite hypersensitivity and food hypersensitivity. Atopic dermatitis is the most commonly diagnosed skin allergy in dogs. The dogs are sensitive to dust mites, moulds, weeds, grasses and trees. A high number of dogs are also affected by an adverse food reaction (Halliwell et al., 2009). When diagnosing a skin allergy, one must always consider the presence of both adverse food reaction and atopy.

Atopic dermatosis results in pruritus, self trauma at the level of the skin, yeast infection or secondary bacterial infection. Chronic otitis externa may also be observed, when diagnosing this condition the history and clinical signs need to be carefully observed. Some breeds are more predisposed than others e.g. Chinese Shar Peis, Irish setters, Dalmations, Labrador Retreivers, several terriers and toy breeds. Clinical signs begin when the dog is exposed to IGE sensitive mast cells which degranulate and release a host inflammatory response. This occurs after exposure to the offending antigen. The inflammatory mediators include histamine, heparin, proteolytic enzymes, chemotactic factors and various types of eicosanoids (Case et al., 2011).

Fatty acid supplementation is recommended in the management of inflammatory skin disease in dogs. Omega three fatty acids used in supplement include polyunsaturated fatty acids, eicopentaenoic acid and docosahexaenoic acid found in fish oil. The omega-six fatty linoleic acid is needed for normal epidermal lipid barrier function (Lloyd et al., 1989). This supplementation has had varying effects when used to manage pruritus and inflammatory responses associated with atopic dermatitis. A small proportion of allergic dogs do not need to be treated with other therapies when a fatty acid supplement is given. Others will not respond which may be due to the fact that different agents induce inflammation and pruritus (Ellis, 2008).

When changing the levels of fatty acids in the diet to control inflammatory disease one needs to ensure that the optimal levels of linoleic acid are supplied to meet essential dietary requirements and to reduce the fatty acid metabolic profile. By controlling the ratio of omega-six and omega-three acids pruritus and tissue eicosanoid profiles reduce in some allergic pets. This may help in controlling atopic dermatitis. New evidence suggests that increasing the polyunsaturated fatty acids in the diet may improve the epidermal barrier in the skin and have a positive effect on the immune system by regulating transcription or transduction (Fuhrmann et al., 2006).

When a dog illustrates signs of an inflammatory dermatological disease as the result of an adverse reaction to ingredients within its diet, it is known as a cutaneous adverse food reaction. This may occur due to a food hypersensitivity, an intolerance to food or an adverse metabolic reaction. A reaction may be non-immune mediated or immune mediated. An immune mediated reaction is caused by a dietary hypersensitivity to several or one components within the diet. Intolerance to food is an abnormal physiological response to a food ingredient which is not mediated by the immune. These can occur due to a food toxicity, a pharmacological reaction to dietary ingredients and a lack of lactose within the intestine. Incidence of occurrence can be seen at any age however the initial signs are usually seen in dogs under one. They can be seen all year round and are not always linked to a recent dietary change. There is no sex or age predilection (Hillier and Griffin, 2001).

The major allergens identified in dogs are proteins with a large molecular weight. In dogs beef, soy and dairy products are the most common food allergens. They also develop reactions to wheat, pork, chicken, corn, horse meat, eggs and fish. These ingredients are common allergens as they are used frequently in pet foods. Therefore there is an increased likelihood of exposure. Clinical signs in the case of an adverse reaction usually manifest as pruritus, which occurs four to twenty four hours after ingestion of the offending antigen. Secondary lesions occur due to intense scratching, biting and self -trauma. Secondary bacterial infections may also occur. A minority of cases presented with a recurrent pyoderma not associated with pruritus. Some dogs may present with gastrointestinal signs including diarrhoea and vomiting.

Three types of elimination diet can be used: a homemade diet, a commercial limited ingredient food or a commercial hydrolysed protein food. A homemade diet should contain one source of protein and carbohydrate. Common protein sources are lamb, rabbit, venison or tofu. Potatoes and rice are the source of carbohydrates. This diet can be expensive and time consuming. It is not nutritionally balanced and should not be given beyond the period required for diagnosis. Commercial limited ingredient foods contain one source of carbohydrate and one source of protein. They can be used during the diagnostic phase and long term feeding. One needs to be aware that not all of the products have been carefully tested as elimination diets. Different sources of protein are used in different products therefore it needs to be selected carefully using the history as a guide. They are used if the dog is too big to make a homemade diet or if the owner does not want to make one. Commercial hydrolysed protein foods are those which contain protein that has undergone hydrolysis to reduce its size and eliminate antigenicity. Chicken, soy and liver are most commonly

used. These diets are complete and can be used for long term feeding in dogs with adverse food reactions (Cave, 2006; Loeffler et al., 2004).

Feeding the elimination diet should be done gradually over a three to four day period. No scraps or treats should be given. Improvements may be seen within a few weeks while others may need to be on it for a six to ten week period .If pruritus does not decrease during the elimination phase then either food allergy is not diagnosed or the diet still contains an offending allergen. Long term-management is achieved by feeding a complete balanced palatable diet without offending antigens. The protein content should be digestible and of a high quality. A reduced omega six omega three fatty acid ratio needs to be used to reduce pruritus. Strict compliance is essential to prevent relapse (Rosser, 1993).

Material and methods

This study investigated the role played by diet in managing skin and hair disorders in four dogs and to see the efficacy of super-premium anallergenic dog food and premium original chicken and brown rice dog food when dealing with these disorders. The trial perod was from April to June 2016.

The first case was that of a 5-year-old male dog, weighing 15 kg, which suffered from dandruff for the last five years. His condition improved when washed with aloe vera shampoo. However, it recurred again whitin a few days. This dog was fed with premium original chicken and rice during the trial period and with super-premium light weight care before the trial period.

The second case was that of a 5-year-old female dog, weighing 30 kg, which suffered from pruritus since she was one. She scratched herself after eating cheese, pork, and food containing eggs and milk. This dog was on a super-premium skin food sensitivity z/d dog food but unfortunately it did not help her, so she was put on a premium food which containes lamb and rice. During the trial period the dog was feed with super-premium anallergenic dog food.

The third case was that of a 6-year-old female Pit Bull Terrier Cross weighin 26.5 kg, which suffered from dandruff since she was four months old. It was diagnosed when she was two years old. This dog scratched herself after eating fresh chicken. She was previously fed with a premium food whichcontain lamb and rice. She was fed with premium salmon and rice before the trial period. During the trial period she was feed with super-premium anallergenic dog food.

The last case was that of a 6-year-old male Labrador weighing 30 kg, which started licking his paws and had hot spots present on his ears in 2012. He was diagnosed with food allergy dermatitis and he was placed on a super-premium anallergenic dog food. The owner changed his diet after a year to premium trainer wet and dry food and his condition flared up again. He also developed urinary calculi. The owner then put the dog back on the super-premium anallergenic food he was fed with during the trial period.

Super-premium anallergenic food is recommended to decrease intolerance to nutrients and ingredients. It contains hydrolysed protein and purified carbohydrates. The benefits are the fact that it contains oligopeptides of a low molecular weight which reduce the risk of an allergic food reaction. It supports the skin barrier, restricts allergens and contains antioxidants to help neutralise free radicals.

Premium original chicken and brown rice is a hypo-allergenic food produced by a veterinary surgeon in Wales. It does not contain added beef, dairy or wheat. It is suitable for sensitive skin and digestion. It is highly digestible as it uses natural ingredients such as whole grains and animal proteins. It has no artificial colours or preservatives which are known to cause food intolerance including itchy skin, digestive upset, excessive moulting, full anal glands and waxy ears.

Results and discussion

Each of the dogs had a clinical examination before the trial began. On being closely examined, case number one showed evident dandruff and no other lesions or modifications were present. When case number two was examined it was evident that she suffered from pruritus and areas of hair loss were visible on her hind legs and around her anus. During examination the dandruff of case number three was very evident on her back but no other modifications were seen. When case number four was examined the only sign of his past condition was a small lesion on his front right paw no other lesions or modifications were present.

During the trial period one dog was fed with premium original chicken and brown rice while the other three were fed with super-premium anallergenic. We noticed that case number 1, case number 2 and case number 3 were reluctant to eat the foods at the beginning of the trial. This led us to believe that they did not find these foods very palatable. However case number 4 illustrated no reluctance in eating this food. The animals were observed very closely during the period to see if any modifications were observed. Case number 1 showed no modifications during the trial period. In fact, his condition remained the same when he received premium chicken and brown rice dog food. Case number 2 scratched more during the trial period and a new lesion was observed on her hind right limb wgen she received super-premium anallergenic dog food. Case number 3 showed no modifications during the period and her condition remained the same when she received super-premium anallergenic dog food. Case number four suffered from hot spots in 2012 and was diagnosed with food allergy dermatitis. He had been feed with super-premium anallergenic dog food since 2013 and he had the sign of an old lesion on his front right paw. When this dog was fed this food he did not suffer from any modifications.

This trial showed a mixture of results as some conditions remain the same while others improved or worsened.

Similar results were observed when a trial was carried out in 2004 on 60 dogs which were fed with a soy hydrolysate and rice based elimination diet.

These dogs also had skin conditions which included localized or generalized pruritus, erythema, self trauma, seborrhea and recurrent pyoderma and/or Malassezia dermatitis as well as otitis. 58 dogs finished the trial. 36 improved during the period but their conditions recurred when the original diets were fed. 20 dogs out of the 36 were diagnosed with an uncomplicated adverse food reaction.

Their clinical signs were either completely regress or were very mild during the trial period. 2 of these 20 dogs did not respond to a soy hydrolysate based diet but did to a soy based home made diet and to rice and rabbit commercially available elimination diets. The remaining 16 dogs improved during the trial period. Their clinical signs remained mild to moderate and the pruritic score was reduced. 22 dogs did not improve when fed the test diet and no improvement in clinical signs or pruritic score were observed. They were diagnosed with atopic dermatitis and did not respond to other elimination diet either (Biourge et al., 2004).

Conclusions

One major finding of the trial was that the commercial diets used were unable to illustrate signs of improvement in three out of four cases.

Moreover, due to the fact that a small number of dogs participated in this trial, it is difficult to assess the efficacy of the diets used.

Furthermore, it was noticed that diet alone cannot treat all dermatological problems. However, if a dog finds a diet which manages its condition, it should not be changed except deemed absolutely necessary. Last but not least, if the animal reacts negatively to any food in their diet, it needs to be eliminated from it.

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Preliminary studies regarding antimicrobial effect of various kuwanon G – antibiotic combinations on some MRSA strains

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Abstract

Methicillin-resistant Staphylococcus aureus (MRSA) is a constant therapeutic challenge in humans and animals, due to the limited range of antibiotics that can be used for the management of infections. This preliminary study is based on the assessment of the antibacterial activity of kuwanon G (a prenylated flavonoid present in white mulberry, Morus alba L., Moraceae) and its interactions with various antibiotics (oxacillin, amoxicillin, erythromycin and gentamicin) against four MRSA clinical isolates (MRSA T1 – T4). The sources of all clinical isolates resistant to cefoxitin and oxacillin were infections (recurrent otitis, pyoderma and laryngopharyngitis) in dogs. Minimum inhibitory concentrations (MICs) for kuwanon G and antibiotics were determined by the microdilution method. Interactions between kuwanon G and antibiotics were evaluated by the checkerboard method and time-kill assay. MICs varied between 6.25 and 12.5µg/mL for kuwanon G alone against all four MRSA clinical isolates. According to the calculated fractional inhibitory concentration index, various combinations were synergistic and additive. Microbicidial time has confirmed the synergy as the logarithmic reductions of colony-forming units obtained for the combinations of kuwanon G and some antibiotics were $2\log_{10}$ lower than the logarithmic reductions obtained for the most potent/active component of the combination. The obtained results are promising, taking into account the antibacterial activity of kuwanon G, as well as its synergistic effects with the most used antibiotics. This study reports on the antibacterial activity of kuwanon G and suggests its ability to act synergistically with antibiotics; combinations effective in combating Gram-positive including MRSA infections might be developed.

Key-words*: checkerboard, kuwanon G, MRSA, synergy, time-kill assay*

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a Gram-positive bacterium that developed drug resistance to β-lactam antibiotics through horizontal gene transfer and natural multiple selections. Infections with MRSA are a real problem for humans and animals and the treatment of these infections is challenging due to the limited range of antibiotics that can be used because of antibiotic resistance (1 - 5). Kuwanon G (KG) is a prenylated flavonoid present in white mulberry (*Morus alba* L., *Moraceae*) leaves, fruits and root bark (fig. 1) (6, 7).

The aim of this preliminary study was to investigate the antibacterial activity of kuwanon G and its interactions with four common antibiotics against MRSA strains.

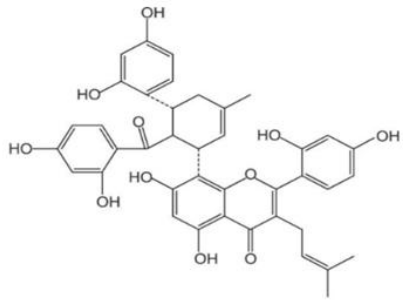


Figure 1. Chemical structure of kuwanon G.

Material and methods

For this study, there were selected four MRSA (MRSA T1 – T4) clinical strains resistant to oxacillin and cefoxitin. The strains were isolated from various infections (recurrent otitis, pyoderma and laryngopharyngitis) in dogs (phenotype being established by the diffusimetric method).

Minimum inhibitory concentrations (MICs) of KG, oxacillin (OX), amoxicillin (Amx), erythromycin (Er) and gentamicin (Gn) against MRSA isolates were determined by the microdilution method according to current Clinical & Laboratory Standards Institute (CLSI) (8) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (9).

Two *in vitro* tests were performed in order to evaluate the interactions between KG and antibiotics: checkerboard method (10) and time-kill assay (11). The experimental design of checkerboard method involves the use of 96-well microtiter plates in order to evaluate the bacterial growth in the presence of the combination of two components (KG and antibiotic) in various concentrations after incubation at 37^0C for 24 hours. The absorbances were determinated spectophotometrically (450/650 nm) before and after incubation. MIC was defined as the concentration that reduced the bacterial growth by 80% compared to the bacterial culture control. Checkerboard method enables the interpretation of the results through fractional inhibitory concentration index (FICI) and isobolograms (12).

$$\mathrm{FICI} = \mathrm{FIC}_{\mathrm{antibiotic}} + \mathrm{FIC}_{\mathrm{kuwanon\ G}} \text{ where:}$$

$$\mathrm{FIC}_{\mathrm{Antibiotic}} = \frac{\mathrm{MIC}_{\mathrm{antibiotic\ in\ combination\ with\ kuwanon\ G}}}{\mathrm{MIC}_{\mathrm{antibiotic\ alone}}},$$

$$\mathrm{FIC}_{\mathrm{kuwanon\ G}} = \frac{\mathrm{MIC}_{\mathrm{kuwanon\ G\ in\ combination\ with\ antibiotic}}}{\mathrm{MIC}_{\mathrm{kuwanon\ G\ alone}}}.$$

A combination is synergistic if FICI value ≤ 0.5, additive when it is > 0.5 and ≤ 1, indifferent when it is 1 – 4, and antagonistic when it is > 4 (11).

The results obtained the checkerboard method were subjected to Bliss independence–based model interpretation with graphical representation of the experimental dose-response surface and theoretical dose-response surface of interaction. Experimental dose-response surface ($E_{measured}$) represents the experimental percentage of growth in the presence of different concentrations of KG and/or antibiotic. Taking into account the non-interactive process between two components, $E_{predicted}$ is the calculated percentage of growth based on the experimental percentage of growth according to Bliss independence–based model. Theoretical dose-response surface of interaction (ΔE) represents the difference between predicted ($E_{predicted}$) and measured ($E_{measured}$) percentage of

growth with KG and antibiotic at various concentrations. Points of difference surface above zero (positive) indicate synergy and below zero (negative) indicate antagonism (10).

In time-kill assay, the bactericidal effect of the combination of KG (at ½MIC_{KG} concentration) and antibiotic (at ½$MIC_{antibiotic}$ concentration) was compared with the bactericidal effect of the antibiotic alone, KG alone and bacterial culture control. After 0, 4, 24 and 48 hours of incubation at 37^0C, aliquots were withdrawn and the colony forming units (CFU) were determined after incubation at 37˚C. Synergy/antagonism is interpreted if the combination increases/decreases by 100 (or $2log_{10}$) times the bactericidal effect, compared to the most potent/active antibacterial agent of the combination after 24 hours or 48 hours (11).

Results and discussion

MIC values of KG alone against all MRSA clinical isolates varied between 6.25 and 12.50 µg/mL and the bacterial susceptibility of MSRA clinical isolates to tested antibiotics is presented in table 1.

Table 1. MIC (µg/mL) of antibiotics and KG*

MRSA clinical isolates	MIC_{OX}	MIC_{Amx}	MIC_{Er}	MIC_{Gn}	MIC_{KG}
MRSA T1	16 (R)	16 (R)	>170.67 (R)	0.25 (S)	12.50
MRSA T2	128 (R)	128 (R)	10.67 (R)	0.25 (S)	6.25
MRSA T3	256 (R)	256 (R)	>170.67 (R)	0.50 (S)	12.50
MRSA T4	256 (R)	256 (R)	>170.67 (R)	1 (S)	12.50

*European Committee on Antimicrobial Susceptibility - Testing Breakpoint tables for interpretation of MICs and zone diameter Version 7.0. Valid from 2017-01-01; Abbreviation: S – sensible, R – resistant

- **KG – OX combinations**

Checkerboard method showed synergies for the combinations KG – OX (FICI= 0.04-0.5; table 2, fig. 2a) against MRSA T1 – T4 clinical isolates. Time-kill assay did not confirm synergy for the combinations KG – OX against MRSA T1 –T4, but excluded the antagonism, because the combination of KG with antibiotics did not decrease, but also did not increase the viable colony count by more than $2log_{10}$CFU/mL compared to the viable count obtained with the most active/potent agent of combination (KG). These differences between the results obtained by the checkerboard method and time kill assay can be explained by the different measured phenomena – the checkerboard method assesses the inhibitory effect while the time kill assay measures the bactericidal process (13).

Table 2. Effects of KG – OX combinations

Strain	MIC_{OX} (µg/mL)	$MIC_{OX–KG}$ (µg/mL)	FIC_{OX}	$MIC_{KG–OX}$ (µg/mL)	MIC_{KG} (µg/mL)	FIC_{KG}	FICI*	TKA**
MRSA T1	16	0.50	0.01	0.20	12.25	0.03	0.04 (S)	NC
MRSA T2	128	0.50	0.01	0.20	6.25	0.03	0.04 (S)	NC
MRSA T3	256	0.50	0.01	1.56	12.5	0.13	0.14 (S)	NC
MRSA T4	256	0.50	0.01	6.25	12.5	0.50	0.50 (S)	NC

Abbreviation: S – synergy, NC – synergy has not been confirmed, $MIC_{OX–KG}$ – MIC of OX in presence of KG, $MIC_{KG–OX}$ – MIC of KG in presence of OX

*effect of the combination determined through checkerboard method, ** effect of the combination determined through time-kill assay

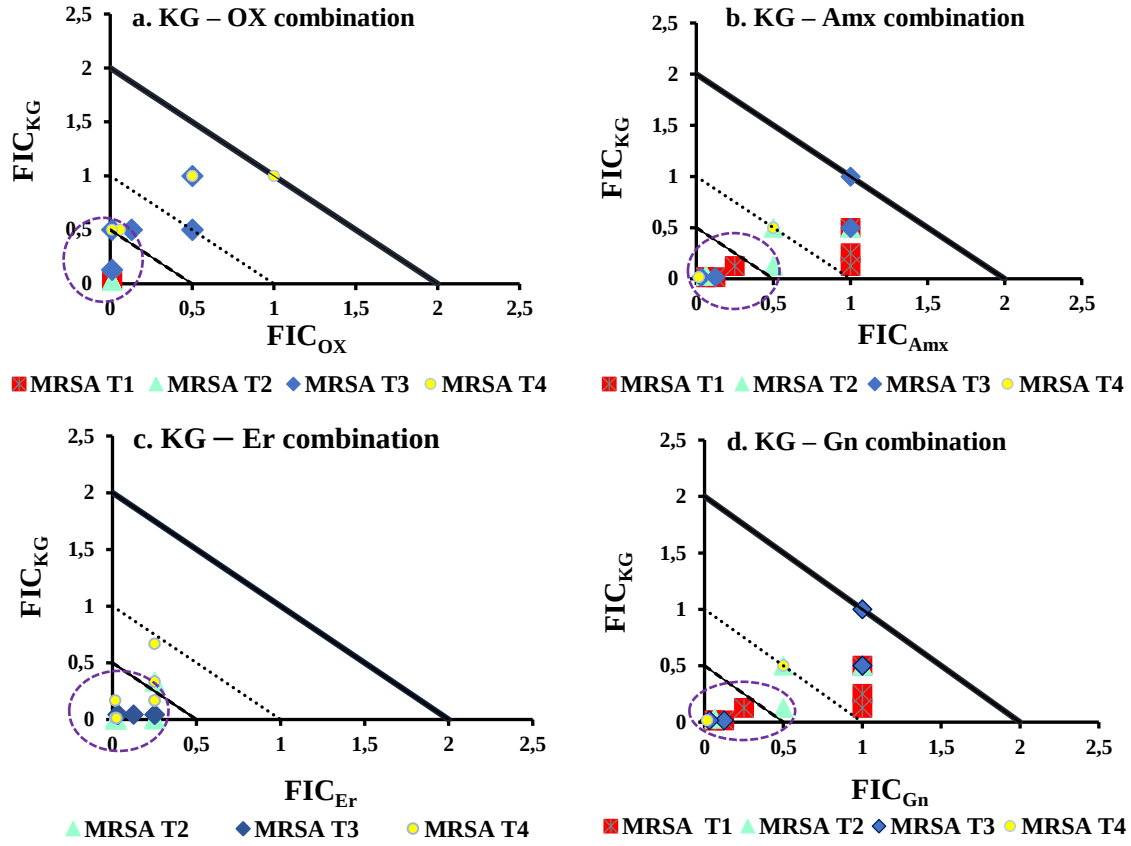


Figure 2. Interactions between KG – OX (a), KG – Amx (b), KG – Er (c) and KG – Gn (d) against MRSA clinical isolates T1 – T4; purple colored dotted circles highlight synergies.

➢ **KG – Amx combinations**

Checkerboard method showed synergy for the combinations KG – Amx (FICI=0.04-0.14; table 3, fig. 2b) against MRSA T1 - T3 clinical isolates and additive effects (FICI=0.51) against MRSA T4. Time-kill assay confirmed synergy for the combinations KG – Amx against MRSA T1 – T2 (fig. 3), but excluded the antagonism against MRSA T3 –T4.

Table 3. Effects of KG – Amx combinations

Strain	MIC_{Amx} (µg/mL)	$MIC_{Amx–KG}$ (µg/mL)	FIC_{Amx}	$MIC_{KG–Amx}$ (µg/mL)	MIC_{KG} (µg/mL)	FIC_{KG}	FICI *	TKA**
MRSA T1	16	0.50	0.01	0.20	12.25	0.03	0.04 (S)	S
MRSA T2	128	0.50	0.01	0.20	6.25	0.03	0.04 (S)	S
MRSA T3	256	0.50	0.01	1.56	12.5	0.13	0.14 (S)	NC
MRSA T4	256	0.50	0.01	6.25	12.5	0.50	0.51 (Ad)	NC

Abbreviation: S – synergy, NC – synergy has not been confirmed, $MIC_{Amx–KG}$ – MIC of Amx in presence of KG, $MIC_{KG–Amx}$ – MIC of KG in presence of Amx

*effect of the combination determined through checkerboard method, ** effect of the combination determined through time-kill assay

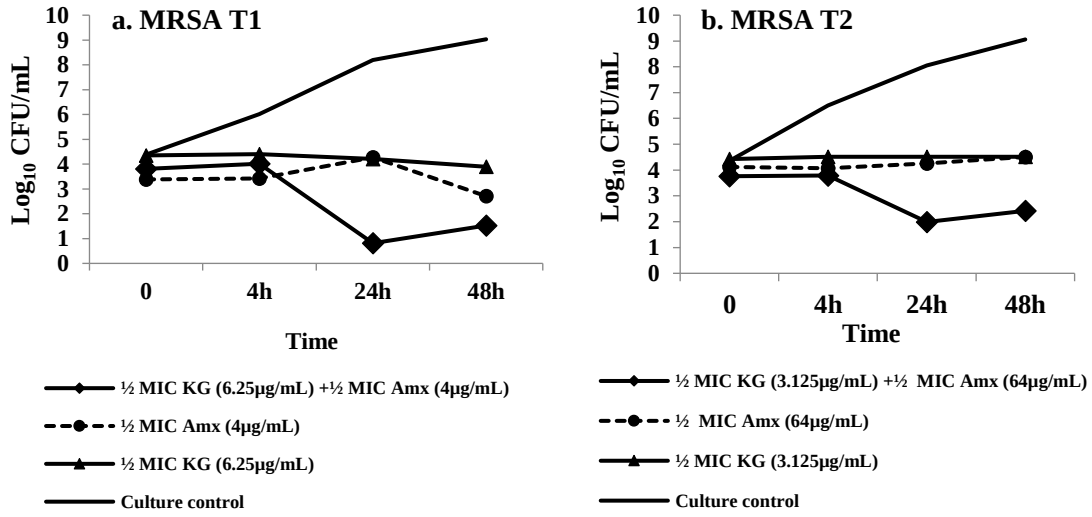


Figure 3. ***Time–kill curves of KG alone, Amx alone and their combination against MRSA T1 (a) and MRSA T2 (b).***

- **KG – Er combinations**

Checkerboard method showed synergies for the combinations KG – Er (FICI=0.03-0.1; table 4, fig. 2c) against MRSA T2 - T4 clinical isolates. Time-kill assay did not confirm synergy for combinations KG – Er against MRSA T2 –T4, but excluded the antagonism. It should be noted that KG did not decrease MIC_{Er} against MRSA T1.

Table 4. Effects of KG – Er combinations

Strain	MIC_{Er} (µg/mL)	$MIC_{Er–KG}$ (µg/mL)	FIC_{Er}	$MIC_{KG–Er}$ (µg/mL)	MIC_{KG} (µg/mL)	FIC_{KG}	FICI*	TKA**
MRSA T1	>170.67¥ (341.33)	>170.67	ND	12.25	12.25	1	ND	NC
MRSA T2		1.00	0.10	0.52	6.25	0.00	0.10 (S)	NC
MRSA T3	10.67	0.33	0.03	0.13	12.5	0.04	0.07 (S)	NC
MRSA T4	>170.67¥ (341.33)	8.00	0.02	12.5	12.5	0.01	0.03 (S)	NC

Abbreviation: S – synergy, NC – synergy has not been confirmed, $MIC_{Er–KG}$ – MIC of Er in presence of KG, $MIC_{KG–Er}$ – MIC of KG in presence of Er, ¥MIC_{Er} >170.67 µg/mL and for calculation of $FIC_{Er,}$ MIC_{Er} was considered as being 341.33 µg/mL

*effect of the combination determined through checkerboard method, ** effect of the combination determined through time-kill assay

- **KG – Gn combinations**

Checkerboard method showed synergies for the combinations KG – Gn (FICI=0.03-0.09; table 5, fig. 2d) against MRSA T1 – T4 clinical isolates.

Table 5. Effects of KG – Gn combinations

Strain	MIC_{Gn} (µg/mL)	$MIC_{Gn–KG}$ (µg/mL)	FIC_{Gn}	MIC_{KG-Gn} (µg/mL)	MIC_{KG} (µg/mL)	FIC_{KG}	FICI*	TKA**
MRSA T1	0.25	0.02	0.06	0.20	12.25	0.02	0.08 (S)	S
MRSA T2	0.25	0.02	0.06	0.20	6.25	0.03	0.09 (S)	S
MRSA T3	0.50	0.02	0.03	0.20	12.5	0.02	0.05 (S)	S
MRSA T4	1	0.02	0.02	0.20	12.5	0.02	0.03 (S)	S

Abbreviation: S – synergy, $MIC_{Gn–KG}$ – MIC of Gn in presence of KG, $MIC_{KG–Gn}$ – MIC of KG in presence of Gn, *effect of the combination determined through checkerboard method, ** effect of the combination determined through time-kill assay

The experimental percentage of growth (fig. 4a) in the presence of different concentrations of KG and/or Gn and the theoretical dose-response surface of interaction (fig. 4b) were represented for KG – Gn combination against MRSA T4 according to Bliss independence–based model interpretation.

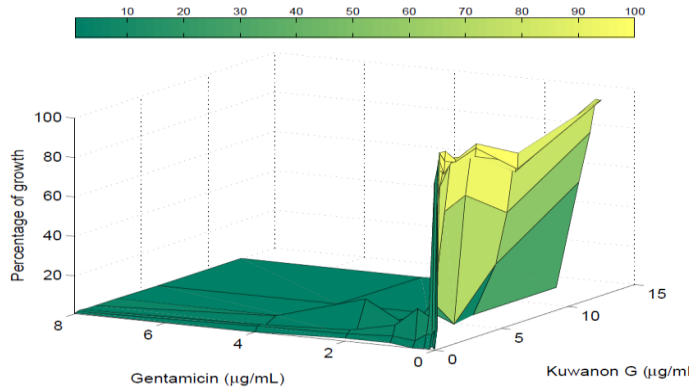


Figure 4a. The three-dimensional plot of the experimental percentage of growth ($E_{measured}$) between KG and Gn against MRSA T4.

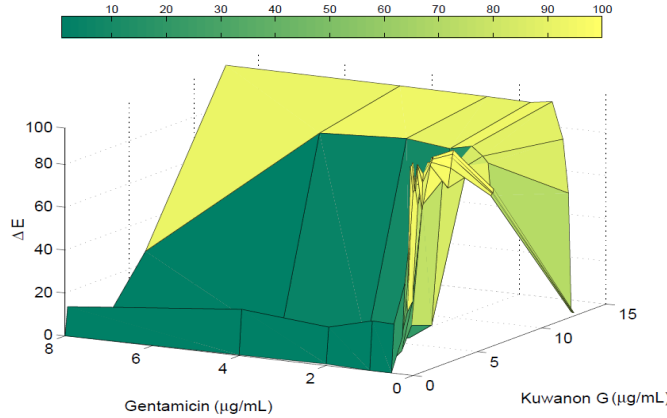


Figure 4b. Theoretical dose-response surface of interaction (ΔE) between KG and Gn against MRSA T4 (ΔE above zero (positive) indicates synergy).

Time-kill assay confirms synergy for the combinations KG – Gn against MRSA T1 (fig. 5a and fig. 6a), MRSA T2 (fig. 5b and fig. 6b), MRSA T3 (fig. 5c and fig. 6c) and MRSA T4 (fig. 5d and fig. 6d).

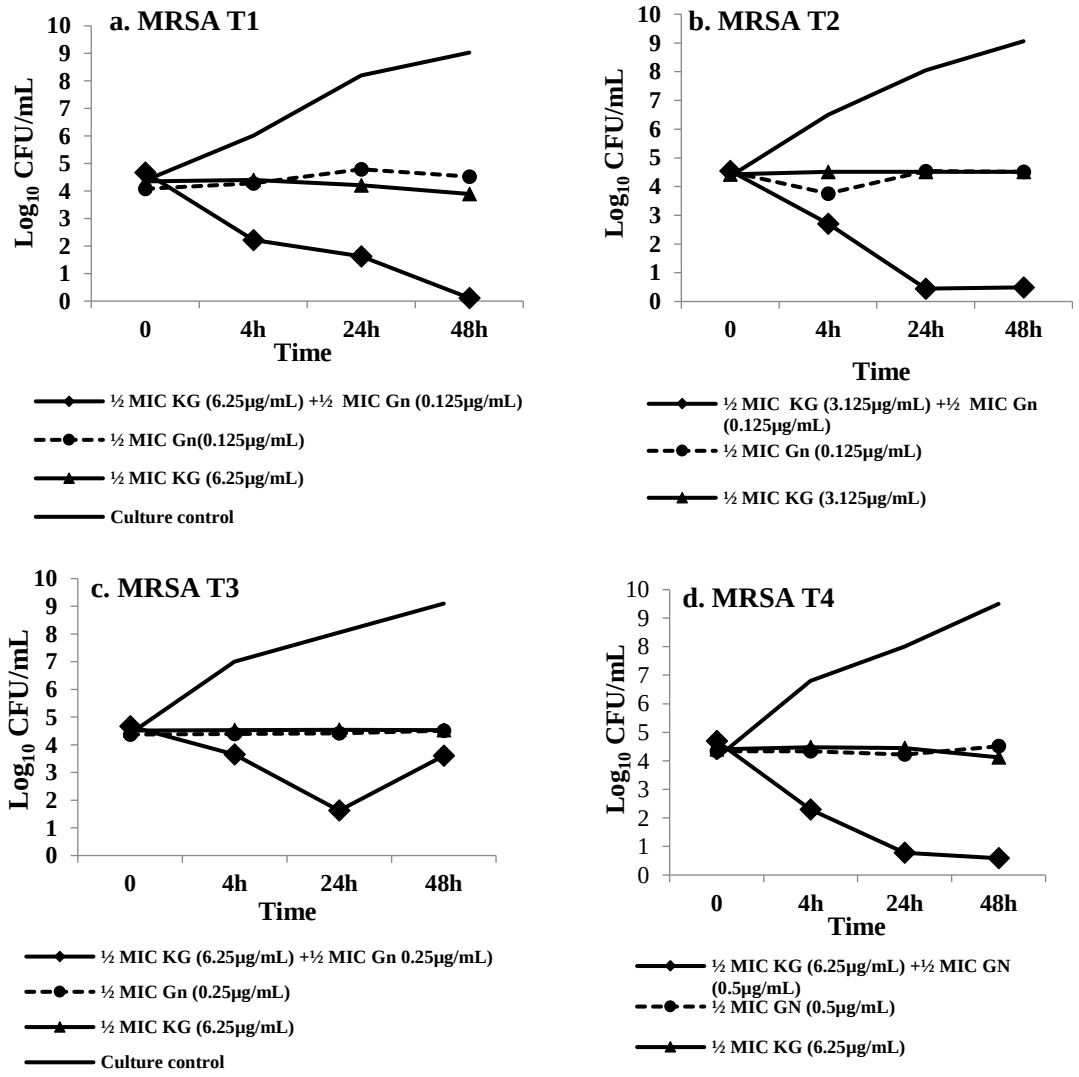
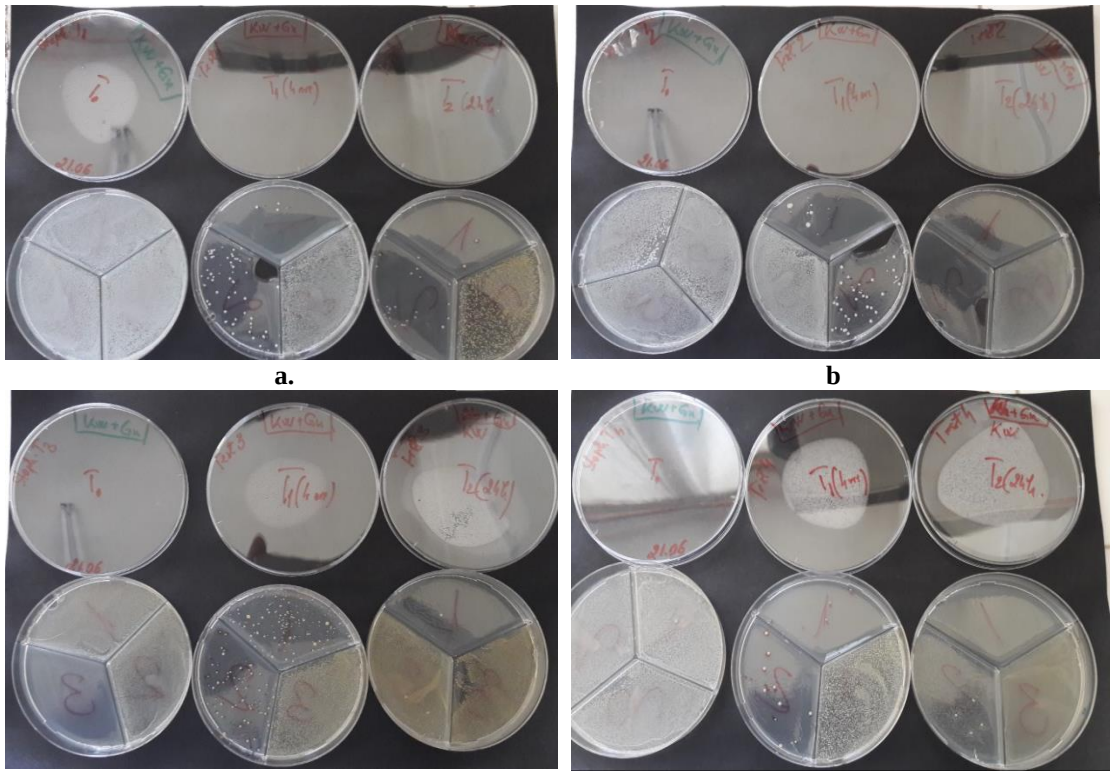


Figure 5. Time–kill curves of KG alone, Gn alone and their combinations against MRSA T1 (a), MRSA T2 (b), MRSA T3 (c), MRSA T4 (d).



a. **b**

c. **d.**

Figure 6. Differences between KG/Gn, Gn, KG against MRSA T1 (a), MRSA T2 (b), MRSA T3 (c), MRSA T4 (d) in time kill-assay determinations.

Conclusion

The results of this preliminary study highlight the antibacterial activity of kuwanon G and its ability to synergize with antibiotics – oxacillin, amoxicillin, erythromycin and gentamicin. The combinations: kuwanon G – oxacillin, kuwanon G – amoxicillin, kuwanon G – erythromycin and kuwanon G – gentamicin tested using the checkerboard method showed synergistic effects against MRSA clinical isolates. The synergistic effects were partially confirmed by the time-kill assay. This study reports on the antibacterial activity of kuwanon G and suggests its ability to act synergistically with antibiotics; combinations effective in combating Gram-positive including MRSA infections might be developed.

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The antibacterial activity and synergies between morusin and some antibiotics against MRSA strains – preliminary study

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Abstract

Mulberry (Morus alba L., Moraceae) is one of the most valuable and rich in phytochemicals plant. Morusin is a prenylated flavonoid present in mulberry roots and leaves. The in vitro antibacterial activity of morusin and its interactions with conventional antibiotics (oxacillin, amoxicillin and gentamicin) were evaluated against four methicillin resistant Staphylococcus aureus clinical isolates (MRSA T1 – T4) with resistance to oxacillin and cefoxitin which had been isolated from dogs with various pathologies. Minimum inhibitory concentrations (MICs) were determined by the microdilution method. The interactions were assessed by the chequerboard method - with interpretation through fractional inhibitory concentration index (FICI) and isobologram analysis. The interactions were confirmed by the time-kill assay. MICs varied between 3.125 and 6.25µg/mL for morusin alone against all four MRSA clinical isolates. Chequerboard method showed synergies for the combinations: morusin – oxacillin (FICI=0.024 - 0.27), morusin – amoxicillin (FICI=0.024 - 0.27) and morusin - gentamicin (FICI=0.05 - 0.12) against all four tested isolates. Time-kill assay determined synergies for the following combinations: morusin – oxacillin against MRSA T1, morusin – amoxicillin against MRSA T2 and morusin - gentamicin against all four isolates. Our preliminary study evaluated the antibacterial activity of morusin and its ability to act synergistically with antibiotics; these results suggest that morusin might be a promising strategy to overcome antibiotic resistence.

Key-words: *bacterial resistance, chequerboard, morusin, synergy, time-kill assay*

Introduction

Mulberry (*Morus alba* L., Moraceae) is one of the most valuable and rich in phytochemicals plant. Mulberry leaves are used for feeding silkworms due to the high content of proteins (1). Numerous reviews have been published on both *in vitro* and *in vivo* studies that assessed antidiabetic, antioxidant, anticancer, hypolipidemic, antiatherogenic and anti-inflammatory activities of mulberry (2 - 4). Mulberry extracts and their isolated compounds showed antimicrobial potential against harmful pathogens: *Bacilllus subtilis, Staphylococcus aureus, Streptococcus faecalis* and *Mycobacterium smegmatis* (5 - 8). Morusin (fig. 1) is a prenylated flavonoid isolated from the root and leaves of mulberry with antibacterial activity against Gram-positive bacteria (9).

The post-antibiotic apocalypse due to the frequent and improper use of antibiotics involves new strategy in overcoming antibiotic resistance (10). Methicillin resistant *S. aureus* (MRSA) is one great concern with challenges because most of the strains are resistant to beta-lactams,

cephalosporins, aminoglycosides, macrolides, fluoroquinolones, but also to other important antibiotics such as glycopeptides (vancomycin and teicoplanin) (11).

A promising strategy in overcoming antibiotic resistance is the synergy between vegetal products and conventional antibiotics (12).

The present preliminary study aimed to assess the antibacterial activity and the interactions between morusin and commonly used antibiotics against MRSA clinical isolates.

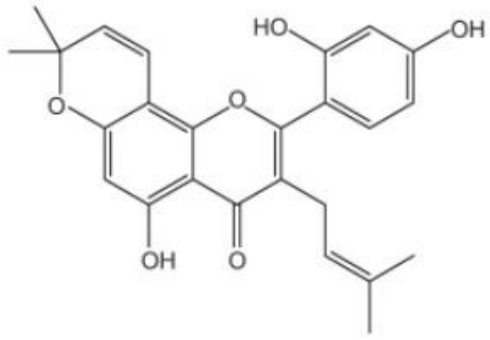


Figure 1. Chemical structure of morusin.

Material and methods

Minimum inhibitory concentrations (MICs) of oxacillin (OX), amoxicillin (Amx), gentamicin (Gn) and morusin (MO) were determined by the microdilution method against four MRSA clinical isolates according to the Clinical & Laboratory Standards Institute (CLSI) (13) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (14). The sources of all clinical isolates resistant to cefoxitin and oxacillin were infections (recurrent otitis, pyoderma and laryngopharyngitis) in dogs.

The interactions between MO and antibiotics were determined using the chequerboard method (15) with interpretation through fractional inhibitory concentration index (FICI) and isobolograms (12).

$$\mathrm{FICI} = \mathrm{FIC}_{\mathrm{Antibiotic}} + \mathrm{FIC}_{\mathrm{Morusin}} \text{ where:}$$

$$\mathrm{FIC}_{\mathrm{Antibiotic}} = \frac{\mathrm{MIC}_{\mathrm{Antibiotic\ in\ combination\ with\ morusin}}}{\mathrm{MIC}_{\mathrm{Antibiotic\ alone}}},$$

$$\mathrm{FIC}_{\mathrm{Morusin}} = \frac{\mathrm{MIC}_{\mathrm{Morusin\ in\ combination\ with\ antibiotic}}}{\mathrm{MIC}_{\mathrm{Morusin\ alone}}}.$$

A combination is synergistic if FICI value ≤ 0.5, additive when it is > 0.5 and ≤ 1, indifferent when it is 1 – 4, and antagonistic when it is > 4 (16).

Graphical representation of experimental dose-response surface and theoretical dose-response surface of interaction were performed according to Bliss independence–based model. Experimental dose-response surface ($E_{measured}$) represents the experimental percentage of growth in the presence of different concentrations of MO and/or antibiotics. $E_{predicted}$ is the calculated percentage of growth based on the experimental percentage of growth according to Bliss independence–based model, taking into account the non-interactive process between two components. The difference between predicted ($E_{predicted}$) and measured ($E_{measured}$) dose-response surface is the theoretical dose-response surface of interaction (ΔE). A ΔE value above zero (positive) indicates synergy and below zero (negative) indicates antagonism (15).

Time-kill assay was performed in order to confirm the results obtained in the chequerboard method. According to the time-kill assay, synergy is considered if the decrease in the viable colony count $\geq 2\log_{10}$ CFU/mL; the combination is evaluated in comparison to the count obtained with the most active single component, after 24 or 48 hours. The antagonism is defined as an increase in the colony count of $\geq 2\log_{10}$ CFU/mL, the combination being compared to the count obtained with the most active single component of combination after 24 or 48 hours (16).

Results and discussion

MIC values of MO alone against four MRSA clinical isolates varied between 3.125 and 6.25 µg/mL. The obtained results were in agreement with the already published results. Sohn HY *et al.* have reported MIC values of 5–30 µg/mL for MO against *Streptococcus faecalis, S. aureus, Mycobacterium smegmatis* and *Bacillus subtilis* (9). Our results confirmed the antibacterial activity of MO against Gram-positive bacteria including MRSA strains.

Table 1. *In vitro* interactions between MO and antibiotics determined by the chequerboard method and time-kill assay

	MRSA T1	**MRSA T2**	**MRSA T3**	**MRSA T4**
MIC_{MO} (µg/mL)	6.25	6.25	3.13	6.25
➢ **OX combinations**				
MIC_{OX} (µg/mL) (susceptibility to OX)¥	16 (Resistant)	128 (Resistant)	256 (Resistant)	256 (Resistant)
$MIC_{OX–MO}$; $MIC_{MO–OX}$ (µg/mL)	0.50; 0.10	0.50; 0.10	2; 0.78	4; 1.56
FICI* / TKA**	0.05 (S)/ S	0.024 (S)/ Nc	0.26 (S)/ Nc	0.27 (S)/ Nc
➢ **Amx combinations**				
MIC_{Amx} (µg/mL) (susceptibility to Amx)¥	16 (Resistant)	128 (Resistant)	256 (Resistant)	256 (Resistant)
$MIC_{Amx–MO}$; $MIC_{MO–Amx}$ (µg/mL)	0.50; 0.10	0.50; 0.10	2; 0.78	4; 1.56
FICI* / TKA**	0.05 (S)/ Nc	0.024 (S)/ S	0.26 (S)/ Nc	0.27 (S)/ Nc
➢ **Gn combinations**				
MIC_{Gn} (µg/mL) (susceptibility to Gn)¥	0.25 (Sensible)	0.25 (Sensible)	0.50 (Sensible)	1 (Sensible)
$MIC_{Gn–MO}$; $MIC_{MO–Gn}$ (µg/mL)	0.02; 0.10	0.02; 0.39	0.02; 0.10	0.03; 0.10
FICI* / TKA**	0.08 (S)/ S	0.12 (S)/ S	0.06 (S)/ S	0.05 (S)/ S

Abbreviation: MO – morusin, OX – oxacillin, Amx – amoxicillin, Gn – gentamicin, $MIC_{atb–MO}$ – MIC of antibiotic in presence of MO, $MIC_{MO–atb}$ – MIC of MO in presence of antibiotic; FICI – fractional inhibitory concentration index, S –synergy, Nc– synergy has not been confirmed

*effect of the combination determined through checkerboard method, **effect of the combination determined through time-kill assay, ¥susceptibility to antibiotic according to European Committee on Antimicrobial Susceptibility - Testing Breakpoint tables for interpretation of MICs and zone diameter Version 7.0. Valid from 2017-01-01.

According to the FICI interpretation and isobologram representation (checkerboard method), synergy was observed for combinations MO – OX (FICI = 0.024-0.27; fig. 2a), MO – Amx (FICI = 0.024-0.27; fig. 2b) and MO – Gn (FICI = 0.05-0.12; fig. 2c) against all four MRSA clinical isolates. Fig. 3 describes the experimental design of the checkerboard method and the

synergy obtained for the combinations MO - Gn against MRSA T4. Table 2 summarizes the results of both the checkerboard method and time-kill assay against all MRSA clinical strains.

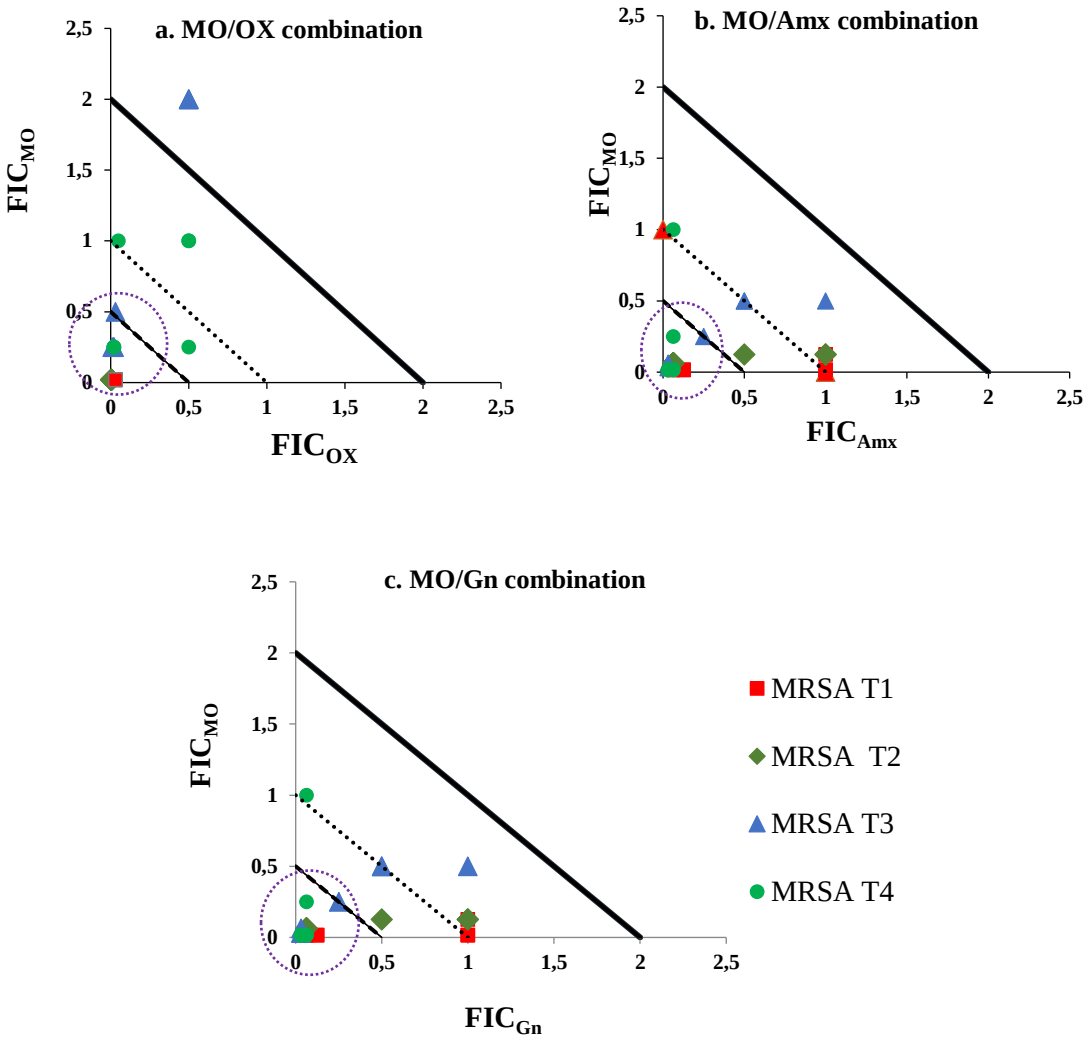


Figure 2. Interactions between MO – OX (a), MO – Amx (b) and MO – Gn (c) against MRSA clinical isolates T1 – T4;
purple colored dotted circles highlight synergies.

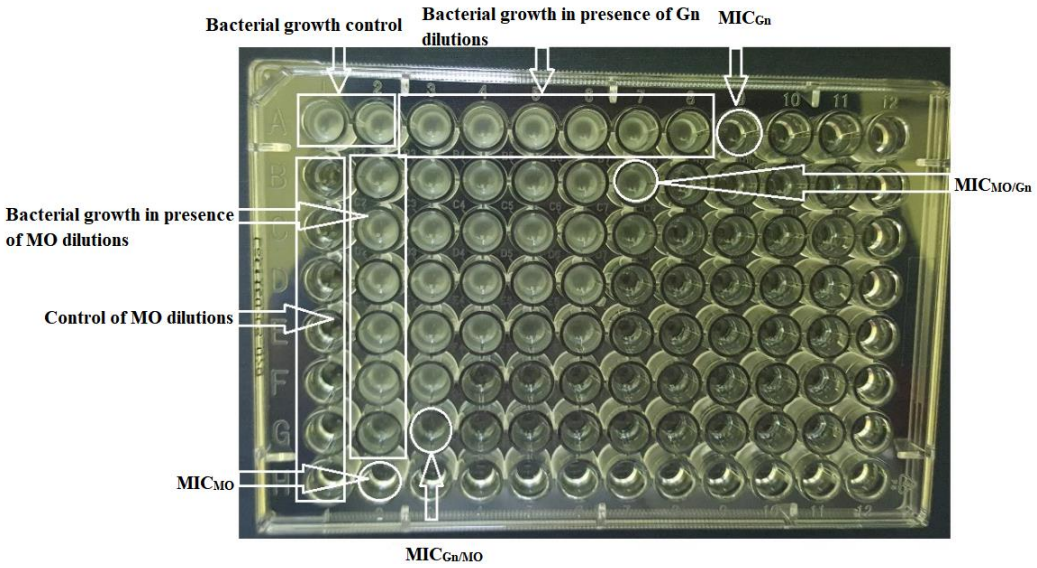


Figure 3. Experimental design of the chequerboard method with the exemplification of the results obtained for the combination MO - Gn against MRSA T4.

The experimental percentage of growth (fig. 4a) in the presence of different concentrations of MO and/or antibiotics and theoretical dose-response surface of interaction (fig. 4b) are represented and synergies have been confirmed through Bliss independence–based model interpretation.

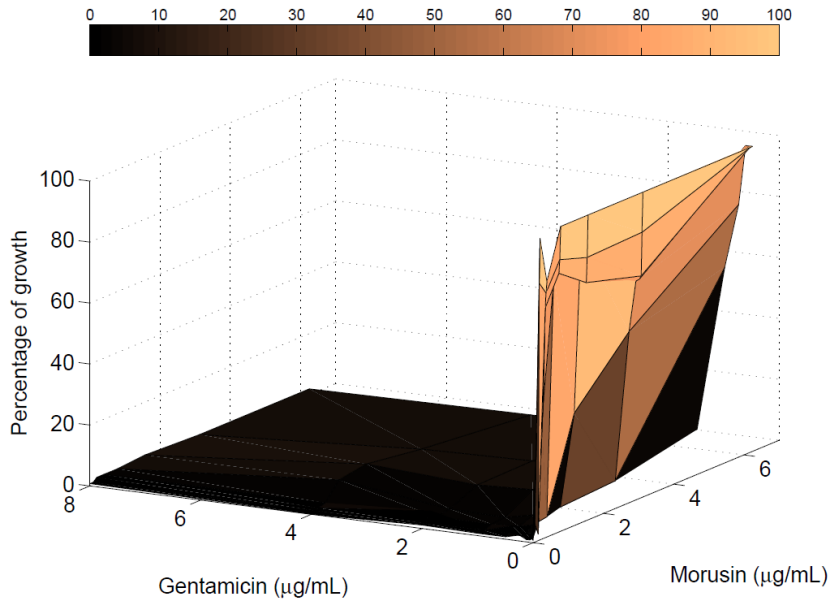


Figure 4a. Three-dimensional plot of the experimental percentage of growth ($E_{measured}$) between MO and Gn against MRSA T4.

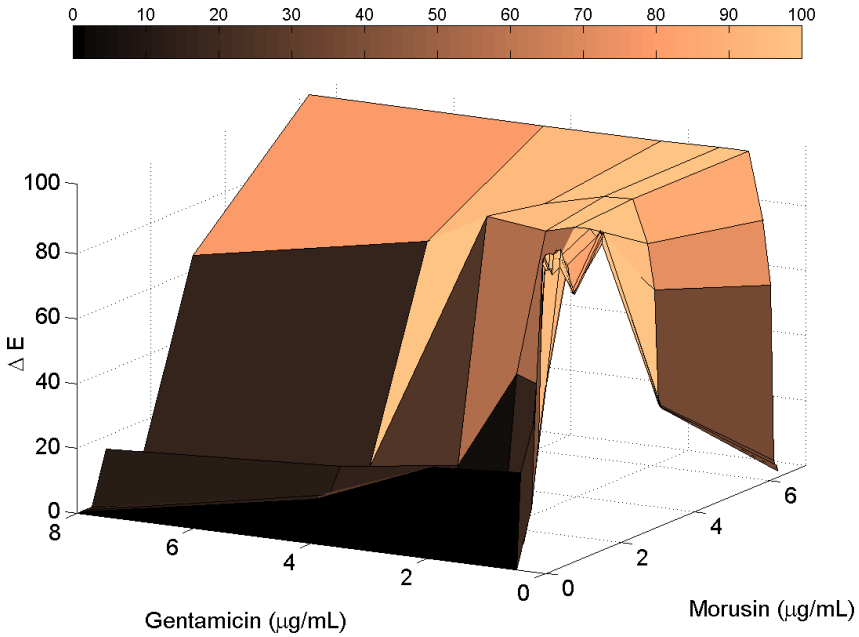


Figure 4b. Theoretical dose-response surface of interaction (ΔE) between MO and Gn against MRSA T4 (ΔE above zero (positive) indicates synergy).

Time-kill assay confirmed the synergy for the combinations MO – OX against MRSA T1 (fig. 5a) and MO – Amx against MRSA T2 (fig. 5b). The results obtained in the time-kill assay method for combinations MO – OX against MRSA T2-T4 and MO – Amx against MRSA T1, MRSA T3 and MRSA T4 were not fully in agreement with those observed when using the checkerboard method because the logarithmic reductions of the colony-forming units obtained for the combinations between MO and antibiotics were not $2\log_{10}$ lower than the logarithmic reductions obtained for the most potent/active component (MO) of the combinations. No increase in the viable colony count of more than $2\log_{10}$ CFU/mL compared to the viable count obtained with the most active single agent of combination (MO) was recorded and the antagonism was excluded for the combinations MO – OX and MO – Amx against MRSA strains.

Differences between the results obtained in the checkerboard method and time-kill assay have been also reported by other authors (12). These differences can be explained by the difference between the measured phenomena - checkerboard method assesses the inhibitory effect while time kill assay measures the bactericidal effect. The concordance between the results given by the two methods has been estimated as being 44-88% (17).

In our study, time-kill assay confirmed the synergy for the combination MO – Gn against all four clinical isolates: MRSA T1 (fig. 6a and fig. 7a), MRSA T2 (fig. 6b and fig. 7b), MRSA T3 (fig. 6c and fig. 7c) and MRSA T4 (fig. 6d and fig. 7d), because the logarithmic reductions of the colony-forming units obtained for the combination MO - Gn were $2\log_{10}$ lower than the logarithmic reductions obtained for the most potent/active component (Gn) of the combination.

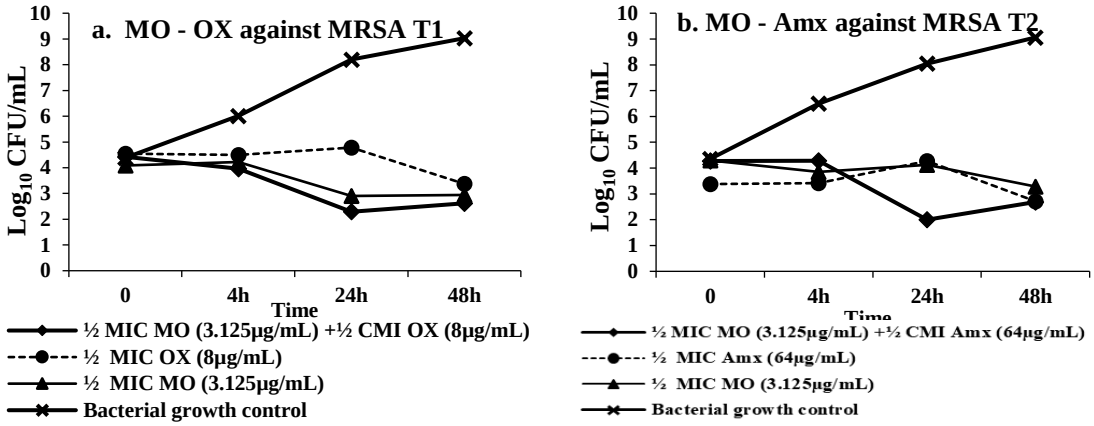


Figure 5. Time–kill curves for the combinations MO – OX against MRSA T1 (a) and MO – Amx against MRSA T2 (b).

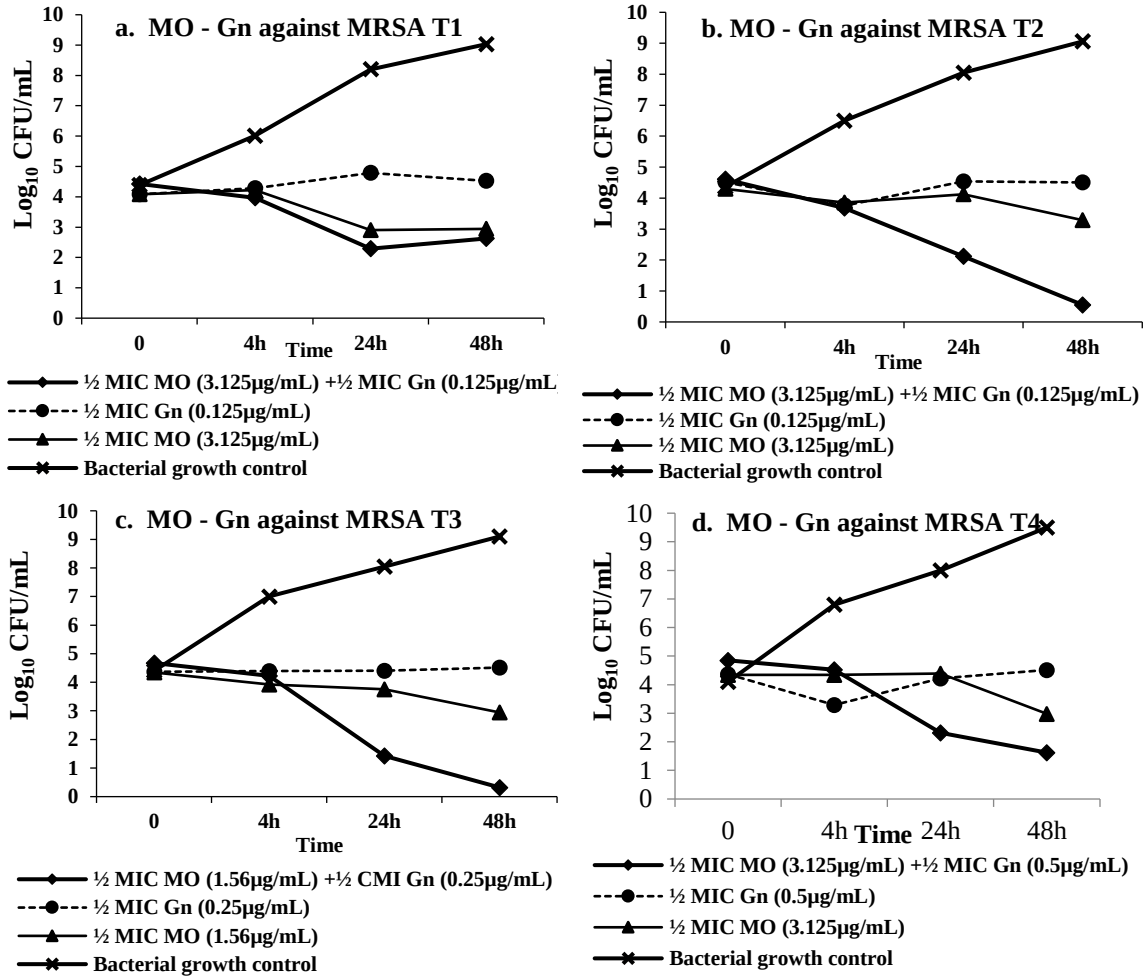
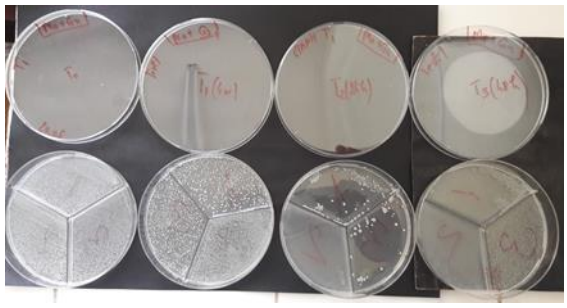
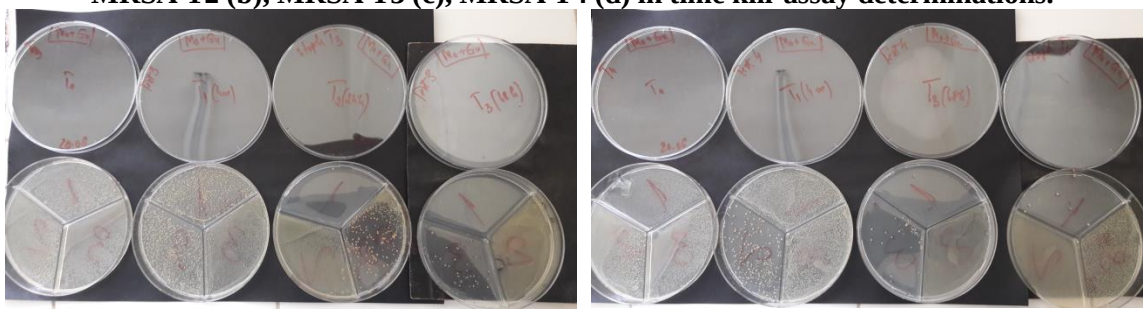


Figure 6. Time–kill curves for the combination MO – Gn against MRSA T1 (a), MRSA T2 (b), MRSA T1 (c) and MRSA T1 (d).



a. **b.**

Figure 7. Differences between MO – Gn (1), Gn (2) and MO (3) against MRSA T1 (a), MRSA T2 (b), MRSA T3 (c), MRSA T4 (d) in time kill-assay determinations.



c. **d.**

Figure 7. Differences between MO – Gn (1), Gn (2) and MO (3) against MRSA T1 (a), MRSA T2 (b), MRSA T3 (c), MRSA T4 (d) in time kill-assay determinations (*cont*.).

Conclusion

Our study reports on the antibacterial activity of morusin alone against four MRSA clinical isolates and its ability to act synergistically with antibiotics. As MRSA has become an increasingly global concern, synergy between phytochemicals and conventional antibiotics is a promising option to overcome antibiotic resistence. This preliminary study showed that morusin has the potential to reverse the bacterial resistence to oxacillin and amoxicillin of MRSA and increase the susceptibility of MRSA strains to gentamicin.

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Copper toxicosis with hemoglobinuric nephrosis in three adult sheep

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Abstract

Acute and, particularly, chronic copper exposures, along with defects in hepatic copper metabolism, altered excretion of copper, and/or nutritional imbalances between copper and other trace elements, can lead to hepatic accumulation of copper and primary copper toxicosis. There is interspecies variation in susceptibility to copper toxicosis, with sheep being the species most likely to develop this condition. The current report is rather unusual in that it describes instances of naturally occurring copper toxicosis with hemolysis and hemoglobinuric nephrosis in 3 adult sheep. In 2 of these sheep, a possible source of excessive dietary copper was investigated but not definitively identified. In the third goat, the etiologic factors associated with the copper toxicosis were not determined. It appears that mature sheep are susceptible to the hemolytic stage of chronic copper toxicosis, which was not observed in a recent, large-scale copper intoxication involving lactating dairy sheep (3, 5, 6, 12). Copper analyses on both kidney samples were necessary to confirm the diagnosis of copper toxicosis in all 3 sheep. All feedstuffs associated with instances of copper toxicosis should be analyzed for iron, molybdenum, sulphur, and zinc as well as copper to determine what nutritional factors are contributing to the pathogenesis of this disease. Consideration also should be given to the ingestion of hepatotoxic plants and other toxic exposures, which could predispose an animal to secondary chronic copper toxicosis (4, 7, 8, 11). It is thought that sheep are predisposed to chronic copper toxicosis because of their reduced biliary and urinary excretion of copper, the distribution of zinc- and copper-binding proteins in the liver, and the relatively small difference between the copper concentrations reported to be adequate for sheep rations (5–10 mg/kg, 7–11 mg/kg, or 10–20 mg/kg on a dry matter basis, depending on the reference) and those dietary copper concentrations considered to be potentially toxic (>15, 20, or 30 mg/kg on a dry matter basis). In contrast, cattle, horses, swine, and poultry tend to be more resistant to copper accumulation and chronic copper toxicosis, with maximum tolerable dry matter concentrations of dietary copper being approximately 50 mg/kg for cattle and horses, 250 mg/kg for swine, and 300–500 mg/kg for poultry. In a previous study, ponies were even reported to tolerate dietary copper concentrations approaching 800 mg/kg for 6 months. However, histopathologic examinations of the kidney were not apparently performed, and it is extremely important to recognize that copper bioavailability and dietary concentrations of molybdenum also play important roles in the pathogenesis of chronic copper toxicosis (9, 10, 13).

Key word: *copper, sheep, kidney*

Materials and methods

It was performed an 3 adult sheep post-mortem examination, following a sudden death. There were taken spleen samples for histopathological examination.

The samples preparation was carried out as follows: 24 h alcohol fixation at room temperature (prevent the tissue alteration due to the enzymes activity; preserve the tissue texture; improves the optical differentiation), alcohol dehydration (five steps: 70, 80, 90, 100% and 100% alcohol, each step for two hours), clearing with benzene, paraffin wax at 56°C, embedding tissues into paraffin blocks, trimming of paraffin blocks (6 μm), sections mounting on the glass slides (using Meyer albumin), hematoxylin - eosin- methylene blue staining.Staining was performed as follows: deparaffination of tissue sections in benzene, rehydration using decreasing concentrations of alcohol, rinsing in distilled water, hematoxylin staining, alcohol, eosin staining and methylene blue staining, water removal using increasing concentrations of alcohol, cover slide mounting. Hematoxylin will stain the nuclei in blue and the mucins in light blue. Eosin will stain the

cytoplasm in pink, collagenin pale pink, red blood cells in bright red, and colloid in red. Methylene blue improves the blue colour of the nuclei, making them more observable.The microscopical examination is useful as differentiating diagnosis method only if chemical preparation of samples is applied (1, 2).

Result and discution

Based on the history and clinical signs, as well as the gross necropsy and clinical pathology results, chronic copper toxicosis was suspected in the 3 goats. Diagnosis was corroborated by the observed histopathologic findings, all of which were consistent with the pathogenesis of chronic copper toxicosis as well as the absence of severe gastroenteritis, which would have suggested a larger and more acute copper exposure. Macoscopic on the renal surface observed black discoloration of the kidneys due to concentration of (met)hemoglobin. Massive acute hemolysis caused by chronic copper poisoning.

Microscopicaly observed proteinaceous material in tubular lumina resulting from hemoglobin filtration. Green-blue homogeneous globules (hyalindroplets) in tubularepithelial cells, are due to reabsorption anlysosomal accumulation of the filtered hemoglobin. Moreover, hydropidegeneration and necrosis of tubularcells.

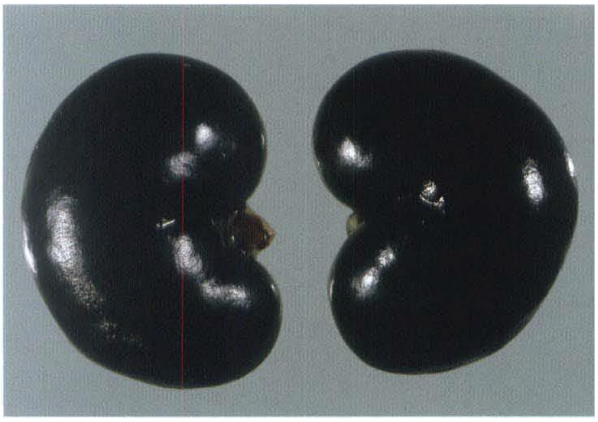

Fig 1. Hemoglobinuric nephrosis,. Massive acute hemolysis caused by chronic copper poisoning. Sheep.

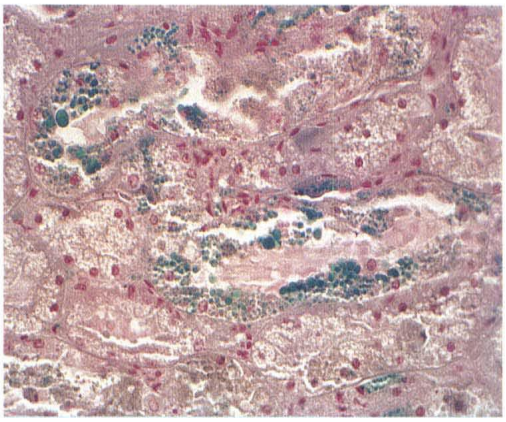

Fig 2. Renal cortex. Hemoglobinuria caused by chronic copper poisoning. Sheep. Fast blue stain for hemoglobin.

Conclusions

1. Chronic copper intoxication was diagnosed in 3 adult sheep based on the macroscopic examination, complete with histopathological examination.
2. Specific lesions were present in the kidneys.
3. Long-term exposure to Copper intoxication induces characteristic kidney damage.

Acknowledgements

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PRRS specific lesions differentiation, from other viral infectious etiology

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Abstract

PRRS syndrome, is an infectious disease found it in intensive rearing of pigs where is producing important economic losses. After 1990, the disease has spread all over the world. In Romania was diagnosed in 1998 by teams led by Dr. STĂNUICĂ and Dr. OLARU (3). The etiological agent is represented by a virus with two genotypes respectively type 1, European, and type 2, American, who have a degree of gene sequence similarity of 50-60% (6.). In Romania, the disease has an evolution characteristic for primary outbreaks affecting all categories of pigs, but also has an endemic evolution, wich is associated with some bacterial infectious diseases (3,4,5,). The aim of this study was to evidentiate some specific lesion for PRRS, and try to establish a differential diagnosis from other bacterial infectious diseases, with viral etiology.

***Key words**: associated disease, PRRS, necropsic exam, lungs, lymphnodes*

Introduction

In intensive swine growth, the pathology of infectious disease has changed significantly due to the emergence of pathological entities us, many of them specific to this growing system.

Depending on the unit who is affected by these entities, were grouped under complex or under syndromes, that include a dominant virosis associated with one or more bacteriosis. Bacterial Infectious diseases evolve as related infectious diseases because the dominant virosis induces immunosuppression, and the bacteries who are commensal on the respiratory and intestinal mucosa is multiplying and produce localized infection or septicemia. These associated diseases may mask both, the symptoms but also anathomopathological lession produced by the dominant virosis.

Together with the state of immunosupression, numerous intrinsic predisposing factors who belong to animals were involved, the most important being: age, breed and hybrid.

Age, is a major factor, in swine these is favoring a lot of infectious disease who are evoluating untill the age of weaning, or infectious disease who evolves after the age of weaning.

The extrinsix favoring factors, are represented by the growing technology, hygiene, nutrition, weaning crisis and stress of transport.

Materials and methods

The research was conducted on the bodies of young swine, the anatomopathological examination were efectuated in laboratory of Infectious Diseases and in the laboratory of Forensic Medicine. The bodies came from two pigs farms from Timis County.

A number of 168 bodies were necropsied, examined anatomopathological and by laboratory tests. The bodies were grouped by age into two groups as follows: group 1, consisting of 124 corpses piglets up to the age of 8 weeks and group 2 consists of 44 youth bodies swine after 8 weeks of age.

From organs with characteristics anathomopathological lesions were taken samples. The laboratory tests were performed: histological, bacteriological, polymerase chain reaction and immunofluorescence.

Samples for histology were represented by the lymph nodes and lungs. The samples were fixed in formalin, embedded in paraffin and stained with hematoxylin-eosin-methylene blue.

For bacteriological exam samples were collected from lung, primary sowings being made in broth and agar with 5% defibrinated sheep blood, and strains who was isolated were identified based on of cultural, dyeing and biochemical characteristics. Examination of samples was conducted in the Laboratory of Bacterial Infectious Diseases in the Department of Infectious Diseases.

Polymerase chain reaction was performed in order to detect the virus PRRS, the Mycoplasma hyopneumoniae and Brachispira hyodisenteriae. This reaction was performed in the Laboratory of Molecular Biology from Pasteur Institute SN Bucharest.

Results and discussions

Necropsy performed on the bodies from the two age categories, has provided conclusive data on the presence of specific lesions for PRRS syndrome and other bacterial infectious diseases associated with the syndrome.

On anatomopathological examination bodies, were found external injuries, represented by weakening, deshydration, congestion of the extremities and enlarged ingvinale lymph nodes.

The results of the anatomopathological examination were processed and given in tables, according to age categories studied.

At the piglets up to 8 weeks of age, were found macroscopic lesions characteristic of the syndrome PRRS, in varying proportions (table 1). The catarrhal and haemorrhagic lymphoreticulitis was present in 33.87% of the bodies examined and pulmonary lesions was represented by congestion were at a rate of 28.22% and interstitial pulmonary edema at a rate of 42.74%.

Microscopic lesions were represented by lymphocytic depletion, outbreaks of necrosis, blastic type lymphocytes and small cysts, located in the cortex.

Microscopic lesions characteristic for interstitial pneumonia were represented by thickening of alveolar walls due to infiltration by macrophages, lymphocytes and plasma cells, hyperplasia of type II pneumocytes and by the presence of necrotic cells in pulmonary alveoli.

At autopsied bodies were discovered and macroscopic lesions with lung and pleural localization, characteristic for enzootic pneumonia and pasteurellosis. A relatively high frequency had fibrinous polyserositis, which was present in 25% of autopsied bodies.

At the digestive tract were present hemorrhagic gastritis (28,22%) and hemorrhagic enterocolitis (57,25%), anatomopathological lesion that is dominant in this category.

Through laboratory tests, who were effectuated, were confirmed next associated disease: enzootic pneumonia, pasteurellosis, Glasser disease and dysentery with *Brachispira hyodisenteriae.*

Table 1. The frequency of pathological lesions in the bodies of piglets up to 8 weeks

Nr. Crt.	Lesion	Nr. Corpses	%
1	Pulmonary congestion	35/124	28,22
2	Interstitial pulmonary edema	53/124	42,74
3	Catarrhal bronchopneumonia	29/124	23,38
4	Fibrinous bronchopneumonia	35/124	28,22
5	Fibrinous hemorrhagic bronchopneumonia	18/124	14,51
6	fibrinous pleuritis	27/124	21,77
7	Pericarditis	13/124	10,48
8	Lymphoreticulitis	42/124	33,87
9	Fibrinous polyserositis	31/124	25

10	Renal dystrophy	36/124	29,03
11	Haemorrhagic enterocolitis	71/124	57,25
12	Haemorrhagic gastritis	35/124	28,22
13	Myocardosis	17/124	13,70

In young swine over 8 weeks of age necropsy examination revealed gross pathological lesions characteristic of the syndrome PRRS and gross pathological lesions characteristic for other associated bacterial infectious diseases, in varying proportions (table 2).

Catarrhal and haemorrhagic lymphoreticulitis was present in 55.1% of corpses, the most affected being ingvinale lymph nodes. These were increased in volume, and on the section were bleeding or marbled. Histological examination revealed in lymph nodes and lungs, the same microscopic lesions.

Macroscopic lung lesions, characteristic of this syndrome was represented by pulmonary congestion (34.09%) and interstitial pulmonary edema (18.8%).

On lungs, pleura and pericardium were present inflammatory lesion like fibrinous in relatively large proportions. At this age category, being present and hemorrhagic pleuropneumonia, caused by A. pleuropneumoniae.

Fibrinous polyserositis were present in a smaller proportion (20.45%) compared with its frequency in age structure presented above.

The dominant anatomopathological lesion at this age structure, was still haemorrhagic enterocolitis (52,27%), accompanied by the hemorrhagic gastritis with a rate of 43,18%.

Laboratory tests have confirmed the following related diseases: enzootic pneumonia, pasteurellosis, hemorrhagic pleuropneumonia, Glasser disease and dysentery with *Brachispira hyodisenteriae.*

Macroscopic and microscopic lesions in the lungs and lymph nodes detected were similar with the lesions reported by other authors in herds where PRRS syndrome evolves both as primary and as evolving disease endemic (1).

Bacterial infectious diseases associated with this syndrome evolves both, in primary outbreaks and in endemic evolution, being produced by commensal bacteria from respiratory or digestive mucosa and are reported frequently and by other researchers as well (1, 2,5).

Table 2. The frequency of pathological lesions in the bodies of piglets over 8 weeks

Nr. Crt.	**Lesion**	**Nr. Corpses**	**%**
1	Pulmonary congestion	15/44	34,09
2	Interstitial pulmonary edema	8/44	18,8
3	Catarrhal bronchopneumonia	6/44	13,63
4	Fibrinous bronchopneumonia	15/44	34,09
5	Fibrinous hemorrhagic bronchopneumonia	9/44	20,45
6	fibrinous pleuritis	16/44	36,36
7	Pericarditis	7/44	15,90
8	Lymphoreticulitis	19/44	43,18
9	Fibrinous polyserositis	9/44	20,45
10	Renal dystrophy	11/44	25
11	Haemorrhagic enterocolitis	23/44	52,27
12	Haemorrhagic gastritis	19/44	43,18
13	Myocardosis	8/44	18,18

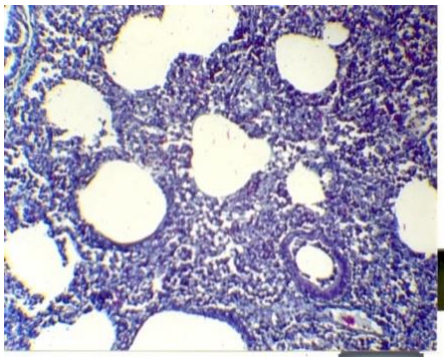

Fig. 1. Thickening of the alveolar septa with lymphocytic infiltration, with epithelial hyperplasia bronchioles

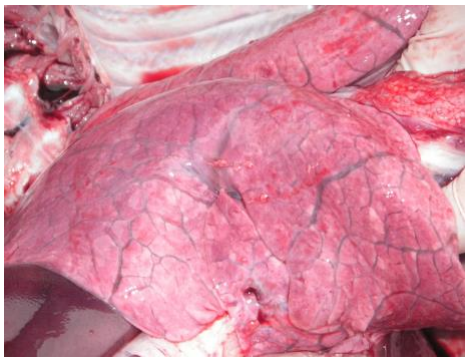

Fig. 2 Interstitial pneumonia

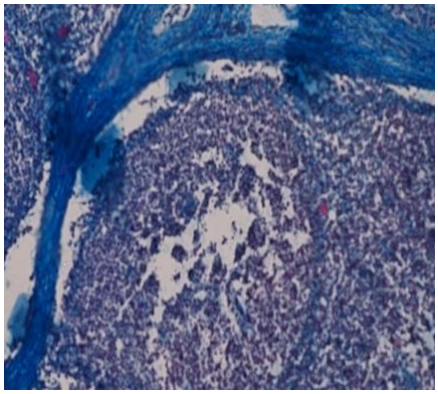

Fig.3 Lymphonoditis –microscopic aspect

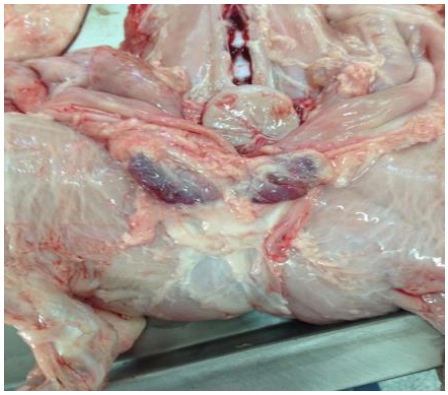

Fig. 4 Lymphonoditis –macroscopic aspect

Conclusions

Necropsy performed on the bodies of the two groups revealed gross pathological lesions characteristic both PRRS syndrome and associated bacterial infectious diseases .

The lesion characteric for PRRS was represented by enlarged ingvinale lymph nodes, and lymphonoditis, lesion that were not characteristic for other bacterial infections.

At necropsied corpses from two farms pigs , PRRS syndrome was confirmed in pigs in the two age groups, and some infectious diseases associated with falling in complex respiratory and digestive complex.

Acknowledgements

This research work was carried out with the support of the project *Dezvoltarea infrastructurii de cercetare, educaţie şi servicii în domeniile medicinei veterinare şi tehnologiilor inovative pentru RO 05, cod SMIS-CSNR 2669. R*

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Molecular studies on *Pasteurella species* isolated from ducks

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Abstract

Duck cholera is a fatal, contagious and septicemic disease of ducks caused by Pasteurella species. A total of 150 ducks were collected from ten farms in Kaliobia Governorate suspected to be suffering from Pasteurellosis that manifested by respiratory signs, sudden death, and nervous manifestation. Collected Samples from these ducks were liver, spleen, heart and lung which subjected for bacteriological examination. A total of 33 Pasteurella strains were isolated, 25 strain were Pasteurella multocida (recovered from liver samples) and 8 strain were Pasteurella pneumotropica (5 strains recovered from lung and 3 strain recovered from heart). Finding of antibiotic sensitivity test showed that Pastreulla isolates were sensitive to florofinicole (80%) and moderately sensitive to ciprofloxacine (60%), enrofloxacin (50%) and followed by tobramycin (40%). Amoxicillin, oxytetracycline and penicillin were less sensitive (30% each) while isolates showed absolute resistance to erythromycin (100%) followed by resistance to gentamycin (90%) and naldixic acid (80%) for both types of Pasteurella. PCR results showed that Cytotoxic protein (toxA) toxcin virulence gene was detected in 4 out of 10 studied strains and fimbrial protein (ptfA) virulence gene was detected in 4 out of 10 studied strains. Sequences of toxA and ptfA genes were submitted to Gen Bank and assigned accession numbers were MF167359 and MF382009, respectively.

Key words: *Pasteurella multocida- Pasteurella pneumotropica- toxA- ptfA -antibiotic sensitivity test- PCR- ducks.*

Introduction

Pasteurella multocida belonging to family *Pasteurellaceae* is a ubiquitous organism affecting many host species, thus causing several diseases like haemorrhagic septicaemia in cattle and buffalo, enzootic bronchopneumonia in cattle, sheep and goats, atrophic rhinitis in swine, fowl cholera in poultry and snuffles in rabbits (Harper *et al.*, 2006 and *Dziva et al.*, 2008). *P. multocida* is identified as a major threat for a poultry industry which hampers the profitable poultry production (Sellyei *et al.*, 2010). Clinically ducks associated with pasterullosis showed anorexia, fever, ruffled feathers, depression, mucus discharge from mouth and nostrils, increase respiratory rate and diarrhea. On postmortem examination: Petechial and ecchymotic hemorrhages were common, particularly in subepicardial (heart) and subserosal (liver) locations, hemorrhages on the coronary band of heart, hemorrhages on air sac membranes adjacent to lungs were evident. The liver was swollen accompanied with multiple, small, necrotic foci (Mohan and Pradeep Kumar, 2008).

Based on capsular antigens, *P. multocida* strains are differentiated into five serogroups. Type A causing fowl cholera pathogen and bovine shipping fever, type B causing hemorrhagic fever in ungulates, type D causing atrophic rhinitis in swine, type E, an African serotype, infecting cattle and buffalo; and type F also causing fowl cholera (Carter, 1955 and Rimler *et al.*, 1987). Ewers *et al.* (2006) studied the virulence profiling of *P. multocida* isolates from different hosts and subsequently it has been used by many authors to understand the diversity of the pathogen recovered from different host origin (Bethe *et al.*, 2009; Tang *et al.*, 2009; Garc´ıa *et al.*, 2011; Ferreira *et al.*, 2012; Furianetal, 2013; Katsuda *et al.*, 2013 and Verma *et al.*, 2013).

Important pathogen factors include capsular and other virulence-associated genes (Katsuda *et al.*, 2013). These virulence factors (VFs) and outer membrane proteins are important for pathogenesis, functionality, protective immunity and vaccine development against *P. multocida* infections (Hatfaludi *et al.*, 2010). The main virulence factors of *Pasteurella was* Endotoxins (lipopolysaccharides, LPS) are particularly important in the septicaemic diseases such as fowl

cholera and bovine haemorrhagic septicaemia. *Pasteurella multocida* serotyes A and D can produce a cytotoxic protein named *P. multocida* toxin (PMT), which stimulates cellular cytoskeletal rearrangements and growth of fibroblasts. Interestingly, a virulent PMT-positive strain and virulent PMT-negative strain have both been reported. However, PMT plays a role in atrophic rhinitis (mild to severe destruction of porcine nasal turbinate bones) and Filamentous hemagglutinins ($PfhB_1$ and $PfhB_2$), surface fibrils (Hsf_1 and Hfs_2), and fimbrial subunits (PtfA, FimA, Flp_1, Flp_2) are adhered to host cells, chemotaxis (Dashe *et al.*, 2015), the ptfA gene of which assemble to form type 4 fimbriae on the bacterial surface (Sellyei *et al.*, 2010).

P. pneumotropica is type of *pasteurella* that its main carriers are rat and mice but the clinical signs are seen if infected animals are stressed, nude mice may developed retrobulbar abcesses in lacrimal gland .*P. pneumotropica* has been associated with conjunctivitis, rhinitis, otitis and cervical lymphadenitis in mice and rat (Baker, 2003).

Aim of the work

Our objective from this study to investigate *Pasteurella* species that isolated from ducks in Egypt and determine the most sensitive antibiotic effective for these strains and throw spot light on the role of the duck in disease transmission as research papers reported. The disease in ducks is sporadic and scarce although *Pasteurella* species is one of important fatal infection in ducks.

Material and methods

Sample collection:

2.1. Samples collection

A total of 150 ducks of different ages and sexes were examined from 10 different duck farms at Kaliobia Governorate for bacteriological examination. Samples were taken from freshly dead ones (liver, heart blood, lung, kidney and spleen from each duck) from suspected clinically affected cases. Each examined organ was taken alone in sterile plastic bag, kept in icebox and transferred with minimum delay to the laboratory for bacteriological examination.

2.2. Phenotypic identification and genotypic determination of virulence factors of *Pasteurella* species:

The surface of organs was seared by hot spatula, and then a sterilized loopfuls were inoculated onto tryptone soya broth and incubated aerobically at 37ºC for 24 hours. A loopful from incubated tryptone soya broth was streaked onto sheep blood agar, baird parker agar with 1ml of 0.1% of crystal violet as *Pasteurella* has ability to grow in presence of 0.1 % crystal violet and egg yolk tollurite (Das, 1958 and Melody *et al.*, 1994); Mac Conkey's agar; (All plates were incubated for 24 hours at 37ºC. The developed colonies were picked up and subculture for purification. The purified colonies were morphologically identified by Gram stain and Leishman's staining technique and biochemical tests (Carter, 1984 and Markey *et al.*, 2013).

2.3. In-Vitro anti-microbial sensitivity test:

The isolated *Pasteurella species* strains were subjected to the sensitivity test against different antibiotics, using the disc and agar diffusion method (Finegold and Martin, 1982) for their susceptibility against 10 anti microbial agents representing classes of different antimicrobial agents (ciprofloxacin, gentamycin, tobramycin, amoxicillin, erythromycin, enrofloxacin, oxytetracycline, penicillin, naldixic acid and florofinicol)

2.4. Detection of toxA and ptfA genes of Pasteurella multocida and pneumotropica by PCR:

PCR was applied on 10 selected *Pastereulla* isolates by using two sets of primers for detection of two virulence genes Cytotoxic protein (toxA) and fimbrial protein (ptfA) that may play a role in virulence of *Pasteurella spp.*

Polymerase chain reaction

DNA extraction: DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at $56^{O}C$ for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer: Primers used were supplied from Metabion (Germany) and listed in Table (1).

PCR amplification: Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmolconcentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Appliedbiosystem 2720 thermal cycler.

Analysis of the PCR Products:

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A gelpilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) and generuler 100 bp ladder (Fermentas, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1): Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extn.	Ref.
				Secondary denaturation	Annealing	Extension		
toxA	CTTAGATGAGCGACAAGG GAATGCCACACCTCTATAG	864	94˚C/ 5 min.	94˚C 30 sec.	48˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.	**16**
ptfA	TGTGGAATTCAGCATTTTAGTGTGTC TCATGAATTCTTATGCGCAAAATCCTGCTGG	488	94˚C/ 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	

Sequencing protocol: By Dye termination method (Sanger *et al.,* 1977).

Steps of sequence analysis*:*

1- The received sequence was imported into alignment window with the downloaded highly similar sequences into BIOEDIT version 7.0.4.1 software.

2- Multiple sequence alignment was conducted using ClustalW application embedded in ***BIOEDIT version 7.0.4.1 software.***

3- Sequence editing, correction, frame adjustment, Amino acid alignment and allocation of antigenic sites were also conducted using different options of BIOEDIT version 7.0.4.1 software.

5- All finely adjusted sequences were exported from BIOEDIT version 7.0.4.1 software as separate FASTA files.

6- FASTA files were inserted into MEGA 5.05 DNA alignment tool and exported into MEGA format (*.meg).

7- MEGA file was used as a base for phylogenetic analysis using neighbor joining method.

8- One handered bootstrap replicates were conducted to assess the statistical support for the tree topology.

9- The resultant trees were saved as photos.

10- Sequence submission was conducted following the instructions offered by the web tool Bankit of GenBank http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank with the following numbers: bankit2012800 seq for TOXA and bankit2026599 for PTF gen of pasteurella multocida,

11- Sequence accession number was received 2 working days from date of submission.

Results and discussion

Clinical cases

The most common observed clinical signs showed by affected ducks were in the form of sudden death, greenish diarrhea, nervous manifestation, locomotory disturbance, depression and mucus discharge from mouth and nostrils. The most observed post mortem lesions were swollen liver with petechial hemorrhages and hemorrhages on heart. Similar clinical signs and postmortem picture were reported in ducks associated with pasterullosis by (Mohan and Pradeep Kumar, 2008).

Isolation and identification: A total of 33 isolates from 150 suspected birds collected from 10 farms (liver; heart blood; lung; kidney and spleen) were identified as *Pasteurella species* on the basis of the conventional bacteriological technique, from 33 isolates *Pasteurella multocida* represent the highest isolation of 76% (25/33) while the *Pasteurella pnemotropica* represent 24% (8/33). Isolated bacterial colonies on blood agar plates were small, glistening, mucoid and dew drop like, and appeared as Gram-negative coccobacilli when stained with Gram's stain and Leishman's staining technique revealed bipolar microrganisms. The isolates failed to grow on MacConkey agar and were found to be non-haemolytic on blood agar. These features were in agreement with previous researches (Akhtar, 2013 and Ievy *et al.*, 2013). Details of cultivation and biochemical tests were showed in Table (2). Similar findings were confirmatory with the findings of Belal (2013).

Table (2): Cultivation and biochemical tests for isolates

Feature	*p.multocida*	*p.pnemotropica*
Macckoncy agar	-ve	-ve
Haemolysis on blood agar	No	No
Catalase test	+ve	+ve
Indole test	+ve	+ve
Oxidase test	+ve	+ve
Urea hydrolysis	-ve	+ve
Growth on TSI	Yellow	Yellow
V.P test	-ve	-ve
Simmon citrate	-ve	-ve
Lysin decarboxylase	-ve	-ve

In the present study *P. multocida* were isolated from ducks by total percent of 22%, (33/150), these result were nearly to that reported by Sayedun *et al.* (2015) and Kumar *et al.* (2004) who isolated *P. multocida* with percentage of 11.42 , 34% and less than Kamruzzaman *et al.* (2016) who isolated *P. multocida* with percentage of 59.72%, respectively . Detection of *P. multocida* infection in ducks indicates its transmitting through nearly established poultry farms as reported by (Botzler, 1991). The present finding of *P. pneumotropica* infection in ducks is the first report in Egypt, *P. pneumotropica* was currently isolated from rat or guinea pig bite wound (Anne-Lise *et al.*, 2005), its occurrence in ducks indicate the role of rodent as reservoir for transmission of the disease to other susceptible flocks.

Antibiotic sensitivity test:

Our findings of antibiotic sensitivity for twenty *Pasteurella* isolates by disc diffusion method revealed that all isolates exhibited variable response to different antibiotics as shown in

Table (3). *Pasteurella* isolates were sensitive to florofinicol (80%) and were moderately sensitive to ciprofloxacin (60%) followed by enrofloxacin (50%), then tobramycin (40%). Amoxicillin, oxytetracyclin and penicillin were (30%) per each, then naldixic acid was 20%, gentamycin was 10%. Whereas, *Pasteurella* isolates exhibited absolute resistance to erythromycin (100%). The obtained results were not in accordance with (Kamruzzaman *et al.*, 2016) who detected that ciprofloxacin was the most effective antibiotic by 95% followed by gentamycin (85%), tetracycline and amoxicillin (75% per each). Also our finding results differed from that obtained by Dashe *et al.* (2015) who showed that ciprofloxacin, streptomycin and gentamycin were highly effective against *P. multocida*. On the other hand, Maity *et al.* (2012) reported that *P. multocida* was sensitive to amoxiclav, chloramphenicol, and moderately sensitive to amikacin, cefotaxime, neomycin and norfloxacin but resistant to ciprofloxacin and lomefloxacin. The variation in the sensitivity grade among various studies may be due to over or limited previous exposure and indiscriminate use of antibiotics as feed additives and/or preventive or curative agents.

Table (3): antibiotic sensitivity for twenty *Pasteurella* isolates by disc diffusion method:

Sensitivity / Antibiotics agent	sensitive	intermediate	Resistance	No. of isolates	Sensitivity (%)
Ciprofloxacin(10µg)	12	-	8	12/20	60%
Gentamycin(10µg)	2	-	18	2/20	10%
Tobramycin	8	-	12	8/20	40%
Amoxicillin(20µg)	6	-	14	6/20	30%
Erythromycin(10µg)	-	-	20	0/20	0.0%
Enrofloxacin(10µg)	10	3	7	10/20	50%
Oxytetracyclin(10µg)	6	-	14	6/20	30%
Pencillin	6	2	12	6/20	30%
Naldixic acid	4	-	16	4/20	20%
Florofinicol(30µg)	16	-	4	10/20	80%

Results of PCR:

In our study two virulence genes were detected by PCR test, *tox*A and *ptf*A genes by 40%, (4 out of 10 samples per each) (Table 4), the obtained results are similar to that obtained by Thales *et al.* (2016) who detected of *ptf*A, *tox*A and other genes in *Pasteurella* isolates.

Sample	Results	
	toxA	***ptfA***
1	-	-
2	+	+
3	+	+
4	-	-
5	-	-
6	+	+
7	-	-
8	+	+
9	-	-
10	-	-

Amplification of ptfA and toxA genes in pasteurella isolates:

The obtained results revealed that *ptfA* gene was detected in four out of 10 *Pasteurella* examined isolates and gave a characteristic band at 488bp (Fig. 1) whereas tox*A* gene was detected

only in 4 isolates out of 10 examined ones and gave positive amplification at 864bp as shown in (Fig. 2).

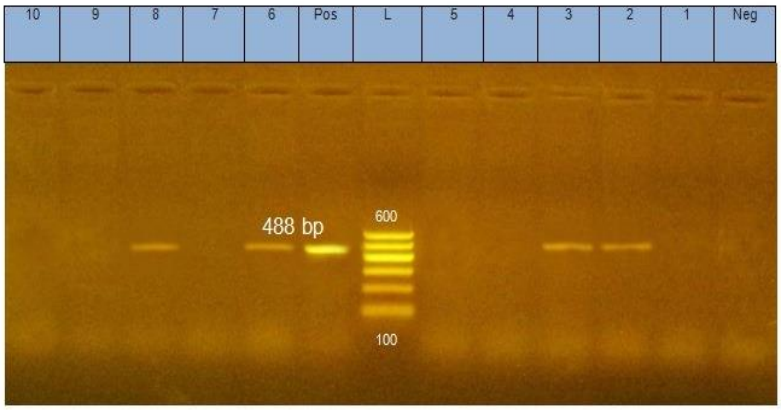


Fig. 1: Agarose gel electrophoresis of *ptf*A gene gene in 10 *Pasteurella* isolates, M: 100 bp DNA marker, lanes (2, 3, 6 and 8): positive amplification of *ptf*Agene at 488 bp, Positive control: standered strain from AHRI Dokki, Negative control.

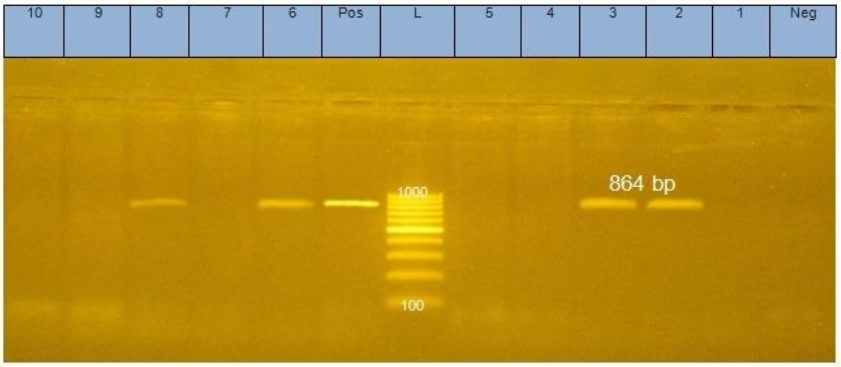
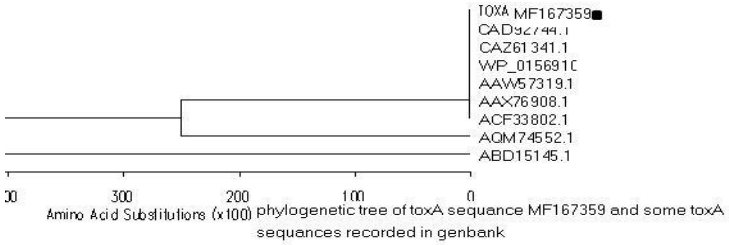


Fig. 2: Agarose gel electrophoresis of *tox* A gene in 10 *Pasteurella* isolates, M: 100 bp DNA marker, lanes (2, 3, 6 and 8): positive amplification of *tox*A gene at 864bp, Positive control: standered strain from AHRI Dokki, Negative control.

Nucleotide sequence accession number

Partial gene sequence of toxA and ptfA of *Pasteurella multocida* isolate was submitted to Gen Bank and assigned accession number were MF167359 and MF382009, respectively.

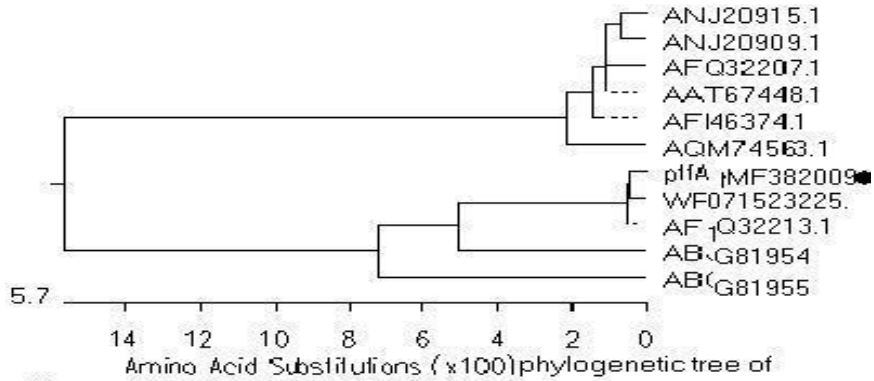


Percent Identity

	1	2	3	4	5	6	7	8	9	
1		98.9	98.9	98.9	98.9	98.9	98.6	3.5	20.0	1
2	0.0		100.0	100.0	100.0	100.0	99.6	4.3	5.0	2
3	0.0	0.0		100.0	100.0	100.0	99.6	4.3	5.0	3
4	0.0	0.0	0.0		100.0	100.0	99.6	4.3	5.0	4
5	0.0	0.0	0.0	0.0		100.0	99.6	4.3	5.0	5
6	0.0	0.0	0.0	0.0	0.0		99.6	4.3	5.0	6
7	0.4	0.4	0.4	0.4	0.4	0.4		4.3	5.0	7
8	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0		5.0	8
9	195.0	195.0	195.0	195.0	195.0	195.0	195.0	195.0		9
	1	2	3	4	5	6	7	8	9	

TOXA MF167359
CAD92744.1
AAX76908.1
CAZ61341.1
AAW57319.1
WP_015691094.1
ACF33802.1
ABD15145.1
AQM74552.1

Precent identefy of pasteurella toxA MF167359 and some strains in gene bank



phylogenetic tree of ptfA accesion number MF382009 of our study and someot other accesion numbers recorded in genbank

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	
1		100.0	99.3	91.0	91.0	79.9	79.2	78.5	78.5	77.8	78.5	1
2	0.0		99.3	91.0	91.0	79.9	79.2	78.5	78.5	77.8	78.5	2
3	0.7	0.7		90.3	90.3	79.2	78.5	77.8	77.8	77.1	77.8	3
4	9.6	9.6	10.4		83.3	73.6	73.6	72.9	73.6	72.9	72.9	4
5	9.6	9.6	10.4	18.9		73.6	72.9	72.2	72.2	71.5	72.2	5
6	23.5	23.5	24.5	32.5	32.5		97.2	96.5	96.5	95.8	96.5	6
7	24.5	24.5	25.4	32.5	33.6	2.8		99.3	99.3	98.6	99.3	7
8	25.4	25.4	26.4	33.6	34.7	3.6	0.7		98.6	97.9	98.6	8
9	25.4	25.4	26.4	32.5	34.7	3.6	0.7	1.4		97.9	98.6	9
10	26.4	26.4	27.4	33.6	35.8	4.3	1.4	2.1	2.1		97.9	10
11	25.4	25.4	26.4	33.6	34.7	3.6	0.7	1.4	1.4	2.1		11
	1	2	3	4	5	6	7	8	9	10	11	

MF382009
ptfA
AFQ32213.1
WP_071523225.1
ABG81955.1
ABG81954.1
AQM74563.1
AAT67448.1
ANJ20915.1
AFI46374.1
AFQ32207.1
ANJ20909.1

Precent identfy between pasteurella ptfAgene (MF382009) and some strains in gene bank

Phylogenetic analysis and nucleotide comparison

The nucleotides sequences of toxA gene and ptfA gene showed percent identity with.1, EGP03065.1) which Submitted (22-JUN-2011). The obtained genetic the selected sequences published on gene bank ranged from 98%-100%. Most of the aligned sequences were isolated from chicken as AQM74552.1, which Submitted (27-AUG-2016) and AFQ32207.1which Submitted

(05-JUN-2012) while others were isolated from wild birds as Anand1_poultry （EGP02957data indicated that application of strategies to control the access of wild birds to duck farms where they act as reservoir for the pasterullosis also the data revealed cross infection between ducks and chicken which give great attention to avoid multi species breading.

Conclusion

We concluded from the present study pay attention of scientist to pasterullosis in ducks as the disease cause deaths in duck flocks and subsequently economic loss. *P. pneumotropica* was firstly isolated from duck in Egypt. Florofinicol is the drug of choice for treatment of *Pasteurella* in ducks.

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A variant of the direct immunofluorescence technique used in the routine diagnosis of PRRS syndrome

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Abstract

Laboratory diagnosis of PRRS syndrome is based on virus detection, isolated strain characterization and antibody detection. Given the severity of the disease, rapid diagnostic methods are used to detect the nucleocapsid viral antigen present in the target organs (lymph nodes, lungs). From swine youth corpses from disease outbreaks, inguinal lymph nodes were taken, and from swine youth with characteristic respiratory symptoms, samples of oronasal fluid were taken. The nucleocapsid viral antigen was detected using the anti PRRSV monoclonal antibody kit labeled with fluorescein isothiocyanate (BIO 268). The smears made of lymph nodes and oronasal fluid to which they were identified, in the microscopic field, the described aspects were considered positive. Thus, 26 samples of lymph nodes (65%) and 9 oronasal fluid samples (45%) were positive, which were controlled to confirm PRRS virus presence by RT-PCR technique. All positive samples of lymph nodes and oronasal fluid positive to the IFD technique in the adapted working variant were confirmed as positive samples by the RT-PCR technique.

Key words: *PRRS, lymphnode, IFD, RT-PCR*

Introduction

Porcine Respiratory and Reproductive Syndrome (PRRS) was diagnosed in Romania in 1998 and is currently being spread in many swine farms (4).

The disease is produced by a RNA virus encompassed in family Arteriviridae, having two genotypes, respectively, type 1 (European) and type 2 (American). There are significant differences between these genotypes, represented by the variability of the gene sequences (5,6).

Laboratory diagnosis of PRRS syndrome is based on virus detection, isolated strain characterization and antibody detection. Given the severity of the disease, rapid diagnostic methods are used to detect the nucleocapsid viral antigen present in the target organs (lymph nodes, lungs) (1,4,5).

Since the immunofluorescence reaction performed on cryosections involves a complex endowment of the diagnostic laboratories, the research sought to develop a direct rapid technique for viral antigen detection, fingerprinting, lymph nodes and oronasal fluid.

Materials and methods

From swine youth corpses from disease outbreaks, inguinal lymph nodes were taken, and from swine youth with characteristic respiratory symptoms, samples of oronasal fluid were taken.

The nucleocapsid viral antigen was detected using the anti PRRSV monoclonal antibody kit labeled with fluorescein isothiocyanate (BIO 268).

The used variant of the direct immunofluorescence reaction had the following steps depending on the pathological material used:

-the removal of glass blades with ethyl alcohol;

- calibration of samples from lymph nodes, on blades by fingerprint;

- centrifuging oronasal fluid samples and showing the sediment on the blades;

- blade welding and fixing in acetone;
- washing the blades with PBS-Blue Evans solution and drying the blades;
- addtion of 0.1 microgram conjugated to fluorescein;
- examination of the fluorescent lightwith Optika microscope.

The confirmation of the obtained results was performed by the RT-PCR Operational Standard Procedure, the Real Time version, used in the Laboratory of Molecular Biology of the Pasteur SA Institute of Bucharest. For this purpose, four extraction kits (Qiagen and Roche, Germany) and two ORF 7-specific primers and the following primers were used.

- Primer PRRS -2 ORF 7: 5 '- GCG AAT CAG GCGCAC WGT ATG-3';
- Primer PRRS-4 ORF 7: 5 '- AGA AAA GTA CAG CTC CGA TGG - 3';

A number of 40 samples of lymph nodes and a number of 20 oronasal fluid samples were examined by this technique.

Results and discussion

Inguinal lymph nodes were taken from fresh, suing youth corpses from farms where PRRS syndrome has evolved as a primary disease. The lymph nodes were increased in volume, with firm consistency, and on the sectional area their color was red due to haemorrhagic inflammation.

Oronasal fluid samples were collected from suing youth where PRRS syndrome clinically evolved in acute form.

A modified version of the immunofluorescence technique performed on cryosections was used in the research to be used as a method of diagnosing of PRRS syndrome because the cryosection technique requires adequate endowment.

On slides with ethyl alcohol, after drying, fingerprints were made on the lymphocyte section of lesions. Oronasal fluid samples were centrifuged and the sediment was uniformly exposed on the glass flaps. After drying, the smears made from the two types of pathogenic material samples were fixed in acetone solution for 15 minutes and then dried for 2 hours at room temperature. In the next step, the lamellae were rinsed with a mixture of saline phosphate buffer solution with Evans Blue and subsequently dried again. In the final step, the smears thus prepared were coated with the fluorescent conjugate, dried and covered with lamellae, and subsequently examined under a UV (20x and 40x) ultraviolet light microscope.

Microscopic lymph nodes have been screened for isolated cells, clustered cells, or large clusters of small, medium, large, plasma, and rare epithelial cell lymphocytes. In lymphocytes, the cellular contour was evident, the nuclei were well individualized, and the cytoplasm was bright fluorescent bright greenish appearance due to the presence of viral nucleocapsid antigens coupled to fluorescein-labeled monoclonal antibodies. Epithelial cells were rare, and the cytoplasmic fluorescence was very obvious.

In the microscopic field, smears of cells, predominantly epithelial with high fluorescence cytoplasm, were detected in oronasal fluid smears.

The smears made of lymph nodes and oronasal fluid to which they were identified, in the microscopic field, the described aspects were considered positive. Thus, 26 samples of lymph nodes (65%) and 9 oronasal fluid samples (45%) were positive, which were controlled to confirm PRRS virus presence by RT-PCR technique.

All positive samples of lymph nodes and oronasal fluid positive to the IFD technique in the adapted working variant were confirmed as positive samples by the RT-PCR technique.

The direct immunofluorescence reaction has been used so far only for the detection of the nucleocapsid antigen of the PRRS virus in cryosections performed from lymph nodes, pulmonary and other lymphoid organs. Our own research has been aimed at developing a simplified method as a routine routine method in the PRRS diagnosis (2).

The results obtained confirm that the IFD technique in the presented variant can be adapted, but more research is required to establish with certainly the degree of sensitivity and specificity of this method.

Conclusions

- The IFD variant used allowed lymphocyte and oronasal fluid smears to be detected with fluorescein-labeled monoclonal antibodies to detect different types of PRRS-infected cells.
- The IFD response following the described methodology detected the presence of viral antigens at 65% of the examined lymph nodes and oronasal fluid samples.
- For the use of the IFD technique as a rapid diagnosis method, it is necessary to continue the research on a much larger number of samples and in comparison to several diagnostic methods.

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Coproscopic identification of *Nosema apis* (Microsporea: Nosematidae) spores in humans

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Abstract

The products of apiculture (honey, propolis, royal jelly, venom, nectar and bee bread) are being used for preventing healing and recovery of humans from various morbid states. In the general context of the bee pathology, the infection of the bees with species from Nosema genus determines loses both for the bees colonies and for the qualities of the bees products. Investigations were conducted in the Parasitology and Parasitic Disease Clinic of the Faculty of Veterinary Medicine Iasi, on the faecal samples from one human, breeder who consumed daily, for a long period of time, as supplement, a bee byproduct called bee bread, derived from their hives. The patient presented an advanced state of weakness, anorexia, headache, listlessness, severe diarrhoea syndrome and oscillating neurological disorders accompanied by aggressive and delusional seizures. Faecal and bee bread samples were assayed by direct and qualitative (Willis) and quantitative (Mc. Master) flotation methods. Examination and microphotography were performed using Leica optical system and Leica Application Suite. The obtained results showed that in both samples collected from the patient and the bee bread samples collected from the hives Nosema apis spores were identified, with a strong intensity (OPG: 20.000-25000, respectively). The results suggest the possible Nosema apis adaptation in humans, triggering an alarm on the consumption of apiculture products and byproducts that are veterinary uncontrolled and warns not to ignore the risk of a possible transmission to humans. It is the first case report of Nosema apis spores in humans, in our country.

Key words*: Nosema apis spores, human faeces, digestive syndrome*

Introduction

Even from ancient times the bees (*Apis mellifera*) represented a rich subject for research but also a profit source for human population, through their products and bee pollination of a great number of plants. The climatic conditions, geographical relief and vegetation from our country are extremely favourable for beekeeping, representing one of the most ancient occupations of the Romanian people from the Carpathian-Danube-Pontic area (1). The products of apiculture are as follows: honey, propolis, royal jelly, venom, nectar and bee bread, all of these being used for preventing healing and recovery of humans from various morbid states (2).

In the general context of the bee pathology, the parasites have an important place due to their severe consequences on economical, hygienic-veterinary and social aspects.

Honey bees, *Apis mellifera*, face different parasite and pathogen challenges against which they direct both individual and societal defences. Effectiveness of bee defense decreases, when the aggression is multiple and concomitant, exerted by parasites, viruses, bacteria, microbes in conjunction with other pathogens that cause bee death and reduce the number of families in different geographic areas (3, 4).

The infection of the bees with species from *Nosema* genus determines loses both for the bees colonies and for the qualities of the bees products (5).

At the moment, *Nosema* genus is part of Fungi kingdom, Microsporidia phylum, Dihaplophasea class, Dissociodihaplophasida order, Nosematidae family, Nosema genus, *Nosema apis* species (6).

Nosema apis (Zender, 1909), was formerly believed to be the only microsporidium to infect epithelial cells of the midgut in adult honey bees (*Apis mellifera* L.). It was, however,

recently discovered that another microsporidium, *Nosema ceranae*, also parasitizes western honey bees in countries of all continents (7). *N. apis* was isolated in the European honey bee (*Apis mellifera*) and *N. ceranae* was isolated from the Asian honey bee (*Apis cerana*) in China. Both species of *Nosema* may be cross-infective on bees, but differ in pathogenity (8). It is considered that *Nosema ceranae* is more pathogen than *Nosema apis*, affecting the honey bees colonies from Europe (9, 10). It is noted, however, that in some geographical areas, *Nosema apis* is replaced by *N. ceranae* (11).

From past and until present day, the apiculture products were not considered responsible for pathological diseases in humans, for this reason being always recommended as energy supplements in most of the diseases, having no contraindications.

This research paper is presenting for the first time in Romania, a case of infection in a human patient with spores from *Nosema apis* species, due to consumption of contaminated honey bee products, with digestive, neurologic and general syndromes, similarly to the ones described in honey bees. The identification of the aetiology for the chronical enteric syndrome in a beekeeper (apiarist) by coproscopic examination and microscopic analyse of the apiculture products ingested by the apiarist.

Material and methods

The analyses were performed in the Parasitology and Parasitic Disease Clinic of the Faculty of Veterinary Medicine, Iasi. The examined material was represented by five faecal samples harvested from an apiarist, aged 32. The faecal samples were harvested during five subsequent days in sterile tubes and coproscopic analyses were performed using Willis flotation qualitative method and Mc. Master quantitative method, the degree of infection being determined in correlation with the number of parasitic elements per gram faeces (OPG).

There were also harvested five bee bread samples, from which the infected beekeeper was ingesting daily as vitamin-mineral supplement, for a year time period. The bee bread was analysed by direct examination on smear, Willis qualitative method and Mc. Master quantitative method. Examination and microphotography were performed using Leica DM 750 optical microscope, Leica ICC 550 Camera and Leica Application Suite (LAS), version 4.2, for image retrieval. The epidemiological, clinical and laboratory data were analysed.

Results

The epidemiological data shows that the infected apiarist had, beside the bee colonies, livestock (horses, cows, pigs, sheep, chickens etc.), fact that leads to both physical exhausting but also permanent and direct contact with animal faeces during the mechanical cleaning inside farm. In the rural areas, the beekeepers are usually and constantly consuming the obtained bee products knowing that they are an energy source and help improving their health status.

The consumption of bee bread was made deliberately, as a supplement to the daily diet with an assimilated product, as a beneficial product for the organism, without considering the hazard for infection. The infection of the apiarist was produced by daily, oral ingestion of the bee bread from contaminated hives of the *Nosema apis* infected bee colonies, for a year time period.

The infected apiarist presented chronic digestive syndrome, characterised by progressively diminished appetite to anorexia, advanced state of weakness, severe antibiotic-resistant diarrhoea, dehydration, diminished working ability and oscillating neurological disorders from apathy and prostration, equilibrium disorders to aggressive and delusional seizures. The macroscopic examination of the faecal samples emphasized the cause of diarrhoea, the liquid consistency, whitish colour and bad smell of faeces.

The microscopic exam underlined the presence of small formations, oval or spherical shaped or even clover-like structures in all faeces samples (Fig. 1).

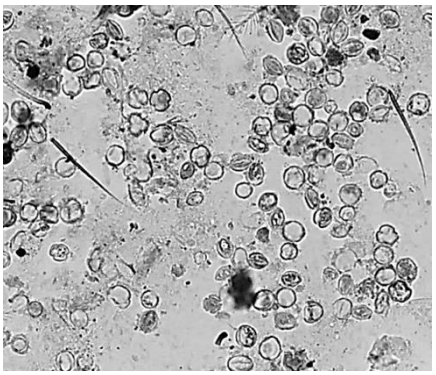

Fig. 1. Oval, spherical shapes, or with clover aspect identified in faecal samples from the infected apiarist, 200x

The microscopic examination of the bee bread samples from the bee hives underlined the presence of similar formations to the ones identified inside the faecal samples (Fig. 2), thus indicating the source of infection of the apiarist.

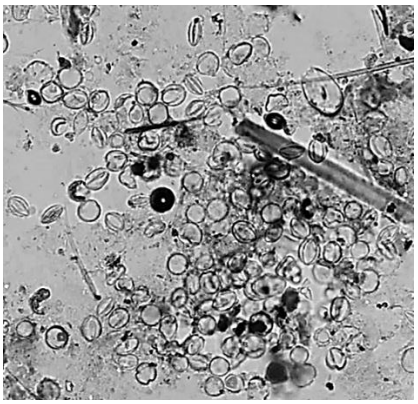

Fig. 2. Oval, spherical shapes, or with clover aspect identified in bee bread samples, 200x

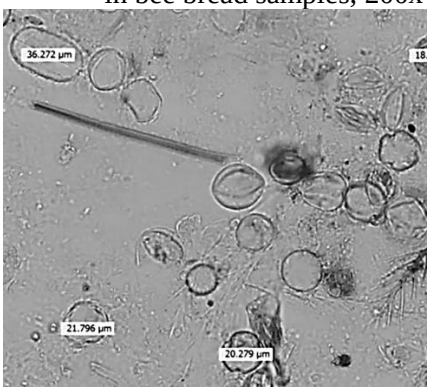

Fig. 3. Spores of *Nosema apis* identified in faecal samples from the infected apiarist. Detail- 400x

The identified spores are oval-shaped, incolor, refringent, shiny corpuscles with dimensions of 3,5-4,6-x 2-2,4 µm with a kitinous wall, a polar capsule, and a long tubular filament which is coiled round the inside wall. The study of the morphological features of the identified spores both from faecal human samples (Fig. 3) and from bee bread samples (Fig. 4), confirms the identification of *Nosema apis* species, compared with the data from scientific literature (2, 12).

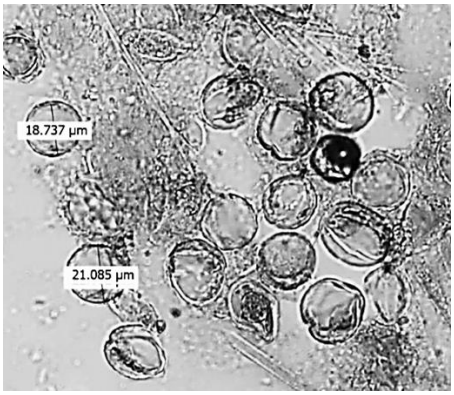

Fig. 4 Spores of *Nosema apis* identified in bee bread samples. Detail-400x

The quantitative determination of the *Nosema apis* spores from the faecal samples underlined a high density with a value of 20.000 OPG. The quantitative determination of the *Nosema apis* spores from bee bread underlined a high density with a value of 25.000 OPG.

Discussion

Nosema apis is an unicellular parasite, localated intracellulary, that multiplies inside the intestinal wall of the bee (*Apis mellifera*), interfering in digestion and food assimilation. It presents two forms: a vegetative and a sporulated form. The vegetative form is multiplying inside the cells of the intestinal epithelium of bee, where, by traumatic, mechanical, irritant and toxic action induces nosemosis. The sporulated form with a reduced metabolism that may be identified usually after the bees death or after environment elimination represents the environmental resistance form. The spore germinates when it reaches the middle intestine of bees, following the normal biological cycle stages (13).

The spores are very resistant to the environmental factors. Suspended inside water or honey, the spores are inactivated at temperatures of 50° C after 15 minutes; at room temperature (22-24°C) they resist for 2 months, and at refrigerating temperature (4° C) they resist for 3 weeks. In dried cadavers, the spores are conserved for a year, inside the dried faeces they resist for 2 years, inside honey approximately 258 days and inside beehive between 3 months and 2 years. The solar rays inactivate the spores from dry environment after 15-32 hours and from wet environment after 37-51 hours (12).

The nosemosis is a frequently encountered invasive disease of adult bees, usually associated with chronical evolution, but also acute, with severe manifestations. It starts more frequently at the end of the winter and beginning of spring, producing the death inside the bee colonies. The rough conditions during winter predispose it, together with existence of weak colonies, less harvest of nectar and pollen, humidity, bad weather conditions etc. It is characterised by diarrhoea syndrome, clinically expressed by lost appetite, abdominal meteorism, liquid diarrhoea, whitish faeces, associated with neurologic syndrome expressed by vertigine, incoordination during flight, falls, paralysis, followed by impossibility of flying and death (12, 14).

There are more genus and species of Microsporidia that infect animals and humans respectively, *Encephalitozoon*, *Enterocytozoon*, *Nosema*, *Pleistophora*, *Septata* genera. In fur animals (rabbits, fur animals, blue foxes, silver foxes), microsporidia from *Encephalitozoon* genus affects the endothelial cells of the blood vessels invading blood and being disseminated in entire organism, with clinical fatal evolution and mortality to 50% of cases (15, 16).

Microsporidiosis have a zoonotic character and the transmission of microsporidies is mediated by biotic (invertebrates, domestic animals, wild animals, etc.) and abiotics factors (climate factors, food, water, precarious hygiene, working utensils, etc.), contributing to parasitic pollution of the environment (17).

The following species are known to parasite exclusively humans: *Enterocitozoon bieneusi* responsible for 90% of human microsporidiosis (18, 19), *Encephalitozoon hellem*, *Encephalitozoon intestinalis*, *Encephalitozoon cuniculi* and *Nosema corneum* isolated from immunocompromised human patients or HIV patients (15, 20). Microsporidiosis is a significant cause of persistent diarrhea, gastrointestinal illness, and weight loss especially for children, immunosuppressed individuals and persons with AIDS (21).

Recently, Carhan and col. (2015) (22) have reported the first case of *Encephalitozoon cuniculli* infestation in an animal keeper, in Turkey, while spores have been identified in the urine. In the scientific literature, there are no information regarding the risk of human infection with spores from *Nosema apis* species followed by illness.

Nosema apis spores, that are present in the feces of the apiarist in such high intensity (OPG: 20000), could be explained either by the adaptation of the parasite and development of the biological cycle in the intestinal mucosa epithelium, or by the passage and storage of the spores resulting from the daily consumption of contaminated bee bread. If the biological cycle has taken place, the gastric juice has released the vegetative form, the sporozoite, which has penetrated into the enterocytes by stadially transforming into trophosoid, pansporoblast, sporoblasts and spores (in 3-4 days, in bees). Clinical manifestations are triggered by the aggression of the parasitic stages on the intestinal mucosa epithelium and the absorption of neurotropic toxins. If the spores passively passed through the stomach and intestine without interfering with the digestive medium, it would probably lack clinical manifestations.

The adaptation of the parasite from the enterocytes of the middle intestine of bees to the ones of small intestine of human with parasitic stages development finalised in spores inside feces, is the possible explanation of the chronical pathological status of the infected patient and all the clinical signs.

The possible passage of the parasite to humans and development of a severe pathological status, similarly to the one in bees, suggests a possible chapter in human parasitic pathology. It seems that the beekeeper, physically exhausted and immunocompromised represented a good substrate for the biological cycle of the parasite that is specific to the bees, in the enterocytes of the small intestine mucosa.

Taking into consideration all the above it becomes very important the control and examination activity of all beekeeping products, regarding the risk of nosemosis. The active medical veterinary activity will succeed to isolate the occurance of nosemosis with serious consequences for reducing bee collectives but also the pathological status induced on persons who ingest beekeeping products contaminated with spores from *Nosema apis*.

Conclusion

This study has as objective to trigger an alarm on the first reported case in Romania, on the risk of human infection with spores of *Nosema apis* through beekeeping products consumption,

that were not previously controlled from hygienic-sanitary point of view and without any parasitological examination.

The development of the vegetative forms of *Nosema apis* in the intestinal mucosa of the human patient, causing the digestive and neurologic syndromes and appearance of the sporulated forms inside the faeces, suggest a possible explanation of the adaptation of the *Nosema apis* species to invade the enterocytes from the small intestine mucosa in humans.

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Haematological diagnosis of anemia in dogs and cats

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Abstract

Anemia is part of the erythrocytic system pathology and is characterized by a decrease in hemoglobin and in number of red blood cells in circulating blood, which is a common disorder both in animals and in humans. This study proposes to identify types of anemia according to morphological and etiopathogenetic criteria in 26 patients. The diagnosis of anemia in dogs and cats was based on anamnestic data, clinical and paraclinical examinations. By quantitative haematological determinations and blood smear examination, there were identified 13 cases of normocytic normochromic anemias, 4 of macrocytic hyperchromic anemias and 9 of microcytic hypochromic anemias. Depending on the number of immature erythrocytes circulating in the blood, were identified 7 cases of hyperregenerative anemias, 6 hyporegenerative, 10 generative and one normoregenerative anemias, 2 of these cases remaining unclassified. Regarding the etiopathogenesis of anemias, were identified 11 cases of parasitic hemolytic anemias, 4 cases of autoimmune hemolytic anemias, one case of infectious hemolytic anemia, 2 cases of posthemorrhagic anemias and 8 hemolytic anemia associated with unknown causes. The results obtained indicate 92.3% of peripheral hemolytic anemias and 7.7% of anemias caused by excessive red blood cell loss.

Key words: *anemia, pets, hematology*

Introduction

Anemia is part of the erythrocytic system pathology and is characterized by a decrease in hemoglobin and in number of red blood cells in circulating blood, which is a common disorder both in animals and in humans. Anemia appears as a result of changes of one or more factors involved in erythrogenesis: marrow, "building materials" of erythrocytes, catalytic or stimulatory factors. Sometimes, although the production of erythrocytes is normal, it can appear destruction or loss of erythrocytes due to other globular or extraglobular causes.

Depending on the morphological criterion, anemia is classified into three types: macrocytic, normocytic, microcytic hypochromic. Macrocytic anemias, characterized by an increased mean cell volume (MCV), hemoglobin (HEM), and reduced red blood cells are less common in animals but may be a transient response to haemorrhage, hemolysis, etc., when macrocytosis is consequence of releasing in the blood of immature erythrocytes, larger than adult ones. Normocytic anemias, where MCV and MCHC are normal , can be commonly caused by hemolysis or bone marrow depression, following inflammatory disorders. Microcytic hypochromic anemias, characterized by small size of erythrocyte, decrease erythocyte number, hemoglobin and HEM, is a frequent deficiency in iron and other anti-anemic microelements (Nicolae Avram, 1999). Normochromic anemia can also be added to this classification, hemoglobin being in normal limits.

Depending on the number of reticulocytes, anemia can be classified as: regenerative, when the bone marrow can respond to anemia and produce new erythrocytes in the blood; hyperregenerative, when reticulocytes are above normal; aregenerative, characterized by absence of immature erythrocytes in anemias; hyporegenerative due to deficient erythropoiesis.

From etiopathogenetic classification, depending on the response of the marrow and the circulating blood, there are:

- central anemia caused by hypofunction of bone marrow, characterized by blocking red cell precursors. Usually are included protein-vitamin-mineral anemias and toxic anemias;

- hemolytic anemia with peripheral origin, caused by excessive lysis of red blood cells and premature destruction. The etiology of these anemias are some endogenous (autoimmune mechanisms) or exogenous factors.

- anemia caused by excessive loss of red blood cells in haemorrhages due to injuries or other causes (Nicolae Avram, 1999). Hemorrhage can be internal or external. Hemorrhage into joints and the abdominal cavity are examples of internal hemorrhage. Hemorrhage from lacerations, loss from the gastrointestinal or urinary tract, or external or internal parasites are examples of external hemorrhage (Maxey L. Wellman).

Size changes (anisocytosis) are characterized by present of red elements in different sizes: megalocyte (12-15 μm), macrocyte (8-12 μm), microcyte (4-6 μm), schizocytes (2-3 μm). Anisocytosis involves abnormal red blood cell regeneration.

Shape changes (poikilocytosis) refer to the detection in smears of different shapes of erythrocytes such as ovalocytes, drepanocyte, rocket, drop, etc. Also, may also appear nucleated erythrocytes, Howell-Jolly corpuscles (indicates exaggerated regeneration), Heinz corpuscles (indicates serious anemias).

Color changes (anisochromia) of RBC are depending on the content and the quality of hemoglobin. RBC with insufficient hemoglobin (hypochromia) appears with a clear central area colored only on the periphery or pale. Hyperchromia is an intense and uniform coloring of the erythrocytes, possibly with a dark hue in center. In some pathological conditions, hemoglobin affinity for acidic is replaced by a more or less pronounced basophilia. Polychromatophilic or basophilic coloration of erythrocytes are aspects found in anemias , indicate a red cell regeneration.

Nucleated RBCs (NRBCs) can indicate active regeneration but are also seen with splenic dysfunction, shock, heavy metal toxicity and bone marrow disorders. The presence of polychromasia, anisocytosis and NRBCs on blood smears may indicate regeneration (Séverine Tasker, 2006).

Although kidney failure and some infections (flea infestation, FeLV infection and hemobartonellosis) are likely the most common causes of anemia, there are many other differential diagnoses to consider, such as bleeding disorders, toxicity, metabolic disturbances, hereditary defects, and immune-mediated hemolytic anemias. It is therefore crucial to carefully assess the feline patient by history taking, physical exam and routine laboratory tests in order to determine the cause and offer the most appropriate treatment (Urs Giger, 2016).

Material and methods

Investigations were conducted on 26 cats and dogs of various breeds and ages that were presented during the March 30, 2016 to March 21, 2017 in the Medical Clinic of Faculty of Veterinary Science from Iași and in private Veterinary Clinic from Iași.

Diagnosis of anemia in animals is based on anamnetic data, clinical and paraclinical examinations. In clinical examination, some common disorders occur in all anemia: pale skin, membranes; red, brown or black urine; hepatomegaly with increased sensitivity, splenomegaly; tachycardia, polypnea or dyspnoea at rest, or at low effort. Paraclinic examinations require haematological determinations and examination of blood smears. Hematologic examination is essential in diagnosis. A low number of erythrocytes, hemoglobin and hematocrit values are the main parameters to diagnose anemia.

Initial diagnostics in an anemic patient should focus on identifying the cause of anemia. A diagnosis of anemia secondary to an underlying immunemediated pathogenesis is based on evidence of accelerated red blood cell (RBC) destruction (Andrew Mackin, Todd Archer, 2014).

The investigation stages to diagnose anemia are:

-determination of hemoglobin (Hb) and hematocrit (Ht) as the main parameters, accompanied by count of red blood cells (E);

-determination of erythrocyte constants: MCV, HEM, MCHC, to specify the morphological type of anemia;

-examination of the blood smear for determining the morphology of the red blood cells: size, color, shape, young elements (Nicolae Avram, 1999).

In order to establish the diagnosis of anemia were used classical methods of haematological analysis such as the hemocitometric method for counting erythrocytes, Sahli colorimetric method for hemoglobin dosing, the microhematocrit method for hematocrit determination, mathematical methods for derived erythrocyte constants (MCV, HEM, MCHC), specific staining techniques with brilliant cresyl blue for reticulocytes, May-Grünwald-Giemsa (MGG) or Diff-Quick (DQ) for erythrocytes.

Hematocrit is the percentage expression of globular blood volume in relation to total blood volume (in other words, as percent of the total blood volume is erythrocytes, since the volume occupied by the other elements is negligible). Determining hematocrit with microhematocrit method uses heparinated capillary tubes. The end of the tube is closed to the flame and then centrifuged at a special centrifuge. To reading a hematocrit it is used a special reading device (fig. 1).

Fig. 1. Janetzky centrifuge (right) and reader for microhematocrit determination (FMV Laboratory Iasi)

Determination of erythrocytes by haemocytometric method uses: the counting chamber, also called hemocytometer (Bürker-Türk, Thoma, Neubauer), Potain pipette for erythrocytes, Hayem dilution fluid and microscope (fig. 2).

Fig. 2. Materials for the haemocytometric method

The Sahli colorimetric method uses: the Sahli hemoglobinometer, hydrochloric acid and distilled water. The Sahli hemoglobinometer contain a comparator and a capillary pipette.

Fig. 3. Sahli hemoglobinometer

The mean cell volume (MCV) is the volume of isolated erythrocyte. It is measured in μ^3 and calculated with formula: VEM = Ht x 10 / E.

Medium erythrocyte hemoglobin (HEM) is the average hemoglobin content of an erythrocyte. It is measured in picograms and calculated with formula: HEM = Hb x 10 / E.

The mean cell haemoglobin concentration (MCHC) is the average hemoglobin concentration in the blood. It is expressed as a percentage or in g / dl red blood mass and is calculated with formula: MCHC = Hb x 100 / Ht (Geta Pavel, Răzvan Mălancuş, 2015).

Results and discussions

By quantitative haematological tests and blood smear examination, were identified 13 (50%) normocytic normochromic anemias, in 5 cats and 8 dogs, 4 (15.4%) macrocytic hyperchromic anemias in 2 cats and 2 dogs and 9 (34.6%) microcytic hypochromic anemias, in 2 cats and 7 dogs.

Depending on the number of immature erythrocytes circulating in the blood, were identified 7 cases of hyperregenerative anemias, 6 hyporegenerative, 10 generative and one normoregenerative anemias, 2 of these cases remaining unclassified. Reticulocytes are erythrocytes with vital grains; the granulocyte substance (identical to the basophilic polychromatic substance) appears colored blue on a pink background, being placed in different positions: sometimes at the edge of the cell in granules, sometimes in the center, or can even fill the whole cell. Increased reticulocyte counts occur in the red cell regeneration phase (adapted from I. Adamesteanu, A. Nicolau, H. Bârză, 1966). Regeneration is evidenced by anisocytosis, polychromatic macrocytes, large numbers of reticulocytes.

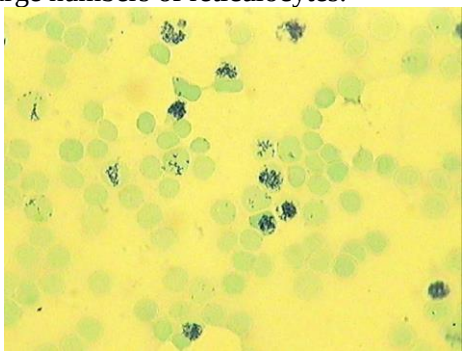

Fig. 4 Reticulocytes, Col. brilliant cresyl blue x1000

Regarding the etiopathogenesis of anemias, were identified 11 cases of parasitic hemolytic anemias, 4 cases of autoimmune hemolytic anemias, one case of infectious hemolytic anemia, 2 cases of posthemorrhagic anemias and 8 hemolytic anemia associated with unknown causes. The results obtained indicate 92.3% of peripheral hemolytic anemias and 7.7% of anemias caused by excessive red blood cell loss. Parasitic haemolytic anemias were caused by Mycoplasma hemofelis in 3 cats and by Babesia gibsoni in 9 dogs. In a study by Shalm (1975) it was found that Mycoplasma hemofelis disease is rare, affecting both sexes but with a higher frequency in males. Infectious feline anemia can affect all ages of animals, most of which are described in cats aged 1-3 years. Based on the findings of the previous study, in the present study only one cases from 3 cases of feline infection were identified. In the blood smear were seen changes of erythrocytes such as: anisocytosis, Jolly bodies, schizocytes, echinocytosis. The analysis of the blood smear in cases with the Babesia gibsoni parasite revealed: intraerythrocytic babesies (fig. 5A), echinocytes (fig. 5B), schizocytes (fig. 5C), Jolly bodies (fig. 5D), target cells, polychromatophiles, young nucleated erythrocytes indicating splenic hypofunction and excessive regeneration, anisocytosis.

The four cases of autoimmune hemolytic anemia (AHAI) were found only in Bichon and American Bulldog dogs. These occurred after post-transfusion reactions, when the donor's incompatible erythrocytes are hemolyzed by the recipient's pre-existing antibodies. Haematological changes characteristic of the disease are the presence of spherocytes, polychromatophilic macrocytes, agglutination and anisocytosis. Recent studies have indicated that all dog breeds are prone to this type of anemia, but predominantly Cocker spaniels, English Springer spaniel, Poodle and English Sheepdogs (Andrew Mackin, 2014). Also, AHAI is more common in dogs than in cats, but recent studies (Husbands, 2002, Kohn, 2006) show that this is also common in cats. IMHA is primarily a disease of middle-aged to older dogs. It may occur at any age but is rare in dogs younger than 1 year (Michael J. Day, 2012).

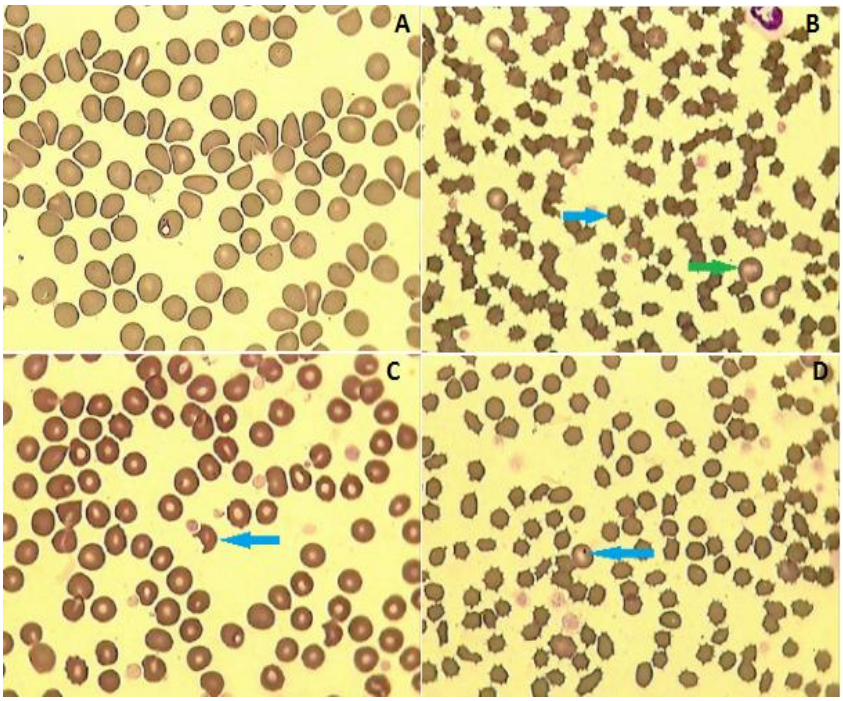

Fig. 5. *Babesia spp.* in red blood cell (A); echinocytes (blue arrow) and macrocytes (green arrow) (B); horn-shaped schizocyte (C), intraerythrocytic Jolly bodies, Col. MGGx1000

Anemias caused by excessive red blood cell loss due to external haemorrhages were found in a dog and was microcytic, hypochromic, normoregenerative anemia (MCV, HEM under normal range), and a cat with a macrocytic, hyperchromic, aregenerative anemia (MCV, HEM over normal limits).

Conclusions

Dysfunction of the red blood cell line, anemia is a common disease in animals. Red blood cells, also known as erythrocytes are important for the transport of oxygen from lungs to all organs in the body. Reducing the number of erythrocytes below the normal limit, the low amount of hemoglobin in anemia has different causes and is associated with many diseases that can be treated more effectively when symptoms are discovered in short time. The diagnosis of anemia in dogs and cats can be based on anamnestic data, clinical and paraclinical examinations. An important role are quantitative haematological tests and blood smear examination. Analysis of haematological determinations leads to a morphological diagnosis, and the analysis of blood smear orientates to an ethiological diagnosis as nucleated cells are indicators of splenic hypofunction in babes and excessive regeneration, spherocytes are characteristic in autoimmune diseases, target cells idicate diseases with hepatic origin, polychromatophiles regeneration, schizocytes and echinocytosis a pathological orientation of the blood vessels.

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Anemia description in *Babesia spp.* infected dogs

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Abstract

Babesiosis is a tick-borne malaria-like illness caused by species of the intraerythrocytic protozoan Babesia. Infection in dogs may occur by tick transmission, direct transmission via blood transfer from dog bites, blood transfusions or transplacental transmission. The most common mode of transmission is by tick bite, as the Babesia parasite uses the tick as a reservoir. The study was undertaken between 2015-2017 in Physiology and Pathophysiology laboratory, Faculty of Veterinary Medicine Iasi on 21 dogs of different breeds and age. Babesia infected dogs represented 4,3% of the total number of investigated blood samples. Due to Babesia spp. affinity for erythrocytes, anemia is the most commonly diagnosed disorder in babesiosis, being observed in 11 patients (52,4%). Direct action of parasites on erythrocytes by producing toxins or indirectly by stimulating an autoimmune response leads to destruction of red blood cells in large numbers according to the degree of parasitemia. The average value of erythrocytes, hemoglobin and hematocrit are inversely proportional to the expressed parasitemia by the studied individuals. As previous studies have shown before, in Babesia spp. infected dogs, anemia is accompanied by monocytosis. The increase in monocyte counts correlates to the leukocyte proliferation found in babesiosis. Monocytosis certifies the chronic evolution of the disease and the autoimmune character induced by the development of the parasitic stages.

Key words*: Babesia spp., dogs, anemia*

Introduction

The primary function of the red blood cells is to transport oxygen to tissues. Anemia is defined as a significant deficit in the mass of circulating red blood cells. As a result, the capacity of the blood to deliver oxygen is compromised. The presence of anemia can be documented by measurement of either the concentration of hemoglobin in the blood or the hematocrit, which is the ratio of the volume of red blood cells to the total volume of a blood sample. A patient is anemic if the hemoglobin or hematocrit value is more than two standard deviations below normal. The lower limits of normal vary with the age of the individual and, in adults, with gender. Occasionally, the documentation of anemia is confounded by a concurrent alteration in the plasma volume. For example, if a patient with a low mass of circulating red blood cells is also hypovolemic, owing to a concurrent loss of plasma volume from dehydration, the blood hemoglobin and hematocrit levels will be falsely elevated and may even be in the normal range. Another case is represented by acute hemorrhage, in which there is concomitant loss of both red blood cells and plasma.[4]

Babesiosis is a tick-borne malaria-like illness caused by species of the intraerythrocytic protozoan Babesia. Infection in dogs may occur by tick transmission, direct transmission via blood transfer from dog bites, blood transfusions or transplacental transmission. The most common mode of transmission is by tick bite, as the Babesia parasite uses the tick as a reservoir.[2]

In babesiosis, the parasite of the *Babesia* genus, *Babesiidae* family, sets in the parasitized organism erythrocytes in variable number (1-4 parasites), putting on different shapes and forms, depending on the species. The parasite has several species that can be found in dogs, like *Babesia canis Babesia vogeli* or *Babesia gibsoni*.[2]

The effect of Babesia spp. *over the red blood cells is their destruction, causing hemolytic anemia.* Animal contamination is achieved by transcutaneous inoculation during the feeding of

infected ticks that inserts the parasites together with saliva. The inoculated parasites initially penetrate red blood cells, they multiply, secret metabolic toxins that causes the lysis of red blood cells, so anemia occurs. Due to these phenomena the entire functioning of the body is disturbed developing liver and kidney disorders, nervous, respiratory and cardiac disease.[3]

Material and methods

The study was conducted at the Faculty of Veterinary Medicine Iasi, over a two-year period, the research being performed on a total of 21 dogs, of different breeds and ages, all these subjects being affected by babesiosis. For each of these cases blood sample collection has been performed using EDTA as anticoagulant.

The investigated hematological parameters have been represented by the number of erythrocytes, hemoglobin, hematocrit, derived erythrocyte constants (mean corpuscular volume - MCV, mean corpuscular hemoglobin - MCH and mean corpuscular hemoglobin concentration - MCHC), reticulocyte count, ESR, both platelets and leukocytes count. The determination of red blood cells parameters have been made by conventional methods.[5]

Determination of the red series main parameters (number of erythrocytes, hemoglobin, hematocrit) can provide relevant data on the existence of anemia, which is common in babesiosis due to destruction of large numbers of red blood cells. However, the persistence of anemia in animals in convalescence may be maintained by the presence of erythrocyte self antibodies and immune complexes, erythrocytes lysis being induced by complement.

The observation of *Babesia spp.* infestation degree has been made by reading the May Grumwald Giemsa stained blood smears and determining of leucocytes formula, the hematological examination allowing to appreciate blood parameters changes, disturbances accompanying *Babesia spp*. infestation. It must be considered that *Babesia spp.* may not always be identified in the blood smear. It is considered that they are visible on the first day after inoculation, and then they disappear until day 10. From day 11 to day 21 after inoculation *Babesia spp.* can be observed in erythrocytes, their presence being directly proportional to the degree of parasitemia.[4]

Assessment of hematologic changes allows to ascertain between different types of anemia, focusing on the infestation development and allowing to appreciate the parasitemia degree. Thus, the determination of leukocyte formula ascertains the evolutionary parasitic forms located in the intermediate hosts blood, these data corroborated with other hematological parameters helping to establish a correct diagnosis that allows precise identification of the starting point of infestation.[3]

The obtained data has been tabulated in contingency tables, statistical appreciation being achieved by using the SPSS Statistics 18 statistical software and Fisher's exact test which illustrates the association between two different categories of investigated parameters

Results and discussions

The conducted study has investigated and allowed to diagnose the types of anemia developed by the *Babesia spp.* infected subjects. The main changes induced by babesiosis had repercussions on the red series regarding the number of erythrocytes, quantity of hemoglobin, hematocrit and derived erythrocyte constants.

Out of 596 laboratory samples examined over the two-year period, 494 represented blood samples. From those, 21 patients have been identified with babesiosis, representing 4,3% of the investigated blood samples. The most *Babesia spp.* infected dogs were identified in 2016 (13 patients) while in 2015 and 2017 babesiosis was observed in 5, respectively 3 dogs (fig. 1). Although a peak was recorded in 2016, the babesia infected patients represented 3,9% of the total investigated patients, while in 2015 represented 4,5% and in 2017, almost 3% (2,8%).

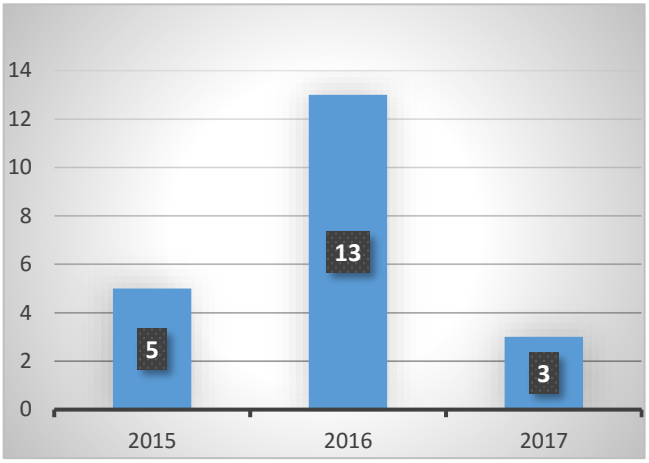

Fig. 1. Babesiosis cases in dogs between 2015-2017

Because of *Babesia spp.* affinity for erythrocytes, anemia is the most commonly diagnosed disorder in babesiosis. The direct action on erythrocytes by producing toxins or indirect action by stimulating an autoimmune response leads to mass destruction of the red blood cells depending on the parasitemia degree. Thus, the reduction of the red blood cell counts, hemoglobin and hematocrit are directly proportional to the expressed parasitemia by the studied subjects.[3]

Patients with mild or moderate anemia are often asymptomatic. Many note breathlessness and/or fatigue only upon strenuous exercise. In severe anemia, dyspnea and fatigue are common complaints. These symptoms reflect limitations in the earlier-mentioned compensations for the tissue hypoxia imposed by a low red blood cell mass. Physical findings also depend on the severity of the anemia. Pallor reflects a compensatory shunting of blood away from the skin to ensure adequate flow to vital organs. Those with severe anemia may have tachycardia at rest, owing to a compensatory increase in basal cardiac output.[2] The hyperdynamic circulation in such patients often gives rise to a systolic "flow" murmur that is transmitted into the neck. In patients with lesser degrees of anemia, the heart rate is normal at rest but, on exercise, increases more than normally. Anemic patients may have many other informative physical findings that depend on specific underlying pathophysiology. For example, those with hemolytic anemia often have splenomegaly, owing to trapping of defective or damaged red blood cells in the spleen, and jaundice, reflecting increased plasma bilirubin levels due to rapid destruction of red blood cells.[4]

The characterization of the anemia tried to assess the following factors: presence of reticulocytosis, dimension of the red blood cells, presence of poikilocytosis and modification in hemoglobin content of the red blood cells in investigated patients.

The anemias can be divided into three broad categories: decreased red cell production, increased red cell destruction, and blood loss. Often, the patient's history and physical examination provide information as to which process is going on. For example, the presence of blood loss is usually apparent from the history. Physical findings such as jaundice and splenomegaly suggest hemolysis.[5] Among the available laboratory tests, the reticulocyte count is the simplest and most reliable way to distinguish among the three major categories of anemia. This laboratory test is a measurement of the fraction of young red cells in the blood (<2.5 days old). In patients with impaired red cell production, the reticulocyte count will be inappropriately low. Despite elevated levels of plasma erythropoietin, the bone marrow is unable to respond to produce adequate numbers of new red cells. In contrast, the reticulocyte count is generally elevated in both hemolytic anemia and in acute blood loss.[6]

We recorded 13 cases (61,9%) that presented reticulocytosis, with the most pronounced values of 78,5%, respectively 610.000 reticulocytes/mm^3 being observed in a patient with severe anemia due to *Babesia spp*. infestation associated to antiparasitic treatment (fig. 2).

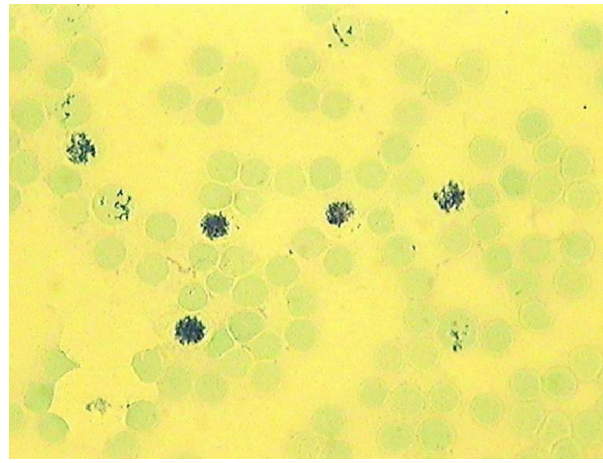

Fig. 2. Increased number of reticulocytes in a *Babesia spp.* infested dog

A wide range of structural, metabolic, immunologic, and mechanical defects can result in premature destruction of circulating red cells. However, irrespective of etiology, uncomplicated hemolytic anemias have a number of features in common. The capacity for efficient erythropoiesis is preserved, and indeed, in response to hypoxia-induced erythropoietin production, red cell production is often markedly increased, an adaptive response that is reflected by an elevation of the reticulocyte count. Usually, destruction of red blood cells is accompanied by a stimulation of erithropoiesis and release of immature red cell precursors in the blood stream, one of the adaptive mechanisms being represented by the merge or skipping of some precursor stages of development and their early release from the bone marrow. Anemia accompanied by reticulocytosis of 5% or greater strongly suggests the presence of hemolysis. However, elevated reticulocyte counts can also be seen in nutritional anemias during the first two weeks of replacement therapy with iron, cobalamin (vitamin B12), or folic acid. Acute hypoxia can also cause a transient elevation of the reticulocyte count. Finally, infiltrative bone marrow disorders such as metastatic neoplasms can also induce a modest sustained elevation of the reticulocyte count due to early release.

Regarding the changes observed in erythrocyte derived constants, 81,0% (17 cases) of investigated dogs presented either decreased MCV, MCH or MCHC, the association being considered statistically significant in dogs with *Babesia spp.* hemolytic anemia , with $p<0,02$. Changes in volume of the red blood cell were noticed in19 patients, the association between the presence of anysocytosis and anemia in dogs being very statistically significant, with $p<0,001$. Changes of MCV and MCHC were recorded in 7, respectively 5 dogs, with no statistical association with manifested anemia.

A statistically significant association ($p<0,02$) was noticed between increased erythrocite sedimentation rate (ESR) and severe infestation with *Babesia spp.*, with 90,9% of the severely affected dogs (10 out of 11 dogs) presenting high values for ESR.

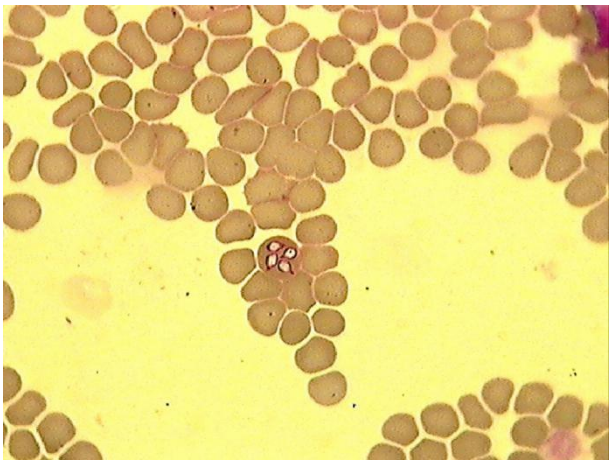

Fig. 3. Massive infestation with *Babesia canis* inside a red blood cell

Although increased ESR has been observed in 14 cases, there is no association between the destruction of red blood cells in moderately infested dogs and the high rate of sedimentation for the red blood cells.

Changes in the shape of the red blood cells, usually associated with anemia, can only been recorded by reading a stained blood film. Microscopic examination of a carefully spread and well-stained blood smear is an important part of the evaluation of any unexplained anemia, but it is particularly informative in identifying the cause of hemolysis.[1] Out of 21 cases, 19 dogs, representing 90,5% of the patients, had poikilocytosis, characterized by the presence of schizocytes (fragments of red blood cells), echinocytes, keratocytes, drepanocytes (sickle cell) or dacriocytes (tear shaped cell) (fig. 4).

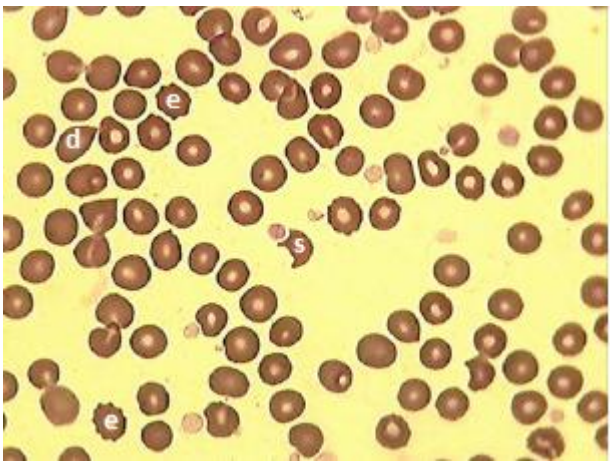

Fig. 4. Poikylocytosis - variety of shapes between red blood cells:
s - schizocyte; e - echinocyte; d - dacriocyte

The changes in shape are considered to be the result of parasite action against the red blood cells and also increased fragility of red cells membrane.

Conclusions

The undertaken study allowed to be drawn some relevant conclusions about the evolution

of anemia in *Babesia spp.* infested animals:

1. *Babesia spp.* infested dogs over a two-year period, between 2015-2017, represented 4,3% of the total number of investigated patients;

2. Thirteen cases (61,9%) presented reticulocytosis, with the most pronounced values of 78,5%, respectively 610.000 reticulocytes/mm^3 being observed in a patient with severe anemia due to *Babesia spp.* infestation associated to antiparasitic treatment;

3. There was identified a very statistically significant association between the presence of anysocytosis and anemia in dogs with babesiosis, with $p<0,001$;

4. A statistically significant association ($p<0,02$) was noticed between increased erythrocite sedimentation rate (ESR) and severe infestation with *Babesia spp.*, with 90,9% of the severely affected dogs (10 out of 11 dogs) presenting high values for ESR

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The use of upper gastrointestinal (GI) endoscopy in dogs

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Abstract

Gastrointestinal (GI) endoscopy is a nonsurgical procedure used to quantify the lesions that occur at the level of stomach and proximal duodenum in patients expressing gastrointestinal disorders. Endoscopic examination also tries to assess the degree of air distension of the stomach and duodenum, gastric and duodenal content and ease of passage through the pylorus, all of these greatly influencing the assessing of gastric and proximal duodenum lesions. Unlike other traditional complementary diagnostic methods, endoscopic examination allows direct assessment of lesions, focal or diffuse changes observation and assessment of the overall appearance of the gastrointestinal tract. Endoscopy is the most recommended imaging method having the ability to visualize changes in the examined levels. Although more expensive than ultrasound and radiological examinations, endoscopic examination is recommended both for its diagnostic and therapeutic value, the use of endoscopy being indicated whenever the use of other imaging methods fails to establish a diagnosis of certainty.

Key words*: endoscopy, dogs, upper GI*

Introduction

Endoscopy is a rapidly advancing technique with applicability to many areas of veterinary medicine. Upper gastrointestinal endoscopy is a noninvasive, atraumatic technique that permits visual examination of esophageal, gastric, and upper small bowel lesions, and allows descriptive or photographic documentation of their severity and extent. Endoscopy provides tissue, cytologic, and fluid samples for laboratory evaluation. It allows therapeutic interventions such as foreign body retrieval, bougienage, and gastrostomy tube placement.[1]

Upper gastrointestinal endoscopy is of most use for the diagnosis of esophageal, gastric, and upper small intestinal disorders with a mucosal involvement or luminal location. Lesions located in the muscular and submucosal layers of the gastrointestinal tract are more difficult to detect with an endoscope.[6]

Unlike ultrasound and radiological investigation, using the endoscopic technique is relatively new in veterinary medicine, this technique involving the knowledge of normal and pathological anatomy as well as learning to handle the endoscope. Endoscopy is currently one of the most important complementary diagnostic methods for gastrointestinal diseases in dogs. In addition to its definite diagnostic value, endoscopy also has therapeutic value, the latter manifesting itself in the presence of various foreign bodies or formations found in the gastrointestinal tract, formations that can be removed, excised, without endangering the patient's life.[4, 5]

Use of endoscopy for pets has increased in the last decade due to the awareness of huge importance in providing a definitive diagnosis. In addition to direct visualization of the gastrointestinal tract, endoscopy allows to take biopsies, gathering of samples for microscopic examination. Thus, endoscopic technique allows to state both a macroscopic and microscopic diagnosis, the technique being the only one that has this capability.[2]

Material and methods

The study was conducted over a 3 months period at the Small Animal Teaching Hospital, Liverpool. Examination of the upper gastrointestinal tract was performed on 51 dogs of different

breeds and ages, the symptoms manifested by those patients covering a wide range of digestive symptoms, from dysphagia, regurgitation, haematemesis, melen to vomiting and chronic diarrhea. Upper digestive endoscopy allowed to identify and assess the changes of color, friability, the presence of lesions and foreign bodies. Endoscopic examination also tried to assess the degree of air distension of the stomach and duodenum, gastric and duodenal content and ease of passage through the pylorus, all of these greatly influencing the assessing of gastric and proximal duodenum lesions. Thus, in addition to the diagnostic value of the endoscopic examination, it also has an important therapeutic value manifested by the removal of the cause that generated the gastrointestinal symptoms.

The endoscopic examination was performed in a specially designated room equipped with anesthesia station (used for animals weighing between 1 and 100 kg), Olympus CV 240 video system, Olympus CLV U40 light source and different endoscopic probes, depending on the type of endoscopy performed and the weight of the patient: Olympus GIF XP240 and Olympus GIF XP260. Monitoring of cardiac and respiratory functions was performed using a digital monitor that allowed observation of CO2 saturation and heart rate.

The anesthesia induction was carried out using an inhaler anesthetic (halothane, isoflurane, enflurane, desflurane). As with any procedure requiring anesthesia, a thorough physical examination with appropriate blood work and diagnostics determines choice of anesthetic protocol. The general condition of the patient is considered, including ongoing disease processes that may or may not be related to the disorder necessitating endoscopic examination. Liver disease is often associated with gastrointestinal disease and can result in detoxification deficiencies, as well as deficiencies in synthesis of such substances as clotting factors and albumin. The nutritional status of the patient is optimized, and dehydration and acid-base disturbances are corrected before anesthesia is given. Renal function and excretion of drug metabolites may be affected by disease or by changes in systemic and renal hemodynamics.[1] Withholding food for 12 hours and water for 2 hours is recommended and may help reduce the incidence of vomiting or regurgitation during the anesthetic period. However, prolonged preoperative fasting has been associated with an increased incidence of gastroesophageal reflux and increased gastric acidity. Complete gastric emptying has been observed in dogs within 10 hours when they were fed canned meat-based food or dry cereal-based food, with complete water emptying occurring in a mean time of 54 minutes.4 To prevent hypoglycemia during or after anesthesia, the clinician should order a shorter fasting interval for animals younger than 3 months old1 or for animals with impaired glucose metabolism.

Stomach examination is a relatively easy procedure, performed by injecting a small amount of air into the esophagus and fixing the tip of the probe to the center of the cardia sphincter. It should always be taken into account that at the end of the insertion tube the video camera is located laterally and not centrally, therefore, on the endoscopic image, the center of the insertion tube will appear to be deflected laterally. After penetrating the stomach, the insertion tube has to follow the large gastric curvature at the base area, making an inflection of about 180 ° (J-shaped maneuver) to visualize the entry into the stomach. After examining all gastric portions, it is possible to pass through the pilor by insuflating a suitable amount of air. As in the case of cardia sphincter passage, the passage through the pilor is performed by mantaining the tip of the insertion tube to the center of the sphincter.[1]

Upper gastrointestinal endoscopy is associated with low morbidity and mortality. Except in animals unfit for anesthesia, there are few absolute contraindications to performing gastrointestinal endoscopy. The procedure is not appropriate when bowel perforation is suspected, because contamination of the surrounding tissues may be increased as a result of the pressurization of the gastrointestinal tract by air insufflation.[2]

Results and discussions

Unlike other traditional complementary diagnostic methods, endoscopic examination allows direct assessment of lesions, focal or diffuse changes observation and assessment of the overall appearance of the gastrointestinal tract. Endoscopic technique has become very commonly used in recent years in order to investigate, as a result of numerous studies on the consequences of feeding on the gastrointestinal tract. Thus, because of the possibility to visualize the gastric and intestinal segments, endoscopic examination has been chosen as the main technique that observes the evolution of these segments in relationship to nutrition.[4]

Regarding the main changes that can be observed when investigating the stomach and duodenum, endoscopy can assess mucosal hyperemia, edema, friability of mucosa, gastric hemorrhage and ulceration.

The study allowed to identify 23 cases of hyperemia (45.1%), gastric edema in 2 patients (3.9%), increased mucosal friability in 12 dogs (23.5%), the presence of gastric hemorrhage in 21 patients (41.2%) and the presence of ulcers in 20 cases (39.2%). Gastroscopy can also be used to remove foreign bodies, this procedure being conditioned by their size and shape as well as the size or type of the available forceps . In many cases, problems arise not when trying to capture the foreign bodies but when attempting to pass through the cardia.[5]

In dogs, the presence and identification of foreign bodies in the stomach through the endoscopic procedure is common, the identified foreign bodies varying: plastic pieces (plastic bottles, balls, toys, etc.), wood or even metal (batteries, wires, etc.).

The presence of a plastic piece, part of a former ball, can be observed in figure 1. Generally, the presence of foreign bodies is associated with specific digestive symptomatology, represented by inapetence and vomiting.

Due to the irritative phenomena caused by the presence of the foreign bodies, moderate gastric hyperemia can be identified, as well as mild haemorrhage and gastric ulceration (fig. 2).

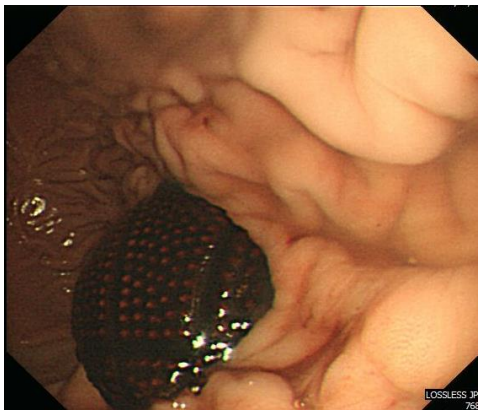

Fig. 1. Gastric foreign body

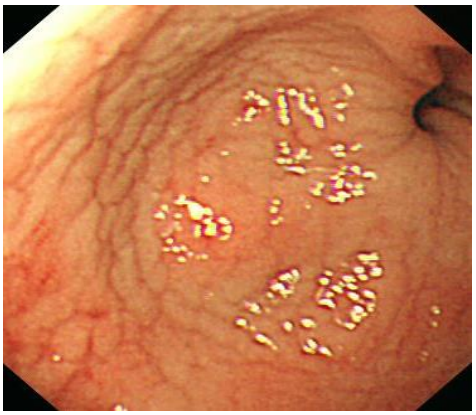

Fig. 2. Gastric hyperemia and small sized ulcerations

Often, in patients who ingest foreign bodies, their irritating action produces pyloric spasm, followed by vomiting, a symptom that is the main reason for seeing a doctor. Usually, the presence of foreign bodies is associated with inflammatory phenomena and mucosal hemorrhage (fig. 3). These lesions can lead to the development of anemia by iron spoliation and chronic loss of blood, often, the animals being in great disconfort.

Stomach inflammation or gastritis is endoscopically characterized by the presence of hyperemic phenomena sometimes accompanied by ulcerous lesions or edema of the gastric wall.

The presence of gastric mucosal hyperaemia is commonly found in patients who suffer from digestive disorders, this being the first observed sign.

A case of generalized hyperemia associated with gastric erythema can be seen in figure 4. It can be highlighted the severe hyperemia of the gastric mucosa, generalized to the entire gastric body.

In the same figure can be noticed the edema of the gastric mucosa, the gastric folds being hardly perceptible. The edema includes even the gastric incision, which should be examined every time when endoscopy is carried out, because both the cardiac and the pyloric openings can be examined on both sides.

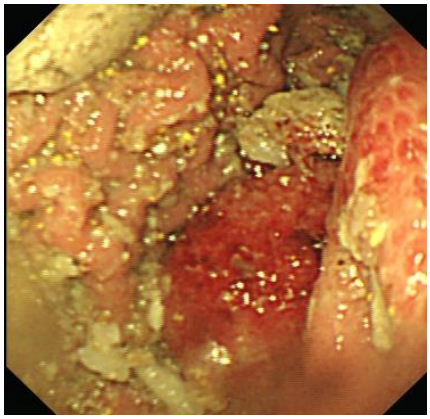

Fig. 3. Gastric hemorrhage

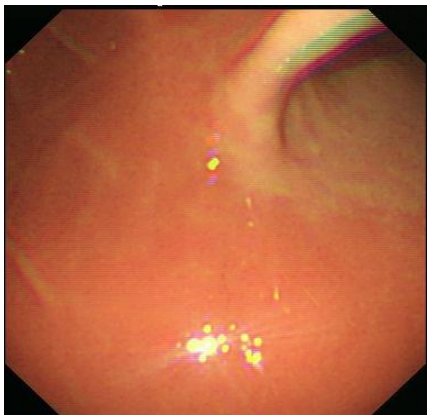

Fig. 4. Gastric hyperemia and edema

Lymphangiectasia and increased mucosal friability is highlighted in figure 5, with evident villi and lacteal dilation. Lymphangiectasia represents superficial lymphatic dilatation caused by a wide range of scarring processes. It is characterized by lymphatic vessel dilation, chronic diarrhea and protein loss. The most common cause of lymphangiectasia is considered to be the congenital malformation of the lymphatics. Secondary lymphangiectasia may be caused by granulomas or neoplasia causing lymphatic obstruction, or increased central venous pressure causing abnormal lymph drainage. Inflammatory bowel disease can also lead to lymphangiectasia by migration of inflammatory cells through the lymphatic vessels. Chronic diarrhea is almost always associated with lymphangiectasia, but most other signs are linked to low serum protein levels (hypoproteinemia), which causes low oncotic pressure. Weight loss is also observed in patients with chronic evolution.[3]

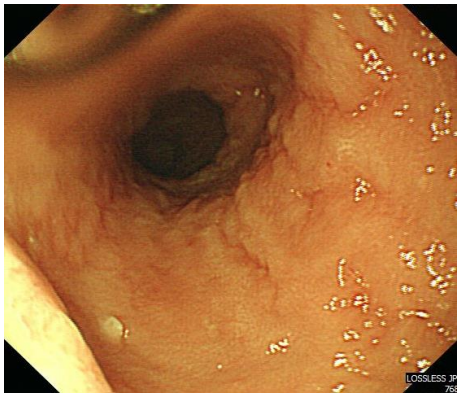

Fig. 5. Lymphangiectasia

Considering the association between the presence of lymphangiectasia and speckles in ultrasound examination, it is recommended to thoroughly examine a patient by using all available tools like endoscopy, ultrasound and radiology.

Conclusions

The study allowed to identify 23 cases of hyperemia (45.1%), gastric edema in 2 patients (3.9%), increased mucosal friability in 12 dogs (23.5%), the presence of gastric hemorrhage in 21 patients (41.2%) and the presence of ulcers in 20 cases (39.2%).

Along with ultrasound and radiological investigations, endoscopic examination is the main diagnostic complementary method that provides data regarding the appearance, structure or size of investigated organs. Unlike other techniques that are non-invasive, endoscopic techniques have some degree of invasiveness because it may cause discomfort to the patient. Taking account of special diagnostic value of this technique, the use of endoscopic examination has expanded in recent years, practitioners supporting the use of endoscopy in pets.

Endoscopic examination helps in formulating a correct, quickly diagnosis, by viewing the changes of the gastrointestinal tract and also by confirming or refuting the initial diagnosis after the microscopic examination of samples.

All these attributes of endoscopic technique have allowed the use of endoscopic examination as a top complementary method to diagnose gastrointestinal disorders in dogs. The use of endoscopic examination have favored the assessment of digestive changes from another perspective, the clinician being now able to observe the gastrointestinal tract lesions directly.

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Lion (Panthera leo) particularities in individuals born and hand reared in captivity

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Abstract

Considering the fact that evolution of species is driven by habitats and the reproduction is a complex phenomenon interfered or influenced by many factors, a reproduction program for captive carnivores is a changeling and many programs cannot afford experimental failure. Captive carnivores pose a challenge to all institution involved in their conservation, presenting a broad pathology from diseases to poor welfare and breeding problems. Infant mortality is primarily caused by inadequate maternal behavior, either active or passive it can be connected to biological factors as well as to individual traits such origin, if they were wild- caught of captive – born. This is the main reason for research team approach in their reproduction program, hand rearing the infants. The present article presents the challenges faced by research team in their efforts to rear two lion infants, from different conceptions. The litters belonged to Barlad zoo, Vaslui County, from eastern part of Romania. Both parents were born and reared in captivity, donated to the institution during year 2014, at 3years of age, both hand reared by donor. During cubs hand rearing we developed a nutrition plan for optimal development of the infants, exposing ours mistakes has educational purpose for others so they avoid them in future.

Key words: *IUCN (International Union for Conservation of Nature), Taurine deficiency, lion hand rearing, retinopathy, Gimcat*

Introduction

The lion (*Panthera leo*) is one of the big cats in the genus *Panthera* and a member of the family *Felidae*, and from immemorial ages has represented one of the ambassador species, kept in captivity from touristic, educational and preservation purposes. At present time more than 1000 African and 100 Asiatic lions are present in zoos and animal parks all over the world.

The IUCN categorizes species according to subtle threat levels and from their perspective the lion is considered vulnerable, mainly because a population reduction of approximately 43% over the past 21years (approximately three Lion generations, 1993-2014).

Considering species decline the research group focused on gathering information's regarding in situ reproduction, and because of limited resources cannot afford experimental failure and losses. Rearing by hand the infants was the optimal approach in order to assure infants survival and reproduction program success. The offspring's were reared by personnel from the age of 2 days respectively 1 week, facing various nutritional challenges.

The decision to let the cubs with the mother as long as possible was a necessary risk, in order to obtain a minimal protection from colostrums, without passive immunization the prognosis during first two months of life is poor.

The nutritional program was formulated step by step, learning from mistakes, and must be noted the fact that on the market there are no available commercial products formulated for lions and the personnel was forced to improvise (Allen, M. E., Ullrey, D. E., & Edwards, M. S. 1999).

Materials and methods

The research group faced the challenges of hand rearing two *Panthera leo* infants from different litters, successfully raising them till maturity. The lions belonged to Zoological Garden Barlad, both adults used for reproduction are 5 year old and entered in zoo collection during year 2014 by donation (Figure 1).

A **B**

Figure 1 The lion´s parents: **A** Female Sheila; **B** Male Isac

The adults are fed with horse flesh, usually fresh meat, the exceeding flesh being stored in frigorific boxes. For dental health the lions are periodically supplied with bones from carcasses (femur, humerus, spinal cord) (Altman J., Gross K., Lowry S.2005).

The cubs belonged to different gestations and for the first days of life stayed with the mother; it was risky but they receive the majority of maternal immunity from colostrums (De Waal H. O., Osthoff G., Hugo A., Myburgh J., & Botes P. 2004), they were closely monitored by personnel and the decision to be removed was based on lack of interest the female had towards the neonates, the first cub had 627g at the time of withdrawal, two days of age (figure 2). The second cub was kept with the mother for a longer period, at the time of withdrawal weighted 1570 g at two weeks of age (figure 3).

After removal of the cubs and first clinical examination it was imperative to ensure the body temperature using infrared bulbs, mainly because the thermoregulatory center is not yet mature (hypothermia can be one of leading causes of death at this age)(Najera F., Revuelta L., Kaufman K.J. 2011).

In order to check the suckling reflex the first feeding of the neonate was done using an electrolyte, reducing the risk of aspiration into the lungs (Hedberg G., Gage L. J. 2008).

In the infant's nutrition were used six different feeding schemes meant to assure optimal nutrition, these formulas varied with age, physiological needs and digestive pathology encountered (Clauss M., Kleffner H., Kienzle E. 2010).

The main difference in formulas is the content in amino acids, more specifically the presence of taurine, essential to the development of felids, deficiency being associated with retinopathy and heart disease.

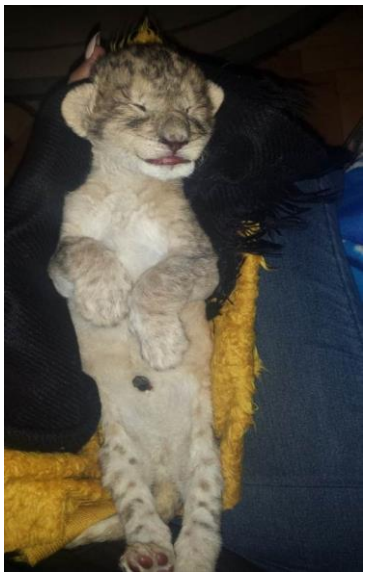

Fig. 2 King Paraschiv

Fig. 3 Thor

Scheme I cow milk 3.5% fat 40ml, cream 20% 10ml, egg yolk 7g, honey 3g (first days of life)

Scheme II Animal milk powder Can Lait (first two weeks),

Scheme III Milk powder for cats with taurine GimCat (alone till 4weeks of life),

Scheme IV Milk powder for cats with taurine Gimcat with cow sweet cheese and powdered Royal canin Babycat (until age of 10 weeks),

Scheme V Milk powder for cats with taurine Gimcatwith cow sweet cheese, powdered Royal canin Babycat and ground horse meat from age of 10 to 13 weeks),

SchemeVI raw horse meat with fresh eggs (from age of 13 weeks).

In the first week of life the feeding interval was 3h day and night. The first feeding scheme was used for the first days of life.

The Can Lait was used for the rest of two weeks, 60-80ml to each 3 hours day and night, the change in diet was necessary due to felines special need in a diet with a higher taurine content (Hedberg G. E., Dierenfeld E. S. And Rogers. Q. R., 2007).

The second choice in milk formula was the Gimcat plus taurine (table 1), used from third week.

Table 1. Nutritional values table in Gimcat -Analytical components (source http://www.gimcat.info/en/Product/vitamins/taurine/cat-milk-plus-taurine.html)

Protein	35 %	**Composition:** Milk and dairy products (63.7%), oils and fats (oil containing arachidonic acid 0.21%), vegetable by-products, lactose derivative with TGOS* (1.0%), minerals *Trans-galactooligo saccharide from milk sugar derivative
Fat content	27 %	
Crude ash	6 %	
Raw fibre	0.1 %	
Moisture	6 %	
Calcium	0.9 %	
Phosphorous	0.5 %	
Sodium	0.4 %	

From 8 weeks of age considering the rising demand for nutrients as quantity and complexity we added cow sweet cheese and powdered Royal canin Baby cat. Because of the increased consistency of portions the feeding intervals were changed to 4h during day time and 6hours during night. The administered quantity was 120-150ml (scheme IV).

After two weeks we added horse meat firstly grounded later diced meat, number of meals decreased to 4, one represented by raw meat. At 3 weeks from diversifying the diet, from 3 feedings with milk and one with meat we reached to a single milk feeding and the rest of them with meat.

From age of 13 weeks the meat was served as big chunks twice daily (500-600g per portion) (Vester B. M., Burke S. L., Liu K. J., Dikeman C. L., Simmons L. G., Swanson K. S. 2010).

Considering the additives contained by the milk, its removal from the diet can prone the developing of organism's to vitamin and minerals deficiency. So the use of vitamin- mineral compounds should be considered to compensate the eventual imbalances (table 2) (Howard J., Rogers Q. R., Koch S. A., Goodrowe K. L., Montali R. J., Bush R. M. 1986).

In the first litter we encountered an episode of juvenile idiopathic panosteitis, around age of 4 month the cub started to limp, accusing knee and elbow joints pain, refusal to move and mourn during movement. The medication used consisted in Arthro vet Complex, Glycoflex and Osteocare syrup.

The treatment was kept for 30 days and resumed after a 14 day pause. Beneficial effects were encountered after fifteen days of treatment, discomfort diminished and the cub resumed physical activity without showing any pain or stress.

Part of the preventive medicine is parasites and infectious disease protection. At the age of 6 week, the first prophylactic deworming was done using, Merial Broadline Spot on containing: Fipronil, S-methoprene, Eprinomectin, Praziquantel (product for cestodes, nematodes and ectoparasites).

We draw attention to the main diseases mentioned to be evolving in captive and wild lion prides: canine distemper, panleucopenia, calicivirus, rhinotracheitis, feline leukemia and immunodeficiency virus (Endo Y., Uema M., Miura R., Tsukiyama-Kohara K., Tsujimoto M., Yoneda K., and Kai C., 2004).

Therefore we used a tetravalent vaccine produced by Merial the PUREVAX feline 4 vaccine, for Feline Rhinotracheitis-Calici-Panleukopenia-Chlamydia Psittaci Vaccine Modified Live Virus and Chlamydia,the inoculations begun at the age of 8week and followed by boosters at 10 week, 12 week, 6month and 1 year. The presented protocol refers to animals that will be kept in captivity and are reared by personnel, in the animals feed by mother the immune response is different because of the interference represented by passive immunity (Hofmann-Lehmann R., Fehr D., Grob M., Elgizoli M., Packer C., Martenson J. S., O'Brien S. J., Lutz H., 1996).

Table 2. Gimcat Additives
(source http://www.gimcat.info/en/Product/vitamins/taurine/cat-milk-plus-taurine.html)

Components	Quantity
Vitamin A	20,000 I.E./U.I.
Vitamin D3	2,000 I.E./U.I.
Vitamin E	100 mg
Vitamin B1	10 mg
Vitamin B2	10 mg
Vitamin B6	8 mg
Vitamin B12	60 mcg

Vitamin K3	0.2 mg
Biotin	200 mcg
Folic acid	2 mg
Niacin	80 mg
Pantothenic acid	20 mg
Vitamin C	100 mg
Choline chloride	2,500 mg
Taurine	1,000 mg
L-Carnitine	400 mg
Copper as copper-(II)-sulphate	5 mg
Iron as iron-(II)-sulphate	90 mg
Zinc as zinc sulphate	50 mg
Iodine as potassium iodide	1 mg
Manganese as manganese-(II)-sulphate	5 mg
Selenium as sodium selenite	0.1 mg
L-Arginine/L-arginine	11.6 g

In the rearing process the success is granted by providing to the cub a proper socialization, once the ear canals are open and environmental temperature allows, the cub is introduced for brief periods of time in the enclosure next to the adult facilities in order to smell and hear the rest of the group. In time the cubs will live with the entire pride. The whole process is meant to assure a safe introduction of the cub in the pride without the risk of being injured by an adult (Read, W. R., and J. E. Meier.,1996).

Results and discussion

Considering researches carried out in Barlad Zoo, county Vaslui, eastern part of Romania, on two lion cubs, from different liters, we managed to obtain following results.

Hand rearing the cubs was not an option at the very beginning, that is why we used the can lait as a substitute till the final milk option arrived (with a more suited and complete formula for lions nutritional challenges).

In the first week of life the feeding interval was 3h day and night.

During the second and third week the feeding intervals are at 3 hours during daylight and at 4 hours during nighttime.

From the fourth week the cubs were fed every 4 hours during the day and every 6 hours at night, six average feedings per day.

In the second litter the difference was obvious, the cub having a better start with an improved weight gain, must be mentioned the fact that period of time spend with his mother was up to one week fact that provided a better immune response. The second cub is more active with an improved weight gain and psychosomatic activity.

The neonate's requirements presume an intake between 10% and 20% of its body weight, a daily ration greater than 35%of body weight can cause digestive disorders.

Even if the second cub spent more time with his mother with a more suited nutrition formula (maternal milk), its weight gain was limited, and at the time of withdrawal he had only 1570g. In his case the Gimcat milk was used from the very beginning, and its qualities are reflected in the weight at 4weeks of age. Must be mentioned that female interests in cub decreased gradually and at the time of withdrawal the infant was dehydrated, but tolerated very well the substitute milk, with a good appetite from the start.

The improved condition of the second cub is presented in table 3 and in the figure 4.

Accumulating experience with two different litters we consider hand rearing of these cubs satisfactory from the point of view of psychological and behavioral outcome. The milk formula and weaning procedure provided good results, correcting the mistakes from the first litter the results were improved in a noticeable manner.

The first feeding scheme was used for the first days of life, with poor results probable because of the high carbohydrates content.

Weaning in captive felines represents one of the critical moments, mainly because some cubs poorly tolerate solid food, this is the main reason for introducing gradually various solid foods.

We believe that postponing the weaning we assured a better start for the infants. In the second litter we avoided homemade milk substitute and royal canin powdered biscuits, with better results and no digestive disturbance.

The team formulated a program able to provide repeatable results in hand rearing large carnivore's infants, in order to provide their survival, under captivity conditions.

We believe in improving the milk formula order to obtain a more suited replacement for maternal milk, as composition and digestibility (the formula used is close but improvable).

Table 3. Weight evolution in different litters

Age	Weight cub litter 1	Weight cub litter 2
At birth	627 g	-
1 week	1450 g	-
2 weeks	2200 g	1570g
3 weeks	3000 g	3800g
4 weeks	4200 g	5100g
6 weeks	5500 g	6580g
8 weeks	6700 g	8000g
10 weeks	7300 g	9007g
12 weeks	8500 g	11000g
4 months	9200 g	13000g
5 months	12 000 g	15400g
6 months	17 000 g	21000g

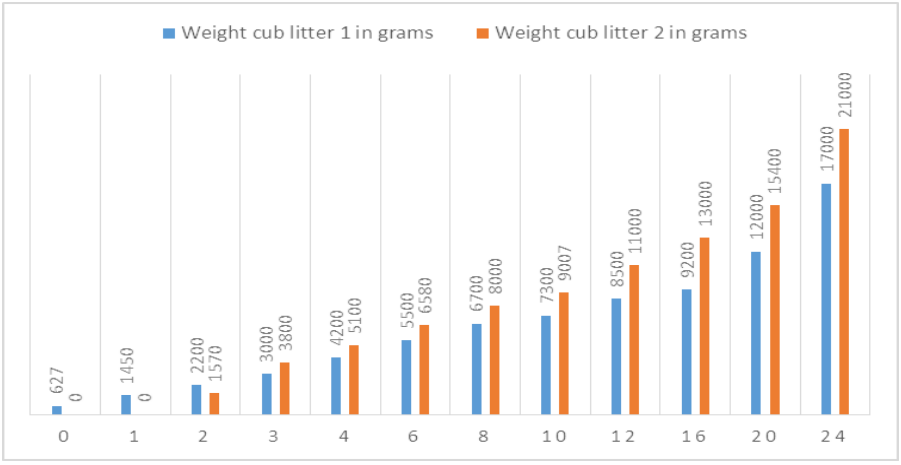

Figure 4 Weight evolution in different litters

Conclusions

1. The most suited method for lion rearing in captivity is hand rearing, this method avoids neonate's death due maternal lack of care.
2. From the products used for hand rearing, the most effective were industrial those with taurine supplementation.
3. The most difficult moment in the feeding program is represented by the raw meat introduction.
4. All feeding programs must be adjusted according individual needs, a proper physiological monitoring is demanded. Weight and neonates continuous evaluation is needed, the most important individual in the scheme is the caretaker.

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Lion Thor Lion King Paraschiv

Lipoma in cockatiel (*Nymphicus hollandicus*) -A case report-

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Abstract

In the past years the cockatiel parrot as pet has risen in number and consequently the pathology related to captivity conditions increased. The present paper describes a lipoma case in cockatiel (Nymphicus hollandicus), a female age 5years old with a formation located in left wing, carpal region. Following clinical exam, the 1x1.5cm tumoral formation was identiffid and surgical excision was recomanded. The owner refuzed the surgergical procedure and returned after 2 months with the bird accusing a deteriorated condition and enlarged tumor measureing 2x3cm. After cockatiel death the tumor was examined histopathologically. The final diagnostic was benign tumor well delimitated by surrounding tissues – a lipoma located in subcutaneous tissue from left carpal region.

Key words: *cockatiel(Nymphicus hollandicus), lipoma, subcutaneous tumors*

Introduction

The name of the nymph parrot comes from the greek "*Nymphicus*" wich means bride. As temperament the nymphs are very gentle, cheerful, affectionate, curious, sociable, loving the company of man but also of other birds and they like to be the focus of attention.

The feeding of captive birds should be similar to that of their natural environment, which is an essential condition.

Water and feed can be a frequent source of bird illness by direct and indirect transmission of pathogens, from diseased to healthy birds, but also through limited intake of vitamins and minerals.

The majority of birds living in captivity are fed with various types of food of plant origin: mixtures of gramineous and oleaginous seeds, greens, fruits but also roots. Gradually animal products such as eggs, cheese, insects, ants can be introduced into the feed.

For a bird's ration to be complete, we can also introduce an assortment of mineral salts, shellfish, bones, and egg shells.

Another important factor in feeding these birds are mineral salts, which are very important for strengthening the bone system and physiological functioning of the body.

These mineral salts are administered using egg shellfish, bone meal, fodder chalk and shellfish (cuttlefish).

Depending on each species, a bird's needs are different, ranging from cage size, nutrition, microclimate, stress factors, and the cage location.

Any microclimate or diet imbalance produces stress and it can be seen in the bird's health condition (Cardoso F. and all, 2013).

Lipoma is a pathology that has as main causes the fat deposits due to excessive nutrition and hipovitaminosis E and A (Tanase I.O., 2016).

In the following work, a case of lipoma localized and diagnosed at a nymph is described.

Materials and methods

Within the discipline "Pathology of exotic and recreational animals" a 5 year old bird from the "*Nymphicus hollandicus*" species was presented for examination, having a formation at the left wing level.

This formation has doubled in size for the last two weeks. There were no feathers on the surface of the formation, because the nymph was pecking and the area was slightly hyperemised.

After the clinical examination, treatment with Clorhexidine aqueous buffer was applied only to ensure good hygiene (Tanase IO, Daraban F., 2015) at the level of the formation, because the owner did not take responsibility for a possible surgical extirpation of the formation.

After a period of two months, the owner returned with the nymph, the general condition of the bird worsened and the wing formation reached the size of a nut.

The second day the bird died, fragments were collected from the formation and a histopathological examination was performed to elucidate the type of lesion (Lightfoot T. and all, 2006).

Results and discussion

Following the clinical examination of the 5 years old nymph, it has been found that, this presented in theskin of the left wing a nodular formation of the size of a peanut (fig. 1).

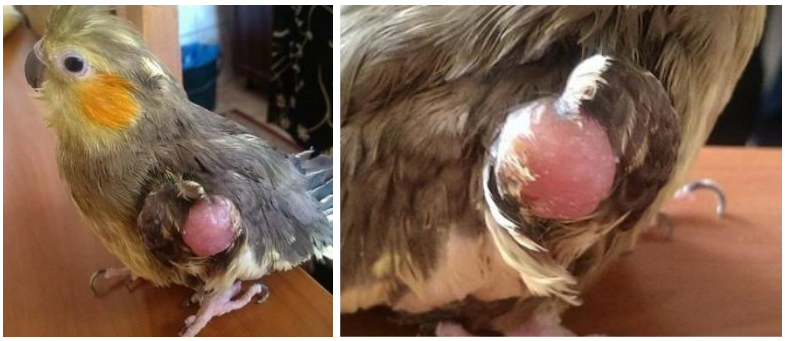

Fig. 1 Nymph with nodular formation on the left wing

Due to this increased formation at the wing level, the bird was apathetic, refused to move and had a capricious appetite.

After a period of two months, during which the affected area was treated conservatively, the owner returned with the nymph, because she stopped eating, was apathetic, presented horiplumation and the formation at the wing level reached the size of a walnut and had a dry appearance (Figure 2, Figure 3).

Fig. 2 Nymph with a modified general condition

Fig. 3 The macroscopic aspect of the formation

After the bird death, a glossy, light-colored appearance with many infiltrations, a fatty aspect and a tough consistency were observed on the sectional area. (Figure 4)

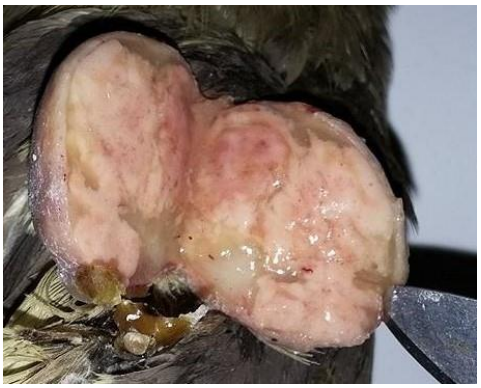

Fig. 4 Macroscopic aspect by section

After the histopathological examination, the presence of crucified tissue and dermo-epidermal inflammatory infiltrate was found on the tumor surface (Figure 5). Figure 6 shows cutaneous hyercheratosis and the corneous layer is well represented and largely desquamated. In cage birds, the main cause of cutaneous hyperkeratotis is represented by hypovitaminosis A and E (Paunescu I.C., 2007).

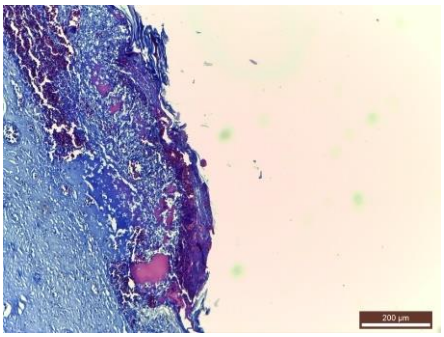

Fig. 5 Epidermal crust – inflammatory aspect dermo-epidermal; x100; HEA staining

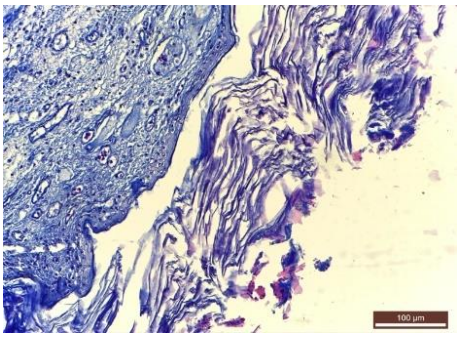

Fig. 6 Skin hyperkeratosis, well represented stratum corneum; x200; HEA staining.

The histopathological examination revealed the structure of a benign tumor of a lipoma (Cowan M.L. and all, 2011). Lipoma is a benign, mesenchymal polygonal tumor consisting of well-differentiated adipocytes and a discrete stroma (Figure 7). Tumor masses showed lymphocytes, eosinocytes and fine neoformation capillaries (Figure 8).

The lesion was represented by the tumor formation, which was placed in the subcutaneous tissue and well delineated by the adjacent structures, through a thick connective capsule consisting of fibroblasts and collagen fibers. Large sized neoformation vessels were found in the capsule (Bradford C. and all, 2009).

Lipo-epidermal prominences were located at the surface of the lipoma, consisting of dermal conjunctival proliferations and epidermal hyperkeratosis. All of these changes are due to hippocampynosis A.

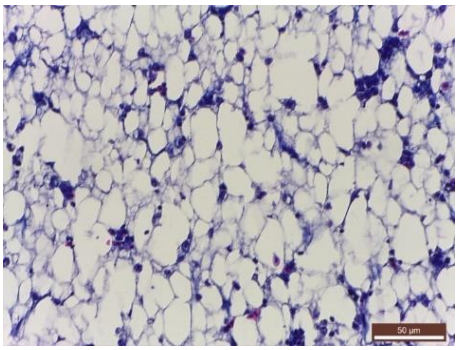

Fig. 7 Benign tumor with well-differentiated adipocytes, discreet stroma; x100; HEA staining

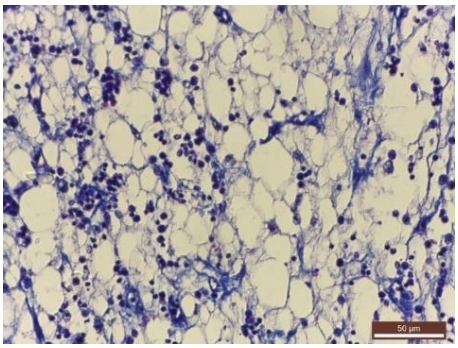

Fig. 8 Lipoma - benign tumor, fine capillaries of neoformation; x400, HEA staining

Conclusions

Following the examination of a cage bird "Nymphicus hollandicus", presented during the consultation in the „Pathology of exotic and recreational animals" discipline, the following can be concluded:

1. The bird presented a nodular formation on the left wing wthat doubled the size in two months, reaching the size of a nut.
2. The macroscopic formation was smooth and glossy on the section, with a fatty aspect.
3. Microscopic epidermal hyperkeratosis with voluminous corneum was identified, the cause being hippocytaminosis A.
4. Following histopathological examination, the diagnosis was lipoma (benign tumor), localized in the subcutaneous tissue of the wings and well defined by the adjacent structures.

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A case of canine malignant histiocytoma

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Abstract

A 13 year-old mixed-breed male dog was presented for a second opinion at the veterinary clinic with a tumor of approximately 15 cm3 on its abdomen. Fine-needle aspiration and cytological examination revealed moderately and distinctly displastic mesenchymal cells. Abdominal radiographs showed the extent of the tumor, which had developed mostly inside the abdomen. Radiography and CT revealed possible metastasis to the lungs. A diagnosis of malignant histiocytoma was made. The tumor was surgically removed at the owner's request, but the dog died 5 days later. We follow with a case discussion, as well as the treatment and prognosis for this type of tumor.

Keywords: *malignant histiocytoma, dog, abdominal radiographs, metastasis, histiocytic sarcoma*

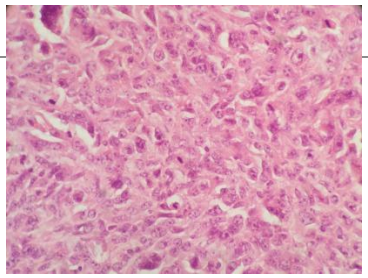
Introduction

The histiocytic sarcoma complex (HSC) is a group of neoplasms characterised by the proliferation of dendritic cells of either Langerhans cell or interstitial dendritic cells lineage that affects both dogs and cats, although the disease is more infrequent in cats (Klopfleisch R, 2016; Moore et al., 2006). Dog breeds more commonly affected by HS are the Bernese Mountain Dog, Flat-Coated Retriever, Rottweiler, Golden Retriever and perhaps miniature schnauzer (North S, Banks T, 2007; Lenz JA et al, 2017; Abadie et al, 2009). The HSC manifests under three forms: as localised lesions of single organs, as disseminated lesions in multiple organs or as hemophagocytic histiocytic sarcoma (HHC), a particular form arising from splenic macrophages (Withrow SJ et al., 2013; Moore PF, 2014).

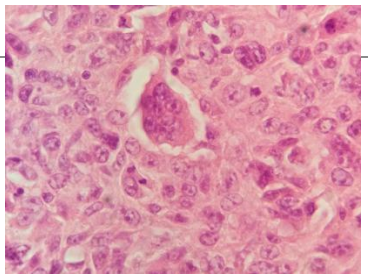
The first form, called histiocytic sarcoma, is usually localised in the spleen, lymph nodes, lung, bone marrow, skin and subcutis, brain or the articular tissue of appendicular joints (Moore PF, 2014). It is composed of highly pleomorphic round cells, varying in cell and nucleus size and ratio (Withrow SJ et al., 2013).

The second form, formerly designated as malignant histiocytoma (currently disseminated HS) occurs as more than one lesion in a single organ that rapidly spread to other locations (Moore PF, 2014). It has a more heterogenous appearance, comprising round, oval and spindle-shaped cells that are less pleomorphic but present more morphological features of malignancy (Withrow SJ et al., 2013; Moore PF, 2014). Other authors describe the disseminated form as the progression of the localised form beyond the regional lymph nodes, commonly to the lung, spleen, and lymph nodes (Klopfleisch R, 2016).

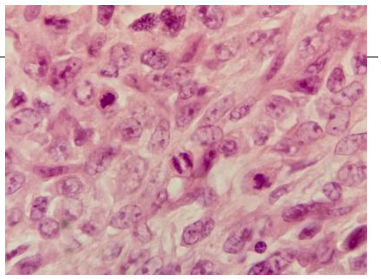
The third form, hemophagocytic histiocytic sarcoma, is the most distinctive; it derives from splenic macrophages and localises in the spleen, liver, bone marrow, and lung (Klopfleisch R, 2016). The cells are hard to differentiate from macrophages found in inflammatory lesions, as they can present little to no malignancy features and it clinically manifests as a hemolytic anemia that does not respond to the use of immunosuppressives (Withrow SJ et al., 2013; Moore PF, 2014).



Neoplastic structure, HE, 400x - Abundant neoplastic cells with marked pleomorphism. Source: prepared by the authors



Neoplastic structure, HE, 1000x - Mesenchymal cells with a malignant morphology - multinucleated cancer cells. Source: prepared by the authors.



Neoplastic structure, HE, 1000x - Mesenchymal neoplastic cells: marked anisokaryosis, euchromatic nuclei, evidently nucleolated nuclei, atypical mitoses. Source: prepared by the authors.

Figures 1-3. Histologic aspects of Gimmi's histiocytic sarcoma. Source: prepared by dr. Teodoru Soare.

Localised and disseminated HS present as white masses with a smooth cut surface, but they can also present red mottling (due to hemorrhage and necrosis), usually with distinct and uncapsulated margins, and differentiate from the hemophagocytic variant, which appears as a diffuse infiltrate in the affected organs (Meuten DJ, 2016; Klopfleisch R, 2016).

Clinical signs depend on the affected organ(s), but are generally non-specific (anorexia, lethargy, malaise, weight loss) (Klopfleisch R, 2016). The mass effect of internal tumors can generate signs from unaffected organs (Klopfleisch R, 2016). Paraclinic findings might include a mild anemia (HS, diffuse HS) or a severe anemia (HHC), thrombocytopenia, hypoalbuminemia and rarely neutrophilia, hypercalcemia or hyper-gammaglobulinemia (Klopfleisch R, 2016, Argyle DJ et al, 2008). As hyperferritinemia seems to be common in dogs with HS, ferritin may be a useful serum biomarker for this neoplasm (Friedrichs et al, 2010).

Treatment options are wide surgical excision and chemotherapy (Meuten DJ, 2016, Klopfleisch R, 2016). The localised form is curable with surgery, if the lesion is detected early; once the disease spreads the treatment is palliative chemotherapy (Meuten DJ, 2016). Chemotherapy for disseminated HS with lomustine, an alkylating agent, at 60–90 mg/m^2 may prolong survival times in responsive dogs (Klopfleisch R, 2016; Skorupski KA et al, 2007; North S and Banks T, 2007). Epirubicin, dacarbazine and other substances can be used in dogs that do not respond to lomustine with variable results (Mason SL, 2017; Kezen KA, 2017, Moore AS, 2017). The prognosis is poor for all forms except localised HS (Klopfleisch R, 2016, Meuten DJ, 2016; Dervisis NG, 2016; Moore AS, 2017).

There is a report on the successful treatment of 4 cases of canine disseminated HS with the human major histocompatibility complex nonrestricted cytotoxic T-cell line TALL-1041 (Vissoneau S et al, 1997), but this is option is not currently widely available.

Materials and Methods

Complete blood counts were performed in-house using a Mindray BC-2800 Vet automatic hematology analyzer. Blood biochemistry was performed in-house using a Rayto RT-1904C semiautomatic chemistry analyzer. Cytology and histopathology were performed Dr. Teodoru Soare at the Faculty of Veterinary Medicine Bucharest. The radiologic and CT examinations were performed by dr. Florin Grosu at 4VET Radiology Center. The ultrasonographic examinations were performed with a portable color Doppler Sonoscape S2 system by dr. Otilia Cristea and dr. Radu Constantinescu.

Case Presentation

A 13 year-old medium-sized mixed-breed male dog was presented for a second opinion at the veterinary clinic for a large tumor on it abdomen (figure 4). The dog had been neutered at the age of 2. The tumor was extremely large (approximately 15 cm^3) and had already invaded the abdomen, making the point of origin impossible to discern. Ghimi manifested an intermittent fever, having evening episodes of pyrexia with a body temperature oscillating between 40-42ºC, registering an optimal temperature during the day). The dog also presented with vomit during the febrile periods and a loose stool the next morning. Ghimi had inspiratory dyspnea, in his attempt to compensate with prolonged, deep inspirations. The body condition score was 2/5 (AAHA Body Condition Scoring Systems, 2010).

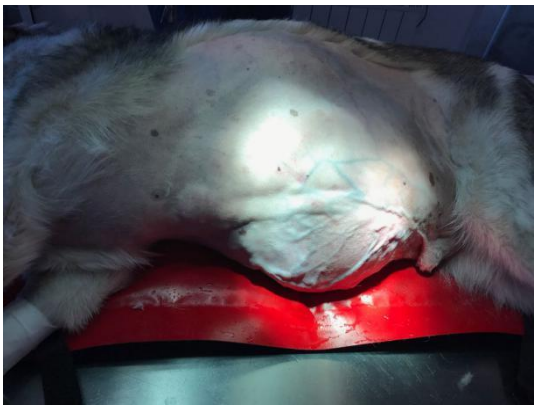


Figure 4. Preoperative aspect of the tumor. Source: from the authors.

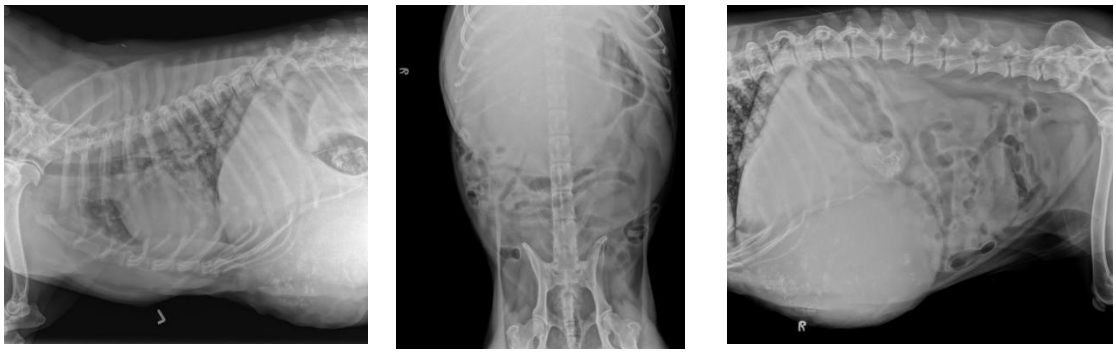
Palpation of the abdomen was impossible due to the extent of the growth. The superficial lymph nodes (popliteal, axillary and prescapular) were reactive; the reactivity of the submandibular lymph nodes could have also been due to the presence of advanced periodontal disease and infection. At this point, the dog was not eating and received supportive treatment (iv fluids, aminoacids), iv broad-spectrum antibiotics (ceftriaxone), pain medication (tramadol) and corticosteroids.

A CBC revealed a leukemoid reaction, as a physiological response to stress and infection (WBC 100.9 - reference values 6-17 K/μL), with mild lymphocytosis and intense neutrophilia, a decreased RBC count (3.64, reference 5.5-8.5 M/μL) and hematocrit (26.8 reference 39-56%) with increased hemoglobin (24, reference 11-19 g/dL). Blood biochemistry was unremarkable except for the alkaline phosphatase (1114.17, reference 10.6-100.7 U/L) and serum amylase (3965.84, reference 269.5-1462.4 U/L). By the second day of ceftriaxone and hydrocortisone hemisuccinate the dog improved, with the disappearance of the digestive signs and the improvement of the respiratory effort. Ultrasound identified a soft tissue mass of variable echogenicity due to areas of necrosis and mineralization.

Two ultrasound guided fine needle aspirates were evaluated cytologically, but due to the presence of inflammatory cells and necrotic debris, they were deemed inconclusive. They did, however, reveal a few moderately and distinctly displastic mesenchymal cells alongside red blood cells, neutrophils, macrophages and lymphocytes. A decision was taken to further investigate the patient in order to establish a conclusive diagnostic.

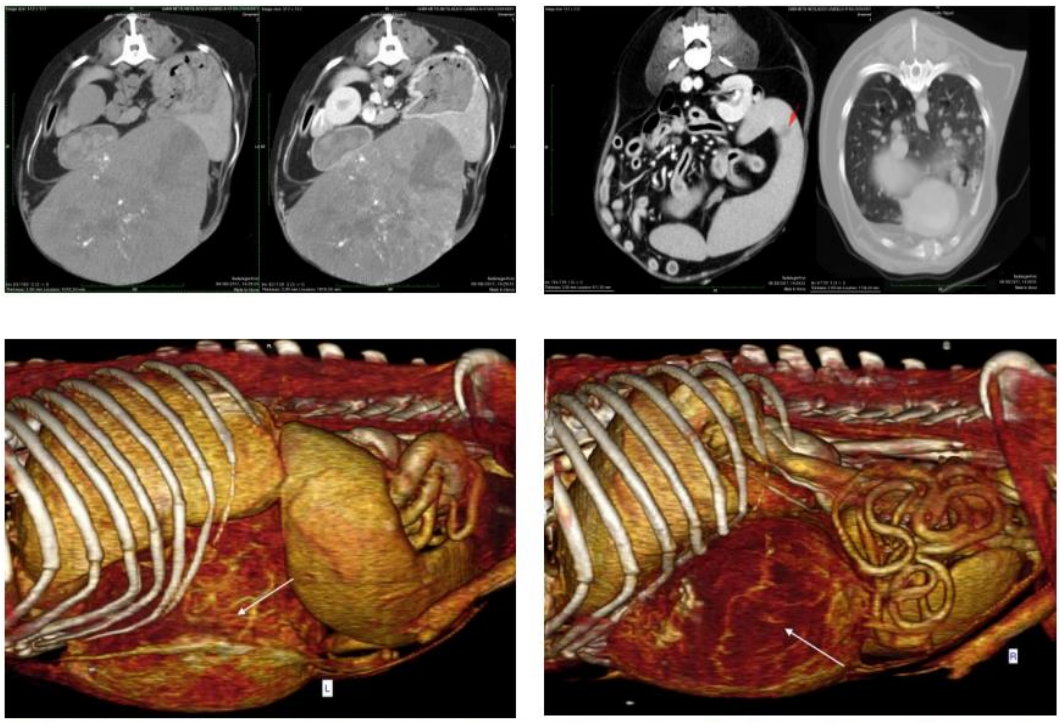
In order to evaluate the extent of the tumor and to identify the presence of any metastases, Ghimi was referred for thoracic and abdominal radiography. The radiographs revealed disseminated nodular densifications in the lung (figure 5) and the magnitude of the abdominal

tumor, which displaced the stomach, lung and intestines. The radiologic appearance of the tumor was of a macronodular densification of soft tissue with areas of amorphous calcification at the right thoraco-abdominal junction of approximately 14 cm/19 cm (figures 6-7).



Figures 5-7. Ghimi's thoracic (left) and abdominal (center, right) radiographs. Note the presence of nodular lesions in the lung, probably lung metastases and the gross displacement of the organs in the abdominal cavity.
Images courtesy of dr. Grosu Florin, 4Vet Radiology Center, Bucharest

At the owner's insistence that the animal be operated and the tumor removed the dog was again reffered for computerized tomography. The surgeon agreed to palliative surgery to remove the large abdominal tumor and improve comfort.



Figures 7-12. Top: CT images of the tumor, spleen and lungs. Bottom: 3D reconstruction of the tumor and surrounding organs. Images courtesy of dr. Grosu Florin, 4Vet Radiology Center, Bucharest.

The CT examination described a heterogenous soft-tissue tumor (figures 7-8, 11-12) located in the cranial and ventral mid-abdomen, with relatively well delineated margins of approximately 20 cm*13 cm*18 cm (L*H*D). The growth has a significant mass effect over the surrounding organs (spleen, liver, gallbladder, small intestine and colon. Its heterogenicity was due to hypoattenuation probably caused by areas of hemmorhagic fluid or necrosis, but also to areas of mineralization/calcification. The tumor filled moderately and irregularly with contrast, which permitted the identification of the tumor's origin to the right ventro-lateral abdominal wall. Its growth had remodelled the orientation of the floating ribs, whose distal half became horizontal. Both lungs presented micro and macro intestitial nodules (figure 10). On the head of the spleen there was a hypoacoustic area of 1.5-2 cm in diameter (figure 9) that did not fill up with contrast (another possible metastasis).

Before the surgery, blood biochemistry revealed an improvement in serum biochemical parameters: serum amylase decreased to half of its initial value and alkaline phosphatase decreased slightly, with the exception of urea, which doubled to 75.75 mg/dL (reference 8.8-25.9 mg/dL). CBC showed continous lymphocytosis (to half the initial value), an increased RBC count and hematocrit with decreased hemoglobin.

According to the owner's wishes, the team proceeded with the surgical excision, despite being warned of the grave prognosis and the small chances of long-term survival. The tumor was removed successfully (figures 12-15), but the dog evolved well for two days but on day three he decompensated (respiratory and circulatory decompensation) and died five days avter the intervention. A histopathologic analysis confirmed the suspicion of histiocytic sarcoma.

Figures 10-13. Intraoperative aspects. Source: from the authors.

Conclusions

Due to the extent of the disease, in Ghimi's case treatment was illusory. Currently, HS is only curable before it metastasises through wide surgical excision. But for the owner's insistence for sugery, the correct approach would have been to treat with palliative chemotherapy. A tumor this size and with such a compressive effect was not well suited for palliative surgery, as the dog decompensated and subsequently died. We recommend that any growth should be investigated as soon as it is detected and ideally, yearly check-ups should include abdominal ultrasonography.

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Diagnosing canine idiopathic hypereosinophilic syndrome

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Abstract

The idiopathic hypereosinophilic syndrome is defined as persistent eosinophilia of unknown origin. It is believed to be a reaction to an unidentified antigen or a inability of the organism to control its eosinophil production. The resultant eosinophilia is a systemic disorder that can be fatal, made manifest through the clinical signs of the affected organs. Eosinophilic invasion of tissues, associated with cytokine release and chemical mediators, determine organ damage and disfunction. Any organ can be affected, thus creating a puzzling clinical presentation. It commonly first affects the gastrointestinal tract, liver, spleen, bone marrow, lungs, and lymph nodes. Less frequently, it involves the skin, kidneys, heart, thyroid, adrenal glands and pancreas. It is believed that the Rottweiler is one of the breeds predisposed to this syndrome, alongside the German Shepherd, Siberian Husky, Alaskan Malamute and Cavalier King Charles Spaniel. We present the case of a Rottweiler with this rare disease and the steps taken to reach this uncommon diagnosis.

Keywords: *hypereosinophilic syndrome, Rottweiler, dog*

Introduction

Eosinophils are polymorphonuclear leukocytes that can be distinguished morphologically once specific secondary granules develop at the progranulocyte stage are nowadays considered pleotrophic multifunctional cells that serve complex physiologic roles (Weiss DJ and Wardrop KJ, 2010). Eosinophils develop in bone marrow and to a lesser extent in thymus, spleen, lung and lymph nodes, depending on the species, and their regulation depends on type 2 helper T (TH2) cells, which secrete IL-5 and IL-13. This includes increased production by bone marrow, mediated by IL-5 and recruitment to tissues by eotaxins, regulated by IL-13 (Weiss DJ and Wardrop KJ, 2010).

Eosinophils differentiate and mature in bone marrow over 2-6 days, depending on the species and comprise less than 10% of bone marrow nucleated cells (Weiss DJ and Wardrop KJ, 2010). The half-life of eosinophils in circulation in healthy individuals is around 1 hour in the dog. Eosinophils migrate into tissues (in particular the gastrointestinal tract and lungs), where they last for about 2 days unless anti-apoptotic factors, such as IL-5, prolong their survival for up to 2 weeks cells (Weiss DJ and Wardrop KJ, 2010; Meler E et al, 2010). Under pathologic conditions, it is possible for eosinophils to re-enter circulation (Dale DC et al, 1976). Activated eosinophils change in morphology, cell surface characteristics and functional activities (Dvorak et al, 1997). These changes usually appear after eosinophils leave circulation, but they may be found in the blood of patients with allergic disease and hypereosinophilic syndrome (Weiss DJ and Wardrop KJ, 2010).

Eosinophilia, defined as >1,500 eosinophils/μL of blood, is a frequent occurrence in dogs (Weiss DJ and Wardrop KJ, 2010). Eosinophilia occurs through inflammation and the elaboration of eosinophilopoietic factors (mainly IL-5) by T cells activated by parasite antigens or allergens (Herndon FJ and Kayes SG, 1992).

Both endoparasites and ectoparasites cause eosinophilia (Weiss DJ and Wardrop KJ, 2010). Chronic eosinophilia is associated with inflammation of mast cell-rich organs – skin, lung, GI tract and uterus, in all species, as well as with eosinophilic myositis, eosinophilic panosteitis and eosinophilic gastroenteritis in dogs (Mansfield C, 2008; Weiss DJ and Wardrop KJ, 2010). Paraneoplastic eosinophilia is caused by a variety of tumors, such as lymphoma, mast cell tumor and solid tumors, in which IL-5 and other cytokines are elaborated (Fernández-Aceñero MJ et al, 2000; Marchetti V et al, 2005).

Rarely, eosinophilia is reported after administration of certain drugs in the dog and has been associated with tetracycline and recombinant IL-2 administration (Weiss DJ and Wardrop KJ, 2010). Other causes of eosinophilia are presented in table 1. Chronic eosinophilic leukemia, a rare disease, must be differentiated from hypereosinophilic syndrome, where mild to moderate blood eosinophilia is accompanied by nonspecific tissue infiltration by eosinophils (Latimer et al, 2011) and the diagnosis depends on ruling out other causes and measuring serum IgE levels (Weiss DJ and Wardrop KJ, 2010).

Causes of Eosinophilia in Dogs and Cats

Hormonal Hypoadrenocorticism Oestrus in some bitches	**Infection** *Bacterial* *Fungal*, e.g. □Aspergillosis □Cryptococcosis *Parasites*, e.g. Aelurostrongylus abstrusus Ancylostoma spp. Angiostrongylus vasorum Capillaria aerophila Dirofilaria immitis
Immune mediated *Allergies* Atopy Feline asthma Flea allergy Food allergies Canine panosteitis Eosinophilic broncho-pneumopathy (dog) Eosinophilic gastroenteritis Eosinophilic granuloma complex Eosinophilic myositis Feline hypereosinophilic syndrome Pemphigus foliaceus	**Neoplastic** Eosinophilic leukaemia *Tumour-associated eosinophilia* □Fibrosarcoma □Myeloproliferative disease □Lymphoma □Mast cell tumour □Mucinous carcinomas □Transitional cell carcinoma

Note. Reprinted from Differential Diagnosis in Small Animal Medicine, Second Edition (p. 360-361), by A. Gough, K. Murphy, 2015, Pondicherry, India: SPi Publisher Services, Copyright 2015 by John Wiley & Sons, Ltd, Wiley-Blackwell

Table 1. Causes of Eosinophilia

Idiopathic hypereosinophilic syndrome (IHES) is described as a persistent eosinophilia of unknown origin and an increased survival of eosinophils in circulation, eosinophilic tissue infiltrates and consecutive organ dysfunction (Weiss DJ and Wardrop KJ, 2010). In humans, idiopathic hypereosinophilic syndrome is defined by sustained (over 6 months) peripheral eosinophilia of >1,500 cells/μL with no discernible cause and multiple organ involvement - gastrointestinal tract, liver, spleen, bone marrow, lungs, lymph nodes, skin, kidneys, heart, thyroid, adrenal glands and pancreas (Lilliehöök I et al., 2000; Weller PF and Bubley GJ, 1994; Sykes et al, 2001). The difference between idiopathic hypereosinophilic syndrome and eosinophilic leukemia is difficult to establish and in some cases differentiation may not be possible (Sykes et al, 2001). One difference to be considered is that the maturation of eosinophils is regular in hypereosinophilic syndrome, while marked eosinophilic left shifts and bone marrow, blood and organ infiltrates are more likely in eosinophilic leukemia (Harvey JW, 2001). One mechanism suggested for eosinophilia is the clonal expansion of T cells generating eosinophilopoietic factors (Weller PF and Bubley GJ, 1994), and this increase in IL-5 levels can prevail over the apoptotic effects of corticosteroids; eosinophilia is sometimes observed in animals with hypoadrenocorticism due to decreased or absent cortisol (Weiss DJ and Wardrop KJ, 2010).

Of all dog breeds, Rottweilers are the most predisposed to eosinophilic diseases, having increased eosinophilic values of no identifiable cause (parasitic, allergic or neoplastic) or age or

sex predisposition (Mansfield C, 2008). There seems to be a heritable component to eosinophilia (Mansfield C, 2008). Rottweilers are also the most frequently affected by hypereosinophilic syndrome. Sykes et al (2001) diagnosed 3 dogs with IHES on the basis of a lack of immature eosinophils and karyotype abnormalities (as opposed to eosinophilic leukemia), increased mean serum IgE concentration and the absence of an apparent cause. The absence of clonal karyotype abnormalities does not rule out underlying neoplasia; in human medicine, some patients with eosinophilic leukemia manifest cytogenetic abnormalities later on (Sykes et al, 2001; Rothenberg ME, 1998), therefore any patient diagnosed with IHES should be regularly monitored.

The treatment of IHES in humans is based on glucocorticoids, which suppress cytokine gene transcription and inhibit cytokine-dependent eosinophil survival (Sykes et al, 2001). Patients resistant to glucocorticoids can be treated with hydroxyurea, vincristine, interferon or cyclosporine (Perkins MC, Watson AD, 2001; Lilliehöök I, Tvedten H, 2003). In veterinary medicine, the disease is more frequently described in cats than in dogs, but due to the small number of cases reported, the prognosis and best treatment options are not yet established (Ferian PE et al, 2017). Although it can be fatal in animals presenting with severe clinical symptoms, spontaneous remission is possible (Ferian PE et al, 2017; James FE and Mansfield CS, 2009). In human medicine, the main cause of death is eosinophilic cardiomyopathy due to infiltration and subsequent myocardial necrosis, mural thrombus formation and eventually subendocardial and endocardial fibrosis, culminating with congestive heart failure due to restrictive cardiomyopathy (Perkins MC, Watson AD, 2001)..

Materials and methods

Complete blood counts were performed at Synevovet Laboratory and in-house using a Mindray BC-2800 Vet automatic hematology analyzer. Blood biochemistry was performed in-house using a Rayto RT-1904C semiautomatic chemistry analyzer and at Synevovet. The cytological examinations were performed by Dr. Teodoru Soare. The cardiac examination was performed by dr. Florin Leca at Doctor's Vet Univers. The radiologic examinations were performed at 4VET Radiology Center and interpreted by dr. Florin Grosu. The ultrasonographic examinations were performed with a portable color Doppler Sonoscape S2 system by dr. Otilia Cristea.

Case presentation

Becko, a 4 year old fully intact Rottweiler, was presented to the vet for malaise and a loss of appetite. The clinical examination revealed fever (40ºC), tachycardia, tachypnea, generalised lymph node reactivity and a distended abdomen. Becko had always been correctly vaccinated and given internal and external parasite preventives.

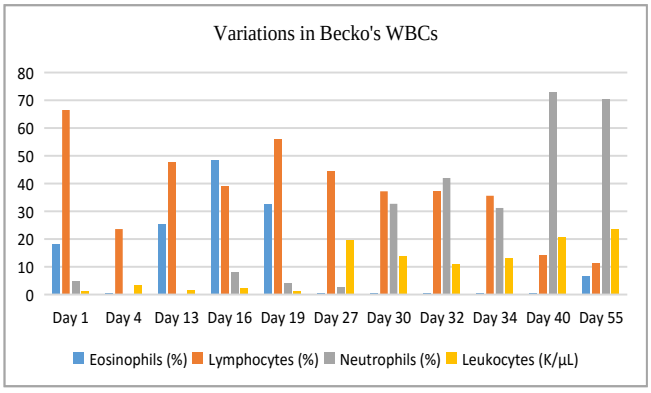

Figure 1. Significant WBC changes over time

An in-house complete blood count (CBC) revealed intense neutropenia and eosinophilia, monocytopenia, lymphocytosis, mild non-regenerative anemia, decreased hematocrit and hemoglobin. The biochemisty revealed decreased albumin, increased total protein (TP) and mild hypocalcemia. Troponin I was 0.01 ng/mL (reference <0.08) showing no signs of myocardial injury. Blood cytology identified no signs of parasites/bacteria or hyperplastic/neoplastic cells.

Ultrasonography of the abdomen revealed an enlarged but homogenous spleen and reactive abdominal lymph nodes. The dog was started on intravenous ceftriaxone and subcutaneous dexamethasone alongside supportive treatment.

The next day, the dog's state deteriorated and a procalcitonin titer of 2.5 ng/mL was obtained (reference value <0.5 ng/mL), supportive of a systemic infection and a high risk of sepsis. The dog presented with fever, lethargy, tachypnea and tachycardia. Becko responded to the treatment (his temperature dropped to 39ºC and he started to eat) after 4 days and the iv antibiotic was replaced with oral cephalexin. Dexamethasone was administered daily for 2 weeks beginning on the first day. Compensatory tachycardia and tachypnea continued despite the absence of fever due to the ensued anemia (figure 2).

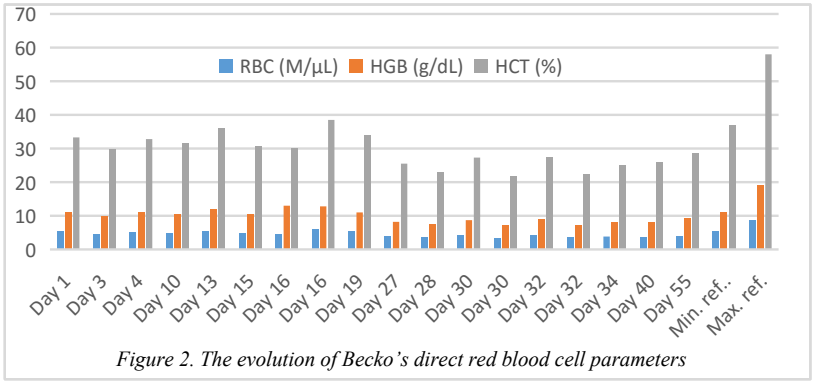

Figure 2. The evolution of Becko's direct red blood cell parameters

The cytologic examination of a popliteal lymph node aspirate revealed a heterogenous population of small, medium and large lymphocytes and plasmocytes and no evidence of neoplastic cells in the examined slides, consistent with a reactive lymph node. The superficial lymph nodes continued to be clinically enlarged and reactive for approximately 5-7 days.

On day 8, after 4 days on oral antibiotics, the fever returned (41ºC). Becko was once again not eating and lethargic and his procalcitonin level was 2 ng/mL. He restarted iv ceftriaxone and was administered one dose of Theranekron, a homeopathic remedy prepared from the spider Tarantula cubensis, for its antiinflammatory properties. Blood biochemistry revealed low albumin, increased total protein, creatine kinase and alkaline phosphatase. The CBC revealed a mild regenerative anemia (RBC 4.9 M/µL, HGB 10.6 g/dL, HCT 31.6%) which gradually worsened over the next weeks (figure 2). Supportive treatment was continued throughout the period the dog was not eating on his own.

As the fever continued, Becko was reffered for thoracic radiographs and a cadiac examination to exclude the possibility of bacterial endocarditis. The X-rays revealed a bronchial pattern indicative of a infectious or inflammatory disease and a physiological vertebral heart score. At the time of examination, the cardiologist identified a heart rate of 137 bpm, a capillary refill time of 2 seconds, normal mucous membrane color, no abnormalities of the peripheral pulse and normal breath sounds. With an increased PQ interval, Becko was diagnosed with a first degree atrioventricular block and scheduled for quarterly examinations. Ecocardiography did not reveal any changes of the heart or its function.

Two weeks after the initial episode, Becko was put on intravenous levofloxacin and meropenem, as his fever stopped responding to ceftriaxone. Despite being treated with dexamethasone, Becko had a marked eosinophilia and dexamethasone was replaced with prednisolone. The eosinophilia was suspicious, as Becko's mother and 3 other male brothers had a history of an unexplicably increased eosinophil counts. We step by step investigated and ruled out the causes of eosinophilia (see table 1). The serum IgE level was determined twice with two week interval and was found to be normal, indicating that an allergic process is unlikely to be present. Toxoplasma gondii IgG and IgM (Synevovet) titers were <1:100 and considered negative. Repeated SNAP 4Dx Plus tests (IDEXX Laboratories, Inc.) were negative to all six vector-borne diseases. Coproparasitologic examinations were performed on three consecutive days and at 7, 10 and 14 days using feces from each stool, from three different areas and from each fragment whose colour or texture were modified and they were all negative. Due to the continuous anemia, we tested a blood sample for the presence of Haemobartonella antigen but the test was negative. The dog had been eating the same food for over a year, there was no sign of gastroenteritis, any difficulty walking or any skin lesions. Eosinophilia was intermittently observed despite no evidence of allergic disease or other causes and treatment with corticosteroids (figure 3). Ultrasonographically, there was moderate hepato- and splenomegaly with a diffuse variation in echogenicity and slightly irregular margins. The abdominal lymph nodes were no longer visibly enlarged.

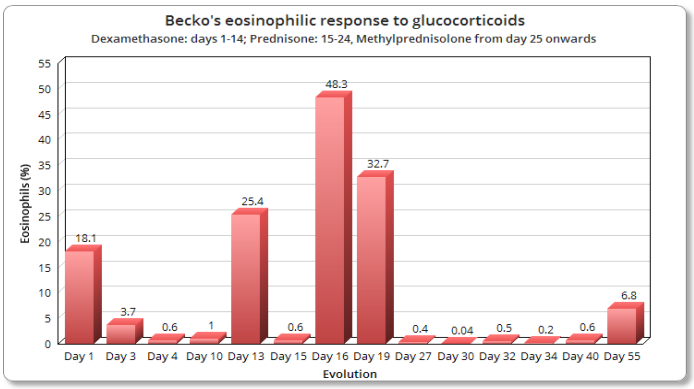

Figure 3. Eosinophilia in response to glucocorticoids. The blood sample on day 1 was obtained before treating with dexamethasone.

The antibiotic was continued for 10 days and Becko showed signs of improvement on the second day of this regimen. His evolution was favourable and after a few days he was given oral methylprednisolone at 0.5 mg/kg, dose which was gradually increased to 2 mg/kg.

Taking into account the history, paraclinical evidence and ultrasonographic changes, and considering Becko's familial history, we suspected a case of idiopathic hypereosinophilic syndrome, overrepresented in Rottweilers. Due to the case's evolution, the previous septic and inflammatory processes, the unexplicable fever and anemia and the continuous CBC variations, a bone marrow aspirate was submitted for a cytologic examination. The slide revealed a normal myeloid:erythroid ratio of 2:1 and the presence of all precursor cells for the erythroid, lymphoid and myeloid lineages.

The confirmation came when the result indicated that over 28% of the myeloid cells were eosinophil precursors (in-house reference: <5%) and no signs of neoplasia in the examined cells. At the same time, concomitant blood cytology did not reveal eosinophilic precursors in the blood stream, which excluded, for now, the possibility of a eosinophilic leukemia. Becko's mother and brothers had had episodes of unexplicable eosinophilia on yearly routine CBCs. In particular, the

mother, which had been tested before each mating, recorded values which were never less than 5% eosinophils, manifesting a seasonal pattern with higher values in spring and autumn, when she would reach > 10% eosinophils.

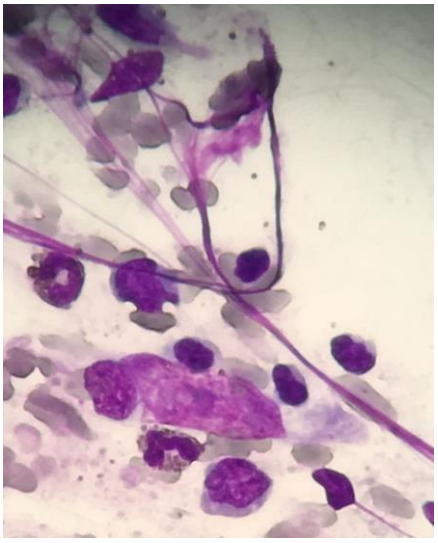

Figure 2. Photomicrograph of bone marrow fine needle aspiration, MGG, 1000x, showing pronounced eosinophilic hyperplasia, undifferentiated myeloid precursors with an eosinophilic differentiation and normal morphology.
Source: prepared by Dr. T. Soare

Unfortunately, Becko's state gradually worsened, manifesting progressive generalised diffuse amyotrophy, including atrophy of the respiratory muscles and finally cachexia. The most significant change in blood biochemistry was hypoalbuminemia. Ultrasonography revealed large quantities of anechoic fluid in both body cavities and diffuse infiltrates of the abdominal organs, lungs and heart.The pleural and peritoneal fluids were examined cytologically and revealed a large number of eosinophils. Becko died of a cardiopulmonary arrest and the owner declined necropsy.

Conclusions

The first clinical sign Becko manifested was the fever of unidentified origin.

The helpful diagnostic clues were the persistent recurrent eosinophilia despite glucocorticoid therapy and the familial history (the mother and brothers always registered over 5% eosinophils and occasionally over 10%, in particularly the male brothers).

These, coupled with the unexplicable pyrexia, led to a suspicion of a genetic disease. Not every dog presenting with eosinophilia has idiopathic hypereosinophilic syndrome and it is essential to rule out the causes of increased eosinophil counts.

If all investigations point to an idiopathic process, the dogs with persistent eosinophilia might benefit from early glucocorticoid therapy, before the onset of clinical disease, and should be subsequently subjected to regular clinical and paraclinical examinations.

We observed that the dog responded better to prednisolone and methylprednisolone than to dexamethasone, as suggested in the literature. In severe cases, treatment seems to be illusive, therefore we recommend that eosinophilia in susceptible breeds, in particular the Rottweiler, be investigated thoroughly and effort be made to examine the other littermates and parents.

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Metabolic researches in Țurcana sheep breeding in different pastoral ecosystems

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Abstract

The health of Tsurcana sheep in different pastoral ecosystems is the result of a continuous adaptive metabolic process to macro and microclimate changes, depending on individual factors and breed characteristics (the rustic, indigenous breeds are better adapted). In this paper, the biological study material were two-year old Tsurcana sheep raised in Fagaraș, Rucar, Bacau (Comanești area); exclusively pasture fed; from each region and from each flock we collected blood samples from 5 sheep and we presented the average of the values obtained. We found: hypercholesterolemia in the Tsurcana sheep in all three regions (Fagaras and Rucar with similar values), hyperglobulinemia in Tsurcana sheep from Rucar; increased GOT activity in all the Tsurcana tested, most notably at Rucar; increased GPT activity, the highest value in those from Bacau; the increase in GGT activity, the highest value in Ţurcanele de Bacau. This increased plasma activity is due to hepatic lesions, hyperuraemia (the highest values being registered for the Rucar and Bacău Tsurcana); hypercreatinemia (the highest value in Bacau). A classification, depending on the affected organs: the liver is affected in sheep in Rucar and in Bacau; - the kidney and implicitly the nucleoproteic metabolism is more affected in Bacău and Rucăr sheep; the proteic metabolism in sheep in Rucar, where the highest globulin value were identified; on the other hand the increased globulins play a role in the host immunity and we must not forget that the research was carried out during lactation and the sheep from Rucar graze during summer at Lake Iezer at an altitude of over 1800 m; as for cholesterol, it is increased in sheep in all three regions; so lipid metabolism is disrupted, implicitly liver function. In conclusion: Fagaras Tsurcana have hypercholesterolemia, but excretion and epuration are less affected; correlating the obtained results, it can be argued that routine explorations can sometimes reveal unexpected and isolated transaminase elevations; these increases may be influenced by excess weight, adaptive liver reactions, cardio-circulatory failure etc.; many of these are not clinically investigated.

Keywords*: pastoral ecosystems, hypercholesterolemia, sheep health*

Introduction

Turcana is a local mountain breed that grows in the hilly regions; Turcana is known in the specialized literature under other names, such as: the Barsana sheep in Barsa Country; *ţuşcă* or ciuşcă, a name under which it is known in Soviet literature; ratzka, Hungarian designation; Zackel, German name, etc. (6).

Sheep health in pastoral ecosystems is the result of a continuous adaptive process to macro and microclimate changes; is influenced by environmental factors (macro and microclimate conditions) and the individual and race factors (the rustic and indigenous breeds are better suited to weather conditions and local food, especially the quality of grass, water and air), the conditions for growth and exploitation (1, 4, 7, 8).

Nowadays in our country sheep breeding is practiced in the mountains, in the mountains and in the hilly area because it is not specifically related to the exclusive existence of the land for the production of the forage; for sheep breeding many sheep owners have leased land. A particularity of sheep raising is due to the fact that sheep are a species that can feed through the practice of transhumance, a very old method applied by shepherds (2, 3, 5). Transhumance is the practice of moving livestock from one grazing ground to another in a seasonal cycle, typically to

lowlands in winter and highlands in summer in areas of the countryside to consume surplus bulk feeds, organised in associative family holdings or by agricultural commercial companies in these areas (9). Although, as a practice, this method is very old, due to the conditions of our country it can be further recommended for the raising and exploitation of the sheep, as during autumn-winter it becomes quite efficient, because it is easier to move the flocks in in different periods of the year depending on the available feed, rather than carrying large volumes of bulky fodder from hill to hill.

Materials and methods

In this research, the biological study material was the two-year-old Turkish sheep raised in Făgăraş, Rucăr and Bacău (Comăneşti area); from each of the flocks we collected blood samples from 5 sheep; in tables 1-4 we present the average of the values obtained from the biochemical determinations.

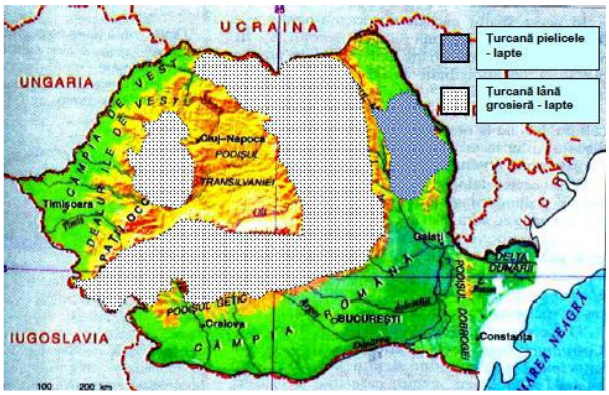

Figure 1. The occupied area and the influence zone of the Turcana breed (Pascal C., 2003)

Results and discussions

Their presentation will be based on the geographical area:

1. Turcana sheep from Fagaras

We took blood samples from the Ramba Iosif farm, from a 300 sheep flock; the results obtained are presented in Table 1.

Figure 1. Turcana breed sheep from Făgăraş

Table 1 shows increased cholesterol, increased creatinine, elevated GOT and GPT

Parameters	*Unit*	*Value*	*Reference*
Glucose	mg/dl	*65,33*	*45-80*
Cholesterol	mg/dl	*178,4*	*52-76*
Total protein	g/dl	*7,29*	*6-7,9*
Albumin	g/dl	*3,50*	*2,4-3,0*
Globulin	g/dl	*3,79*	*3,6-4,9*
Urea	mg/dl	*21,35*	*8-20*
Creatinine	mg/dl	2,84	2-2,7
GOT	UI/L	*332,54*	*307+/-43*
GPT	UI/L	*42,12*	*30+/-4*
GGT	UI/L	*58,57*	*20-52*
Calcium	mg/dl	*11,77*	*11,5-12,8*

Table 1. Variation of biochemical parameters in lactating sheep (Turcana breed), age 2 years, Făgăraş

2. Turcana sheep from Rucăr

We have collected blood samples from the Andreescu Dragoş farm, from a 432 sheep flock; the results obtained are presented in Table 2.

Figure 2. Turcana breed sheep from Rucăr

Table 2 shows increased cholesterol, hypergammaglobulinemia, increased creatinine and urea, elevated transaminases

Parameters	*Unit*	*Value*	*Reference*
Glucose	mg/dl	*53,5*	*45-80*
Cholesterol	mg/dl	*174,5*	*52-76*
Total Protein	g/dl	*7,54*	*6-7,9*
Albumin	g/dl	*2,35*	*2,4-3,0*
Globulin	g/dl	*5,19*	*3,6-4,9*
Urea	mg/dl	*22,40*	*8-20*
Creatinine	mg/dl	2,87	2-2,7
GOT	UI/L	*368*	*307+/-43*
GPT	UI/L	*52,8*	*30+/-4*
GGT	UI/L	*58,0*	*20-52*
Calcium	mg/dl	*12,34*	*11,5-12,8*

Table 2. Variation of biochemical parameters in lactating Turcana sheep, age 2 years, Rucar

3. Turcana breed sheep from Bacău

We collected blood samples from the Constantin Becaru farm, from a flock of 323 sheep in lactation; the results are shown in Table 3.

Table 3 shows: increased cholesterol, creatinine and urea increased, elevated transaminases

Parameters	***Unit***	***Value***	***Reference***
Glucose	mg/dl	*51,90*	*45-80*
Cholesterol	mg/dl	*165,6*	*52-76*
Total Protein	g/dl	*6,89*	*6-7,9*
Albumin	g/dl	*3.32*	*2,4-3,0*
Globulin	g/dl	*3,57*	*3,6-4,9*
Urea	mg/dl	*22,3*	*8-20*
Creatinine	mg/dl	2,9	2-2,7
GOT	UI/L	*347*	*307+/-43*
GPT	UI/L	*58*	*30+/-4*
GGT	UI/L	*61*	*20-52*
Calcium	mg/dl	*11,90*	*11,5-12,8*

Table 3. Variation of biochemical parameters in Turcana sheep in lactation, age 2 years, Bacau

Region	***Cholesterol***	***Globulin***	***GOT***	***GPT***	***GGT***	***Urea***	***Creatinine***
Făgăraș	178,4	3,78	332,5	42,12	58,57	21,35	2,84
Rucăr	178,5	5,19	368	52,80	58,00	22,4	2,87
Bacău	175,6	3,57	347	58,00	61,00	22,3	2,90
Reference and unit	52-76 mg/dl	4,9 g/dl	307+/-43 UI/L	26-34 UI/L	20-52 UI/L	8-20 mg/dl	2-2,7 mg/dl

Table 4. Comparative biochemical values (abnormal values) from Turcana sheep in different regions

Cumulatively, table 4 shows hypercholesterolemia in the Turcana sheep from the three regions (in Făgăraș and Rucăr, about the same value), hyperglobulinemia in Turcana sheep from Rucăr, increased GOT activity in all sheep, mostly at Rucăr; increased GPT - the highest value in Bacau; increased GGT activity, the highest value in Bacau; hyperuricaemia, the highest values in sheep from Rucar and Bacau; hypercreatinemia, the highest values were recorded in Bacau.

As for creatinine, it is a 'waste' product of the body that is transported to the kidneys by blood from where it is filtered and removed from the body through the urine. The amount of creatinine produced each day depends on the muscle mass; the blood creatinine level usually goes down as a result of poor kidney function (kidney infection, dehydration, decreased blood flow to the kidney - difficult to diagnose in Veterinary Medicine); therefore paraclinically evidenced hypercreatinemia is difficult to diagnose etiologically.

From the above it is observed that at the functional, hepatic level there are problems; laboratory data exploring for liver disease, called liver function tests; actually represent a 'battery' of biochemical analyzes that support the diagnosis of hepatopathy. The liver is the site of complex

biochemical processes so there is no test that can be considered as a unique indicator for hepatic dysfunction.

In connection with liver enzymes, we mention that GGT catalyses the transfer of the γ-glutamyl group from peptides such as glutathione (GSH) to other amino acids; is the only enzyme that cleaves significant amounts of GSH and GSH conjugates into the γ-glutamyl (GSH is transported to the extracellular surface of the membrane, where it is cleaved by GGT in cysteinyl-glycine and y-glutamyl residues, which are transferred to other amino acids). GGT plays an important role in the metabolism of inflammatory mediators, such as leukotrienes, carcinogenic and toxic substances. In hepatobiliary disease, GGT correlates with alkaline phosphatase levels. Increases are, however, not specific and can also be associated with pancreatic, cardiac, renal, etc. GGT dosing is also useful for the diagnosis of a hepatopathy in the presence of a bone disease. GOT (ASAT) and GPT (ASAT) dosing is the most useful and sensitive biochemical investigation for hepatocellular disease.

If we make a classification, depending on the biochemical parameters investigated and which, in part, represent the optimal functionality of some organs it is observed that:

- ✓ **the liver** is affected in the sheep from Rucar and Bacau;
- ✓ **kidney and implicitly nucleoproteic metabolism** is affected in Bacau and Rucar;
- ✓ **protein metabolism is abnormal** in sheep from Rucar, where the highest globulin value was found (globulin plays a role in the immune system); we must not forget that the research was carried out during the lactation period and the sheep from Rucar paste in the summer at Lake Iezer at an altitude of 1825 m. That is why in the future we will have to hematologically investigate this effect as it is also possible for an increased erythropoiesis disturbed by altitude (oxygen scarification).
- ✓ in terms of cholesterol, is increased in sheep in all three regions; so lipid metabolism is disrupted, implicitly liver function.

Conclusions

1. Clinically, Turcana sheep, regardless of the region where they are raised, are healthy.
2. Paraclinically, the sheep in the present research are affected by changes in the main biochemical parameters investigated.
3. Turcana breed sheep in the three regions have liver disease (liver transaminases and other parameters that are part of liver function tests)
4. The respective altitude, the summer habitat from Turcana breed from Rucar influences the body's homeostasis; the effort to go to these places and the eventual ‘fatigue’ caused by lactation, by daily food searches, act as stressors on the main organs, although at this altitude the quality of the grass is incontestable.
5. Turcana breedsheep from Făgăraș have hypercholesterolemia, but the excretion and purification of the body are less affected.
6. Turcana breed sheep from Bacău have a ‘renal laboratory pathology’ without a clinical correspondent.

By correlating the results obtained, it can be argued that routine explorations may sometimes reveal unexpected and isolated increases in the main biochemical parameters, increases that may be due to overweight, adaptive reactions, cardio-circulatory insufficiency etc .; many of these are not clinically and paraclinically investigated.

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The metabolic status of goats from Târnava Farm, Sibiu County

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Abstract

Târnava farm is located in Sibiu County, 12 km from the town of Mediaș and in 2017 owns 450 goats (740 goats in 2016, 420 in 2015). The farm is based on a reproductive core of different goat breeds: Saanen, French Alpine, Carpathian, cross bred Boer goats, both domestic and acclimated breeds. In establishing the metabolic status of these goats, we took blood samples from 10 2-year-old lactating goats, representing each breed. For each breed we averaged the values obtained and used as reference values the values provided by the equipment manufacturer; the samples were processed in the Laboratory of the Internal Medicine Department of the Faculty of Veterinary Medicine Bucharest. From the research we carried out, what we found metabolically in all the goat breeds on the farm was: normal proteic profile and lipid metabolism, normal enzymatic profile except for an increased alkaline phosphatase; hyperbilirubinemia; creatinemia and normal urea levels. As for the alkaline phosphatase – the orthophosphoric-monoester-phosphohydrolase has three isoenzymes: hepatic, bone, intestinal and during gestation, there is also a placental form. The hepatic alkaline phosphatase, which has major implications in veterinary pathology, plays a role in transport at the biliary and sinusoidal poles of the hepatocyte; in our research we observed that the hepato-biliary alkaline phosphatase is increased and accompanied by hyperbilirubinemia. Small non-specific increases may also occur in heart failure, possibly through intrahepatic biliary duct obstruction, all of which are difficult to follow pathological phenomena in veterinary medicine, so we can discuss about hepato-biliary dysfunction in the goats in this farm. The largest increase in alkaline phosphatase and bilirubin was registered in the Saanen breed, more pronounced in males than in females, followed by the French Alpine breed, while in the Carpathian the growth is moderate. We consider that this is a problem of functional adaptation in these imported breeds, one of the aspects observed during our research, constituting a part of a complex metabolic adaptation syndrome of imported goat breeds.

Keywords*: hepatic alkaline phosphatase, hyperbilirubinemia, metabolic syndrome of functional adaptation*

Introduction

Goat farming in an extensive system, under the geoclimatic conditions of our country, represents a continuing challenge for both the farmer who seeks the most profitable profits and the veterinarian who supervises the health of the livestock. In the present research we have monitored some biochemical parameters by which the veterinarian and the owner can supervise the health of the livestock. The goats harvest the crops obtained from the natural pastures and mountain meadows, which can not be used for the crops and can also utilize a range of industrial waste from large bakery businesses, from alcohol, beer, starch, vegetable and sugar factories (1, 4, 8).

The issue of feeding goats, through the level and quality of fodder administered during stabling and grazing during the warm season is one of the factors under the control of the grower, and who can ultimately decide the economic profitability of the goat breeding and exploitation.

In some countries in Europe like France, Switzerland, Austria, England, etc. effective programs of breeding and amelioration, nutrition, sanitary-veterinary etc. have been developed and implemented, which have imprinted with capriculture a special note of industrial exploitation by exploiting the lactogenic capacity and diversifying and selling the obtained products.

Implications of milk and meat productions obtained from goats in human food:

Goat milk is a good food, but can also be considered a preventive and curative medicine which is recommended for children, the elderly and the sick. Due to the calcium and phosphorus content, the consumption of goat milk contributes to the prevention of osteoporosis; balances blood pressure and relieves muscle and joint pains; helps regenerate cells; increases immunity, helping people with respiratory problems, especially TB; reduces the risk of breast cancer by 45% -65%; prevents colon cancer (5, 6).

Goat cheese has the property of treating pulmonary, cardiovascular and intestinal diseases; with a low fat content, goat cheese is digested more easily than other cheeses.

Goat meat is healthier than other types of meat. The calorie level reaches the 122 percent, similar to the non-skinned (120 calories), calculated for 100 grams. Furthermore, goat meat is 50-65% less fatty. It is also distinguished by the high nutritional value due in part to the high level of protein compared to other red meat, but also essential amino acids and iron and potassium salts. It has low cholesterol, a lipid level eight times smaller than beef and 40% lower than chicken (2, 7).

In our country there are two native breeds of goats: the Carpathian breed and the White Banat race (9). *The Târnava Farm, where the research was conducted, is located in Sibiu County, 12 km from Mediaş; holds a total of 450 goats in 2017 (in 2016 there were 740, in 2015 to 420 goats).*

Material and methods

The biological core was made up of goats of different races, indigenous races or acclimatized in our country.

We took blood samples from a total of 10 goats, 2 years old, from the Carpathian, French Alpine and Shanen breeds, lactating; for each race we made an average of the values obtained.

As reference values or used the values provided by the apparatus with which it was worked; the samples were processed in the Internal Medicine Laboratory of the Faculty of Veterinary Medicine Bucharest.

Results and discussions

We initially present the location where the research was conducted:

2.1. Presentation of the farm

In the Târnava farm, the biological nucleus is complex, represented as follows:

1. The Carpathian breed predominates; Carpatina X Shanen has a production of 3 l milk / day, while Carpathian native breed has a production of 1.8-2 L milk / day.

2. On the farm there are also the Metis of the French Alpine.

3. In 2017, on the farm there are goats - Alba de Banat (only pure races exist); usually 4-5 births; maximum yield (3 l milk / day) is obtained between 2nd and 4th calving.

4. There are also Boer race breeds that are a meat breed; at calf lambs have 6-7 kg; 1 month have 12-13 kg; at 2.5 months have 13 kg of meat and at 6 months have 20 kg of meat.

5. Breed Shanen; the Shanen breed was made up of Shanen goats from Austria and Germany; there are 16 Shanen males out of which 4 are pure breeds and the rest are meticulous; initially there were 11 Shanen goats out of which 8 died (did not adapt) and 3 remained, as the farm owner performs intense activity for improvement.

Figure 1. The Goat Effect

Figure 2. Shanen breed

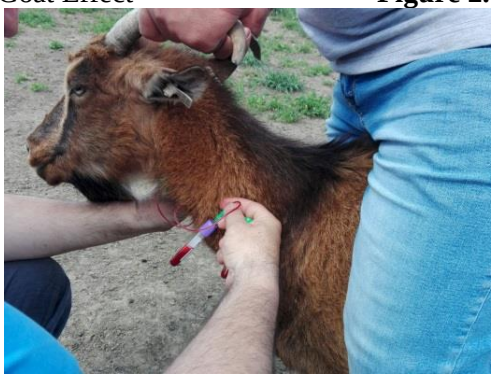

Figure 3. Taking samples of blood from Alpina French

2.2. Reproduction

Mount is natural. The herbs are introduced into the flock on August 25; in. December to January is a 60-day rest period; 300 days / year goats are lactating.

With regard to the half-cattle, half of the produce is male and half are female; on March 1 begins the sacrificing of the fawns.

2.3. Nutrition.

Summer is grazing in the field; in winter, lucerne hay, corn meal and wheat 1.4 kg/goat/day are administered. The owner uses only maize of native varieties that has 14% protein against maize hybrids that have only 7%; for all cereals used, laboratory analyzes are performed.

In the Târnava farm, between 10 December and 10 April the goats are not grazed except on warmer days. For feed, 20 maize trailers for 500 goats are needed compared to sheep where 30 t corn / 500 sheep are needed.

2.4. Pathology in the farm

1. There are 40-50 abortions / year.
2. Lesions of the limbs are common; most are of a mechanical nature due to thorn prickles over which bacterial infections overlap.
3. External parasites with lice and ticks were found; In this regard, baths are made at the beginning of summer and Ivomec is given in winter and spring.
4. Regarding parasites, the frequent findings of cases of Fasciolosis etc. on the farm are successfully treated with Bermectin, with the specification that carcass is not to be consumed for 66 days.

2.5. Therapy and immunoprophylaxis. Vaccinate against anaerobiosis and sputum (2 times / year); antiparasitic treatments and other supportive therapies are performed.

2.6. Biochemical Investigations in Goat (2017)

We determined the following biochemical parameters:

Biochemical parameters	**V.N**	**A1**	**A2**	**B1**	**B2**	**C1**	**C2**
Total protein (g/dL)	5,8-8,5	7,2	7,5	6,9	7,3	7,4	7,7
Albumin (g/dL)	2,5-3,7	2,7	3,0	2,6	3,0	2,8	3,1
Globulin		4,5	4,5	4,3	4,3	4,6	4,6
Cholesterol (mg/dL)	70-280	50,0	87,0	69,0	80,0	77,0	87,0
Triglycerides (mg/dL)	25-500	73,0	37,0	67,0	43,0	83,0	54,0
ASAT (UI/L)	0-82	16,0	24,0	18,0	25,0	14,0	23,0
ALAT (UI/L)	78-132	109,0	121,0	124,0	132,0	187,0	173,0
LDH (UI/L)	692-1445	863,0	766,0	784,0	744,0	848,0	763,0
Creatinine (mg/dL)	0,4-1,0	1,0	0,9	1,1	0,9	1,0	0,9
Urea (mg/dL)	10-25	14,0	17,0	18,0	21,0	16,0	19,0
Total Bilirubin (mg/dL)	0,2-0,3	0,4	0,4	0,4	0,5	0,4	0,6
Alkaline phosphatase (UI/L)	0-80	87,0	135,0	185,0	215,0	215,0	223,0

Table 1. Biochemical parameters determined in lactating goats (different breeds), Tarnava Farm 2017. Labels: Goats A - Carpathian breed, Goats B - French alpine breed, Goats C - Shanen breed; A1, A2, A3 - males; B1, B2, B3,- females; VN- normal values

Table 1 shows that in all breeds of goat farmed on the holding we obtained:

- ✓ normal protein and lipid profiles;
- ✓ normal enzymatic profile except for phosphatase which is increased;
- ✓ moderate hyperbilirubinemia;
- ✓ creatinemia and normal urea levels.

In relation to alkaline phosphatase (orthophosphoric-monoester-phosphohydrolase, FA) it is known to have three isoenzymes: hepatic, bone and intestinal; in gestational conditions, a placental form may also occur transiently. As far as hepatic alkaline phosphatase (this has major implications in veterinary pathology) plays a role in the transport of the bile and sinusoidal hepatocyte poles; of our research we noticed that FA (hepato-biliary) origin is accompanied by hyperbilirubinaemia; small non-specific increases may also occur in possible cardiac failure through intrahepatic biliary duct obstruction.

Interpretation of results depending on race:

Carpathian breed: proteinemia and high albuminemia in females; the same globulinemia in females and males; cholesterol increased in females; increased triglycerides in males; ASAT and ALAT increased in females; LDH increased in males; increased creatinine in males; urea increased in females; the same bilirubin in males and females, alkaline phosphatase increased in females.

French alpine breed: proteinemia and high albuminemia in females; the same globulinemia in females and males; cholesterol increased in females; increased triglycerides in males; ASAT and ALAT increased in females; LDH increased in males; increased creatinine in

males; urea increased to female; increased bilirubin in females; alkaline phosphatase increased in females.

Shanen breed: proteinemia and high albuminemia in females; the same globulinemia in females and males; cholesterol increased in females; increased triglycerides in males; ASAT increased in females; *ALT and LDH increased in males, increased creatinine in males; urea increased in females; increased bilirubin in females, elevated alkaline phosphatase in females*

From the above interpretations, in the three races, we found:

- ✓ the protein profile is normal; depending on sex - in females is higher;
- ✓ cholesterol increased in females; triglycerides grown in males;
- ✓ ASAT and ALAT grown in female Charpatina and French Alpine breeds;
- ✓ increased creatinine in males; urea increased in females;
- ✓ increased bilirubin in females at French Alpina and Shanen;
- ✓ high alkaline phosphatase in females.

So, the biochemical differences are:

- ✓ **Shanen breed**- enzymatic profile (ASAT increased in females, ALT and LDH increased in males);
- ✓ ASAT and ALAT increased in females of the Carpathian and French Alpine breeds;
- ✓ increased bilirubin in females in the French Alpine breed and Shanen.

Conclusions:

1. Clinically, goats are healthy.
2. Changes in liver transaminases occur in all three races.
3. In functional adaptation processes, the liver is one of the required organs, with malfunctions as before.
4. Paraclinical, we can discuss at this stage of cellular biochemical lesions without clinical expression.
5. Metabolically, females are more affected than males (protein metabolic changes, lipid, enzymes at the hepatocellular level).
6. Increased creatinine in males compared to females (within the normal range) may be genetic as human creatinine is also increased in males.

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Holocrine secretory mechanism in granular ducts in Brown Norway rat. Histological study

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Abstract

Mandibular glands in rodents contain a particular type of ducts, namely granular ducts. The cells lining these ducts present granules in their cytoplasm and secrete different substances. Our study aimed to assess these granules and the secretory mechanisms of these cells. The biological material was represented by 5 adult males Brown Wistar rats. We harvested the mandibular glands and processed them for histological examination. The slides showed that the cells lining the granular ducts present granules of different sizes with a spherical shape. They can occupy up to half of the cell cytoplasm and sometimes even more, forming large aggregates. The secretion of these large aggregates takes place through a holocrine secretory mechanism. The cells presenting this type of mechanism can be easily identified on the slides because they show discontinuity of the apical pole and a tendency of dispersion of the cellular contents. The other two types of secretory mechanisms are also present. In other words, the cells lining the granular ducts from Brown Norway rats mandibular glands, present merocrine, apocrine and holocrine secretory mechanisms. This is the first evidence of the holocrine mechanism cells from granular ducts in mandibular gland in this species.

Keywords*: granules, holocrine, mandibular, Brown Norway rat.*

Introduction

Granular ducts from rat mandibular glands are lined by cells containing obvious granules (Matthews, 1974a). Along time, researchers considered that these granules are not secretory, because they did not observe signs of their exteriorization nor abundant endoplasmic reticulum, which would suggest the fact that the cells are secretory. Recent publications mentioned that there are signs of exocytosis observed quite often, which confirment the secretory nature of the cells lining the granular ducts from rat mandibular gland (Giebisch, 2013).

In rodents (mouse, rat, hamster etc.), granular ducts from the mandibular gland are interposed between intercalary and striated ducts. They secrete proteases and bioactive polypeptides (different growth factors) (Mori et al., 1992; Tandler et al., 2001; Cha, 2017). These ducts are encountered in large numbers in males because they are androgen-dependent. Thus, the granular ducts present sexual dimorphism (Cha, 2017), being much more developed in males (Frith and Townsend, 1985; Amano et al., 2012). At birth though, the gland is immature and does not contain granular ducts, which form only later (somewhere in the interval 1-3 months of life) (Hecht et al., 2000, Coire et al. 2003).

Granular ducts are lined by more types of cells as follows: dark narrow, light granular and dark granular. The dark narrow cells contain a lot of free ribosomes (free-floating), but no endoplasmic reticulum or granules. The second cellular type, the light granular cells present a variable endoplasmic reticulum quantity and granules, while the third type is filled with granules, and the cellular organelles, the cytoplasm, and nucleus are pushed towards the basal zone. It seems that these cellular types would be secretory stages of the same cellular type, fact that would sustain

the affirmations according to which, the secretion of this cellular type is cyclic and not continuous (Tamarin and Sreebny, 1965).

Material and methods

The experimental study unreeled with the accord of the Bioethics Committee of the University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca. The utilized animals were kept in the biobase of the Faculty of Veterinary Medicine in Cluj-Napoca and were represented by 5 Brown Norway rats.

Immediately after sacrification, the mandibular glands were harvested for histological investigations. The samples were immersed in 10% buffered formalin immediately after harvesting and maintained in the fixation solution at room temperature, for 5 days. The utilized formalin was prepared 5 days before using it, from 20 ml concentrated formalin and 180 ml distilled water. During the fixation period, we changed the fixation solution 3 times, so that the fixation would be appropriate. The proportion of the volume of the sample and the one of the fixation solution was 1:40 (Kiernan, 1990).

After the fixation period was finished, the samples were immersed in successive baths of alcohol, in increasing concentrations, as follows: 70^0, 95^0 and absolute. At the end of the dehydration period, the samples were clarified with n-butanol. The paraffin infiltration was achieved at a 56^0C temperature, after which the samples were immersed in melted paraffin and were left at the laboratory temperature to solidify. After shaping the paraffin blocks in which the sample was included, we obtained seriated sections of 5 µm thickness with the aid of a Leica rotary microtome. After mounting on histological slides, the contrasting technique used was Goldner's trichrome staining procedure.

The histological slides were examined under an Olympus BX41 light microscope and the photographs were taken with a photo camera (E-330), attached to the microscope. The subsequent processing of the photographs was performed with the aid of Adobe Photoshop CS2 software.

Results and discussions

The mandibular gland in Brown Norway rats resembles the one in albino Wistar rat and albino laboratory mouse from a histoarchitectural point of view, in the sense that it presents very well developed granular ducts. The cells lining these ducts are tall and have a cytoplasm filled with acidophilic granules with different sizes and spherical shape (Fig. 1).

What comes forward, is the presence of very large granules in some cells, occupying a major part of the cytoplasm (Fig. 2).

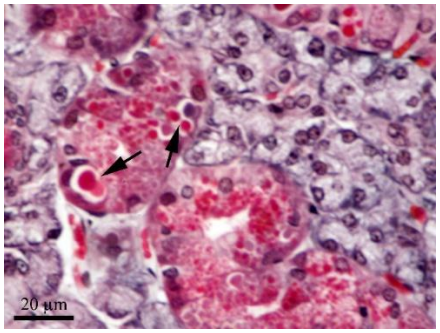

Fig. 1. Mandibular gland in Brown Norway rat – Acidophilic granules in the cells lining the granular ducts (black arrows)

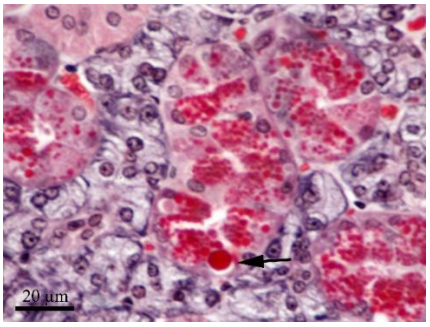

Fig. 2. Mandibular gland in Brown Norway rat – Large granules in the cells lining the granular ducts (black arrow)

These cells can present one or more such granules, which suggests that they form by fusion of smaller granules. The aspect was signaled by other authors who sustain that the granules from the cytoplasm of cells in granular ducts existent in some rodents can fuse in order to form larger granules or even aggregates of large and sometimes very large dimensions (Thomopoulos et al., 2002).

In mandibular gland from Brown Norway rats taken into study, we highlighted a remarkable polymorphism of the intracytoplasmatic granules, which suggests the fact that the granular aggregation process is not only present, but also very intense. Moreover, the evolution of granule fusion until large size conglomerates (aggregate) formation is frequent. One or more such aggregates can be found in the cytoplasm of one cell so that in some cases, they can occupy up to half of the cytoplasm or even more (Fig. 3). In most of the cases, the large conglomerates are accompanied by a certain intracellular oedema, materialized on the microscopical image through a clear halo, surrounding the structure (Fig. 4).

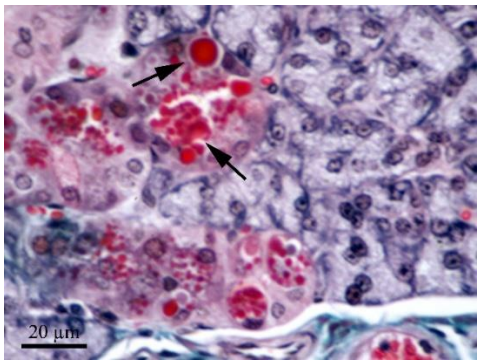

Fig. 3. Mandibular gland in Brown Norway rat – Aggregates in the cells lining the granular ducts (black arrows)

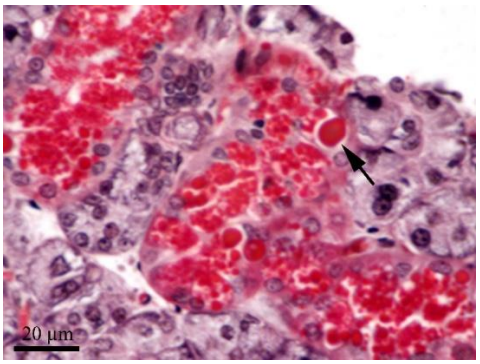

Fig. 4. Mandibular gland in Brown Norway rat – Clear halo around an aggregate (black arrows)

Needless to say that the presence of such structures in the cytoplasm of the cells disturbs the normal unreel of the cellular metabolism. Moreover, these cells have a secretory activity and the secretory products accumulated in the intracytoplasmatic secretion granules have to be eliminated from the cells when they are needed. Elimination of the secretory products from the small sized granules is possible through a merocrine secretory mechanism (reversed pinocytosis) (Amano et al., 2012). Also, these granules and even the larger ones (medium size) can accumulate in the apical pole of these cells in order to be eliminated through an apocrine secretory mechanism, also signaled before in the cells from granular ducts (Messelt, 1982; Messelt and Dahl, 1983). Some authors signal the formation of these aggregates even in the striated ducts of the mandibular gland in slow loris (*Nyctecebus coucang*) (Tandler et al., 1996; Tandler et al., 2006). The authors mention that filaments are present in the apical pole, which associate with the membrane surface and help the large granules move towards the surface in order to be exocitated (Tandler et al., 1996; Tandler et al., 2001). The question arises whether large conglomerates can be eliminated through one of the two secretory mechanisms (merocrine and apocrine) signaled in the scientific literature. We do not think such a thing is possible because the dimension of some granules is too large for the two secretory mechanisms to be functional. Moreover, their presence and persistence in the cytoplasm of the cells elicit an imbalance which will lead at some point to dysfunctionality of the cell, which will eventually disintegrate. By rupture of the cellular membrane, the conglomerate (or conglomerates) will be eliminated along with the other granules present in the cell. In this situation, these cells eliminate their secretory products through a holocrine secretory mechanism

(disintegration of the cell which produced them), aspects present on the sections we made in the mandibular gland in Brown Norway rat. The aspects we intercepted clearly suggest that besides the secretory mechanisms signaled in the scientific literature (merocrine and apocrine), in Brown Norway rat, the holocrine secretory mechanism is also present. We did not find any information in the scientific literature regarding the presence of holocrine secretory mechanism in the mandibular gland in Brown Norway rat. Given the situation, it seems like this is the first evidence of the presence of holocrine secretory mechanism in the mandibular gland, in Brown Norway rat. We have to mention that this mechanism is not necessarily predominant in the mandibular gland in Brown Norway rat, but is relatively well represented. It is present as mentioned before in cells in which large granulations or conglomerates are formed, but also in other cells which present small or at most large granulations. These cells are relatively easy to identify in a histological investigation because they present discontinuities (ruptures) of the apical pole and a tendency of dispersion of the cellular contents. Such phenomena can be observed in either isolated cells, larger or smaller groups of neighbouring cells, presenting clear signs of structural disintegration. Some are intercepted when only a part of the granules, regardless of the size, were eliminated, but others appear void of contents. The situation highly differs from one duct to another, which determines us to think that the secretion is not synchronized not only from one duct to another but also from one area to another of the same duct. This asynchronous secretion is mentioned by Tandler et al. (2001) in striated ducts, who state that it can be encountered in different species. Also, they mention the fact that the secretory granules differ a lot between the cells. It seems that the secretion rhythm and the mechanisms through which the secretory product is eliminated from the cells are adjusted to the functional necessities of the gland and maintained between physiological limits. If the histological investigation allows the assessment of the size and aspect of the granules in the cytoplasm of the cells lining the granular ducts in Brown Norway rat, it does not offer information on the phenomena determining the fusion of granules with the formation of large granules and conglomerates. In this situation, we are bound to only signal their presence, without being able to state if their formation is an advantage or disadvantage for the animal. It is possible that they do not have any functional meaning, especially if the substances they contain do not suffer structural changes. In such a situation, the substances will be liberated through the disintegration of conglomerates in the lumen of the excretory ducts, which transport the secretion products and subsequently released for the organism to use, same as the small sized granules.

The absorption of tritiated tryptophan in the cells lining the granular ducts was studied and a slow turnover process of the secretory proteins was observed (Matthews, 1974a). The sympathetic stimulation led to cell degranulation, while stimulation of parasympathetic nerves did not yield any response regarding the secretion (Matthews, 1974b). It seems that the granules form again 8 hours after degranulation.

The authors mention that the secretory granules seem to be serous because they are electrono-dense and contain glycoproteins in small quantity. The granules are polymorph, suggesting a fluid consistency. The membrane of two granules can fuse on a larger or smaller distance or they can fuse with the cell membrane, forming a pentalaminar membrane. Yet, the authors did not observe other signs of exocytosis (Matthews, 1974b; Giebisch, 2013).

Our study revealed that the cells lining the granular ducts in mandibular gland of Brown Norway rat release their secretory granules in three different ways. We found evidence of holocrine secretory mechanism, which was not mentioned in the scientific literature so far.

Conclusions

The cells lining the granular ducts in the mandibular gland in Brown Norway rat present the three types of secretory mechanisms, but only two of them are mentioned in the scientific

literature: merocrine and apocrine. Some cells present a holocrine secretory mechanism materialized through the disintegration of the cells, with the elimination of the cellular content. This is the first evidence of the holocrine secretory mechanism in cells from granular ducts in mandibular gland in Brown Norway rat.

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Comparative stereological study of granular and striated ducts in mandibular glands in Wistar and Brown Norway rats

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Abstract

Mandibular glands in adult rats contain granular ducts, derived from the striated ones, which enrich the saliva with different components. The aim of our study was to conduct a comparative stereological study of the striated and granular ducts in two rat strains (albino Wistar and Brown Norway). With this purpose in view, we harvested salivary glands from 5 animals from each strain in part and histologically processed them. We analyzed fields with the total surface of 1699510 µm^2 with the aid of AmScope software and the statistical analysis was performed with GraphPad Prism 6.01 program. The results showed that in both species, the number of sections through the granular ducts is higher than the one of striated ducts and the total surface occupied by the granular ducts is higher than the one occupied by the striated ones. The statistical analysis revealed the fact that there are no significant differences between the number of striated or granular ducts from one species to another, nor between the surface occupied by each type of duct (striated or granular) on the sections taken into study in the two rat strains. Thus, the mandibular gland in Wistar and Brown Norway rat resemble one another regarding the surface occupied by the striated and granular ducts and also the number of the two types of ducts on the section taken into study.

Keywords: Brown Norway, granular, mandibular, striated, Wistar.

Introduction

The parenchyma of the mandibular gland in rodents contains two secretory compartments. They are represented by acini and ducts (Coire et al., 2003). The ducts present in the mandibular gland of rodents divide in: intralobular (intercalated, granular and striated), excretory and main excretory ducts (Amano et al., 2012). Among the ducts, the granular and striated ones can be secretory, but not in all rodent species (Tandler et al., 2001). Moreover, the granular ducts are not encountered in the mandibular gland of all rodent species (e.g. they are not found in chipmunks (*Tamias striatus*), antelope squirrels (*Citellus tereticaudus*) and guinea pig (*Cavia porcellus*) (Flon et al., 1970; Tandler et al., 2001), but in adult rats they are found in great numbers (Tandler et al., 2001).

At birth, the mandibular gland of rats is not completely developed. It grows in volume along with the development of the acinar cells, as well as the ones lining the granular ducts. The increase in volume takes place due to the cell hypertrophy and their hyperplasia (Enesco and Leblond, 1962; Pardini and Taga, 1992).

The granular ducts are present in the mandibular glands in rodents and are majoritary (Tandler et al., 2001), being sometimes mistaken for mucous acini (Amano et al., 2012). Thus, the structures present at birth in the mandibular gland of rat are transient (Hand et al., 1996; Denny et al., 1997; Hecht et al. 2000., Coire et al. 2003) and reach maturity after passing through two phases, namely: acinar and ductal (Coire et al., 2003). It seems that the grainnular ducts arise from the striated ones, starting with the third week of life (Cutler and Chaundry, 1975; Gresik, 1980) and reach maturity after 3 months (Srinivasan and Chang, 1975; Coire et al., 2003). Some authors write that the ducts can form starting from the cells lining the intercalated ducts (Denny et al., 1993;

Srinivasan and Chang, 1975; Zajicek et al., 1985). In mouse, the granular ducts form earlier than in rat (Pardini and Taga, 1997).

The salivary glands broadly differ from one species to another, depending on the environment they live in and the food type. We set out to assess if there are intraspecific differences regarding the number and surface occupied by the striated and granular ducts in albino Wistar and Brown Norway rats, by conducting a morphometrical study.

Material and methods

The present study unreeled in the University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca and was approved by the Bioethics Committee. The biological material was represented by 5 albino Wistar rats and 5 Brown Norway rats, all males. The rats were sacrificed by prolonged anesthesia with isoflurane and the mandibular glands were harvested as soon as possible. The samples were fixed in 10% buffered formalin for 7 days long and renewed the fixation solution 3 times. The next steps consisted in dehydration with ethanol in increasing concentration, clarification with n-butanol and paraffin embedding. Alternate 5 µm thick sections were cut with a Leica rotary microtome and subsequently stained with Goldner's trichrome method.

Determination of the surface of striated and granular ducts

The histological slides were examined with the aid of Olympus BX41 light microscope using the 10x objective and the images captured with the photo camera attached to the microscope (Olympus E-330). The measurements were made with AmScope software and we measured one field from each rat in part, with an area of 1699510 μm^2. We determined the number of striated and granular ducts and the section surface of each duct in part, and then determined the percentage of each type of ducts on the surface taken into study.

Statistical analysis

The total surfaces obtained for striated and granular ducts were compared between the two rat strains with the aid of GraphPad Prism 6.01 program for Windows, using Unpaired t test and the level of significance was set at 5%.

Results and discussion

Both the mandibular gland in albino Wistar rat (Fig. 1.) and the one in Brown Norway rat (Fig. 2.) contain numerous granular ducts (whose cells are filled with future secretion products) and striated ducts in obviously smaller numbers.

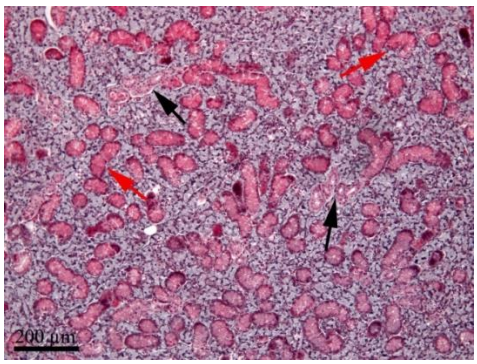

Fig. 1. Mandibular gland in albino Wistar rat – striated ducts (black arrows); granular ducts (red arrows)

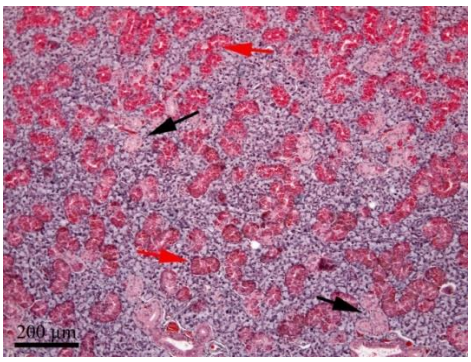

Fig. 2. Mandibular gland in Brown Norway rat - striated ducts (black arrows); granular ducts (red arrows)

The data regarding the number of striated and granular ducts on the section surface taken into account, as well as the total surface of striated and granular ducts in each rat in part (albino Wistar and Brown Norway) are presented in Table 1.

The average number of sections (transversal or oblique) through the ducts present on the surface taken into study, was represented by 46 striated ducts and 169.8 granular ducts in albino Wistar rat and 41 striated ducts and 157.8 granular ducts in Brown Norway rat.

In the case of the mandibular gland in albino Wistar rat, the average total surface of the sections through striated ducts was 87985.35 μm^2 and 552455.65 μm^2 for granular ducts, which represents 5.18% from the total surface taken into study (1699510 μm^2) for the striated ducts and 32.51% for the granular ones, respectively (Chart 1).

Table. 1. Number of ducts/picture and total surface of ducts

Rat strain	No. of striated ducts/picture	Total surface of striated ducts (μm^2)	No. of granular ducts/picture	Total surface of granular ducts (μm^2)
W1	46	132499.19	145	569354.83
W2	38	64264.98	167	570983.41
W3	33	59238.48	175	557325.57
W4	57	90138.36	187	510577.53
W5	56	93785.71	175	554036.86
BN1	40	95588.94	158	583563.94
BN2	38	124312.90	157	575768.66
BN3	38	180799.08	137	454496.19
BN4	34	91001.38	166	552408.29
BN5	55	137414.40	171	582920.96

W – Wistar albino rat; BN – Brown Norway rat.

The results were comparable in the case of the mandibular gland of Brown Norway rat, in which the total average surface of the sections through the striated ducts was 125823.34 μm^2, as for the granular ones 549831.61 μm^2, the striated ducts representing 7.40% from the total surface taken into study (1699510 μm^2) and the striated ones 32.35% from the total surface of the section (Chart 1). Thus, we can state that in section, the granular ducts occupy approximately the same percentage out of the mandibular gland surface in the two rat strains taken into study, while the striated ones present an insignificat difference of 2.23% in the favor of the granular ducts in albino Wistar rat.

The differences recorded in both the surfaces of granular ducts (p value = 0.5317) and striated ones (p value = 0.0952) in the two rat strains were not statistically significant. Upon statistical analysis of the difference between the number of sections of the striated ducts on the section surface of the mandibular gland in albino Wistar rat and Brown Norway rat, respectively, it turned out to be insignificant (p value = 0.5476). Similarly, $p > 0.05$ in the case of the number of sections through the granular ducts in the two rat strains (p value = 0.1349), which signifies that in this case, the differences were also statistically insignificant in the two rat strains taken into study.

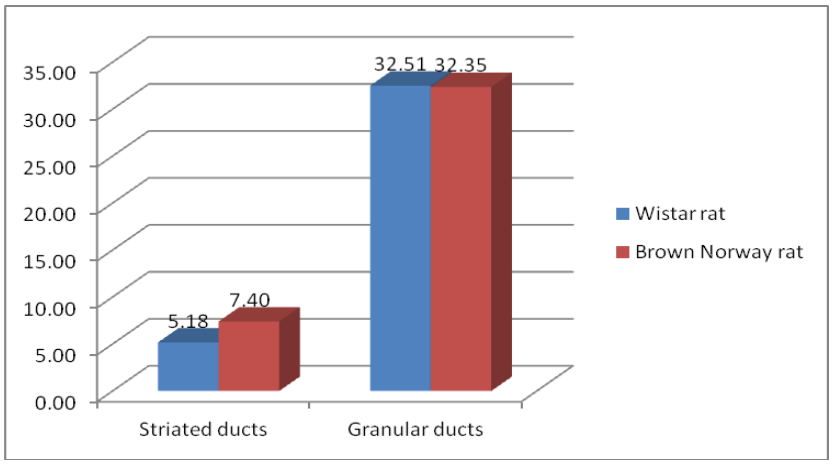

Chart 1. Percentage of intralobular ducts in Wistar and Brown Norway rat

In the scientific literature, authors mention the fact that the granular ducts occupy a moderately large volume in adult rats, increasing by 132% between the first and third month of life (Coire et al., 2003). The rats taken into the present study were mature males, thus the mandibular glands from both rat strains contained a large number of granular ducts, which occupy 32.51% from the section surface in albino Wistar rat and 32.35% in the case of Brown Norway rat.

Amano et al. (2012) write that the striated ducts are well represented in rodents, aspect confirmed in our study as well, occupying 5.18% from the section surface in albino Wistar rat and 7.40% from the section surface in Brown Norway rat, with an average number of 46 sections of striated ducts/surface taken into study in albino Wistar rat and 41 for Brown Norway rat, respectively. The granular ducts are sinuous (Taga and Pardini, 2002; Greaves, 2012), thus the number of sections through these ducts is larger than the one through the striated ducts. The explanation of the better representation of this type of ducts could be the importance of the products synthesized and eliminated by the cells lining it. Among these substances, there are different bioactive polypeptides, hormones and growth factors (Amano et al., 2012).

Conclusion

Both studied rat strains contained better represented granular ducts than the striated ones in their mandibular glands, occupying a more significant surface and the intraspecific differences of number and surface occupied by the ducts (striated and granular) were not significant among albino Wistar and Brown Norway rat.

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Comparative morphometrical study of the acini in parotid gland in Wistar and Brown Norway rats

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Abstract

Morphologically, the salivary glands consist of acini and duct system. The acini have a different structure depending on the salivary gland, differ from one species to another and being in relation to the diet. We did not find enough information about the appearance of the acini for each species, that is why we considered it appropriate to convey morphometric investigations on acini in parotid gland from two strains of laboratory rats. For this study, we used five male Wistar rats and five male Brown Norway rats, euthanised by prolonged exposure to isoflurane. The parotid glands were harvested for histological investigation. For measuring and counting the acini we used AmScope program and the obtained data were analyzed with GraphPad Prism 6 software. The investigation showed that the number of the acini/studied surface (1699509.677 µm^2) turned out to be different in those two rat strains taken into study. In Brown Norway rat, from the total surface taken into study, the acini occupy 80.52% and 75.55% in Wistar rat. In Brown Norway rat, the acini were found to be 1.58 times bigger than in Wistar rat, but they are 1.46 times more numerous in Wistar rat. The difference is given by the larger average size of the acini in Brown Norway than in Wistar rat.

Keywords: *acini, Brown Norway, morphometry, parotid, Wistar.*

Introduction

Salivary glands are paired organs that secrete saliva in the oral cavity. They are grouped into major and minor salivary glands (Tandler, 1993; Wolfgang 2003; Tucker and Miletich, 2010). The size of the major salivary glands, the structure of the acini and the particularities of secreted saliva (serous, mucous or mixed) differ from one species to another and are in direct relation to the diet (Sisson and Grossman, 1964; Da Cunha Lima *et al*., 2004; Treuting and Dintzis, 2012). Morphologically, the salivary glands consist of secretory units (acini) and duct system. The acini have a different structure depending on the salivary gland. Most of the authors claim that in mammals, including human, the parotid gland is serous. Thus, there are authors that affirm that it is seromucous because, besides some enzymes, the secretory granules of the acinar cells contain carbohydrates, sialomucins and sulfomucins. Mucosal cells which produce secretory granules rich in neutral glycosides and acids have been found in the developing of the parotid gland in humans and rats (Kiyomi *et al*., 2001).

All salivary glands are tubuloalveolar glands and have several types of ducts. Intercalated ducts collect saliva directly from the acini, and by merging they form the striated ducts. Between those two types of ducts, in rodents appear a third one, the granular duct. The striated ducts cohere to form interlobular ducts, which eventually open into the excretory duct. The excretory ducts of the parotid gland open into the oral cavity at the upper molars (Gresik, 1994; Taga and Sesso, 1998; Al-Saffar and Simawy, 2014).

In special literature, we have not found enough information about the size, shape and appearance of the acini for each species, nor if it exists or not differences between animals belonging to different strains of the same species. In this context, we considered it desirable to conduct morphometric investigations on parotid gland acini from two strains of laboratory rats in order to capture some possible differences.

Material and methods

The biological material used in this study was represented by five male Wistar rats and five male Brown Norway rats. The investigation was approved by the University of Agricultural Sciences and Veterinary Medicine Bioethics Committee of Cluj-Napoca and was carried out in accordance with the legislation of the Ministry of Health. Animals were euthanized by prolonged exposure to inhaled anesthesia (Isoflurane). The parotid glands were harvested for histological and morphometrical investigations. The harvested pieces were fixed in 10% buffered formalin, dehydrated in ethyl alcohol (70°, 95°, absolute), clarified with n-butanol and included in paraffin. Sections of 5 μm thickness were stained with hematoxylin-eosin and examined with an Olympus BX41 optical microscope equipped with a digital camera. For measuring and counting the acini we used AmScope program, we photographed five fields/section with a 10X lens objective from each animal. The obtained data were analyzed with GraphPad Prism 6 software. We determined the values: minimum, maximum, average, standard error of the mean and standard deviation. We also calculated the percentage occupied by the acini on the section area taken into study. The surface difference is occupied by other structural elements (excretory ducts, other types of acini, connective tissue, blood vessels).

Results and discussions

The histological examination revealed differences between the acini in parotid gland in the two rat strains regarding the dimensions and the aspect of the cytoplasm of the acinar cells (Fig.1, Fig.2, Fig.3, Fig.4). In order to quantify these differences, stereological investigations on the acini size and number were necessary, as well as the surface occupied by them related to the other structural components.

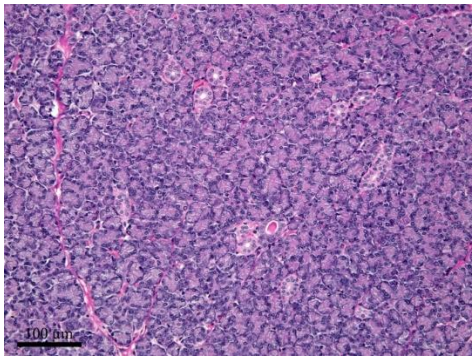

Fig. 1. Parotid gland in Wistar rat (H-E)

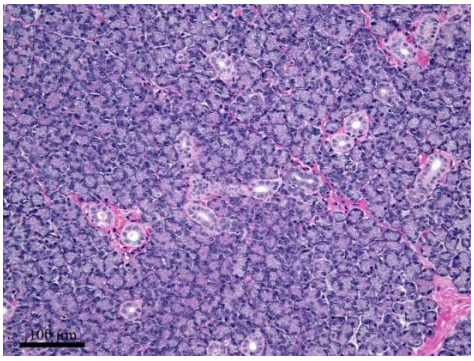

Fig. 2. Parotid gland in Wistar rat (H-E)

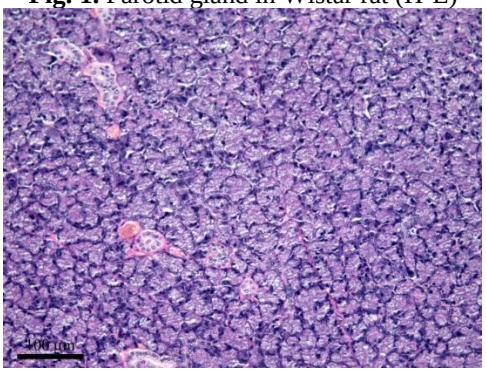

Fig. 3. Parotid gland in Brown Norway rat (H-E)

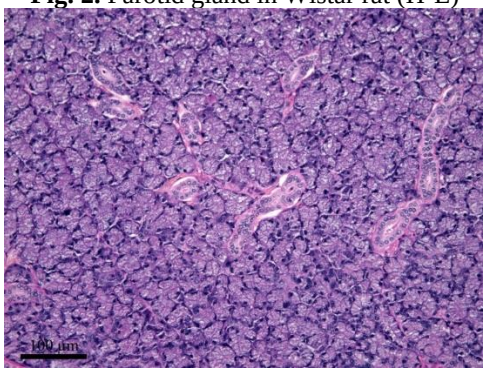

Fig. 4. Parotid gland in Brown Norway rat (H-E)

The number of the acini/studied surface (1699509.677 μm^2) turned out to be different in the two rat strain. Thus, the average number of acini/studied area was 618 in Wistar rat and 417 in Brown Norway rat (Table 1).

Table 1 The average number of acini/studied surface (1699509.677 μm^2) in Wistar rat and Brown Norway rat

	Wistar rat	*Brown Norway*
N=	618	417

Areas occupied by the acini from the total surface (1699509.677 μm^2) are comparable but not identical. Therefore, from the surface taken into study (1699509.677 μm^2) the acini occupy 80.52% (1368413.14 μm^2) in Brown Norway rat and 75.55% (1283980.47 μm^2) in Wistar rat. It is found that, although the number of acini on the studied surface is different in those two rat strains (617 compared to 417), the surface occupied by acini related to the other components (excretory ducts, other types of acini, connective tissue, blood vessels) is relatively close.The fact that 617 acini occupy the same surface as 417 acini are due to the fact that in Wistar rat the number of acini/surface is 1.46 times more numerous than in Brown Norway rat. The difference is given by the larger average size of acini in Brown Norway than in Wistar rat.

The size of the acini was appreciated by measuring their surface area/section, in all the acini on the total surface taken into study. In this way, both the size of the acini and their polymorphism could be appreciated. There were differences between these two strains. In Brown Norway rat, the acini average area was 3285 μm^2, and in Wistar rat 2079 μm^2. It is found that the acini in Brown Norway rat parotid gland are 1.58 times bigger than in Wistar rat.

Table 2 Dimensions of the acini. Min – minimum; Max – maximum; $\bar{x}$ - mean; SD – standard deviation; SEM – standard error of mean; CV - Coefficient of variation

Species	Wistar rat	Brown Norway
Min (μm^2)	335.6	608.1
Max (μm^2)	4785.5	10125.5
$\bar{x}$ (μm^2)	2079	3285
SD (μm^2)	665.9	1311.5
SEM (μm^2)	26,8	64,3
CV (%)	32,1	39,9

In terms of coefficient of variation, the highest value is in Brown Norway rat with 39.9% (minimum 608.10 μm^2, maximum 10125.50 μm^2, average 3285 μm^2), and then Wistar rat with 32.1% (minima 335.60 μm^2, maxima 4785.50 μm^2, media 2079 μm^2) (Table 2). The smaller the coefficient of variation, the less polymorphic are the acini. Appreciating the polymorphism of the acini in parotid gland in the studied species, we find out that the most polymorphic acini are in Brown Norway rat followed by Wistar rat.

In terms of multiple comparisons (using ANOVA One-Way Test), between Brown Norway rat and Wistar rat we found statistically significant differences ($P < 0.001$).

The statistical results demonstrate the existence of relatively large differences between the acini of the parotid gland in the studied rat strains, in terms of size and number of acini.

Conclusions

The stereological study of the parotid gland revealed differences between the two rat strains. In Brown Norway rat, the acini were found to be 1.58 times bigger than in Wistar rat, but they are 1.46 times more numerous in Wistar rat.

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Histological and histochemical study of the granules in granular ducts cells in mouse and Wistar rat mandibular gland

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Abstract

The mandibular ducts system is different in rodents, having, in addition, a specialized type of secretory duct, situated between the intercalated and the striated ones. The granular ducts cells have granules that are generally round, but not all of the same size. The aim of our study was to conduct histological and histochemical investigations on these granules. We used three white laboratory mice and three Wistar rats, males. The animals were sacrificed by prolonged exposure to inhalatory anesthesia. Immediately after the euthanasia, we harvested the mandibular glands and then processed them for histological and histochemical investigations. Our study highlights the fact that in white laboratory mouse, the polymorphism of the granules is highly pronounced. The granules appear more polymorphic and with an obvious higher tendency to form large conglomerates. In Wistar rat, the cells contain granules of relatively same size and the polymorphism is relatively discreet. The cytoplasm of the mandibular gland cells of both species taken into study presented PAS+ materials, but there are quantitative differences. Thus, in mouse are present very small amounts, while in Wistar rat they are slightly larger.

Keywords*: histochemical, mandibular, mouse, Wistar.*

Introduction

In rodents as in humans, major salivary glands are represented by the parotid, mandibular and sublingual glands. Histologically, they are formed of acini and ducts system (Pritam *et al*., 2013; Ruberte, 2017). In mouse and rat, the mandibular gland ducts system is different from other mammals (Young and Van Lennep, 1978; Jayasinghe *et al*., 1990; Coire *et al*. 2003; Tsuboi *et al*., 2004; Moghaddam *et al*., 2009; Al-Saffar and Simawy, 2014; Yamagishi *et al*., 2014). In rodents mandibular gland there is a specialized type of secretory ducts situated between the striated and intercalated ones, called granular ducts (Young and Van Lennep, 1978; Jayasinghe *et al*., 1990; Bazan *et al*., 2001; Tsuboi *et al*., 2004 Moghaddam *et al*., 2009). They develop postnatally from striated ducts cells (Cutler and Chaudhry, 1975; Gresik, 1994) and contain columnar or pyramidal cells arranged around a distinct lumen. Occasionally, granular ducts cells are associated on the basal side with myoepithelial cells (Mori *et al*., 1992).

In mouse, the size of the granular ducts cells varies with different strains (Tom-Moy and Barka, 1981; Gresik, 1994). Some authors claim that in mouse and rat, granular ducts are fully developed only at sexual maturity. The same authors argue that age, gender and hormonal disturbances are often responsible for biochemical and histological changes in mandibular glands of these species (Srinivasan and Chang, 1975; Mori *et al*., 1992; Gresik, 1994).

Granular ducts in rodents (mouse, rat, hamster, gerbil) have 4 cell categories: granular, dark granular, pillar cells and transition cells. Granular cells are the majority and contain many secretory granules located at the apical pole of the cell. These granules are generally round, but not all of the same size (Mori *et al*., 1992; Gresik, 1994; Bazan *et al*., 2001) and they have the same electron density (Bazan *et al*., 2001). In special literature consulted, we have not found clear indications if there are differences between the granules of the granular ducts cells in mouse and rat. That is why

we considered it appropriate to conduct histological and histochemical investigations on these granules in this two rodent species.

Material and methods

The investigation was approved by the Ethics Committee of University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca and was carried out in accordance with the legislation imposed by the Ministry of Health. For this study, we used three mice and three Wistar rats, adult males, coming from the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca biobase. The animals were sacrificed by prolonged exposure to Isoflurane. Immediately after the euthanasia, the mandibular glands were collected in order to perform histological and histochemical investigations. The samples were fixed in 10% formalin for 3 days, with daily changing of the fixator, then dehydrated in alcohol with increasing concentration (70°, 95°, absolute), clarified with n-butanol and included in paraffin. We made 5 μm thickness sections, which were contrasted using two techniques: Tricrom-Goldner staining and PAS reaction. The histological samples obtained were examined with an Olympus BX41 microscope equipped with a digital camera, and for the photo process, we used Adobe Photoshop CS2 software.

Results and discussions

On Goldner's trichrome staining, the mandibular granular cells'cytospasm appears loaded with acidophilic granules in both strains. They appear spherical or oval in both species but have a high degree of polymorphism in size.

In white laboratory mouse, the polymorphism of the granules is highly pronounced, from small to very large granules, sometimes with large differences from one granular duct to another (Fig. 1). In some sections, there are present only small and medium sized granules, but this does not mean that this appearance is maintained on the whole length of the examined duct. The aspect can be traced on sections that capture the duct over a longer section (oblique or longitudinal sections). In other sections, among small and medium sized granules there are large granules that result from merging the smaller granules. By merging several granules, it sometimes forms conglomerates of such size that they occupy a large part of the cell. Most of the times, in cells containing very large aggregates there is a clear tendency to fuse other granules existing in the cell cytoplasm with the formation of new aggregates. In mouse, such situations are relatively common, but often contain isolated cells or small groups of 2-4 cells, rarely more. It is certain that in mouse mandibular gland, the granular polymorphism in granular ducts cells is so developed that rarely the section through a duct appears very close to that of the adjacent ducts (Fig. 2). Giving the fact that the shape of the granular duct is contorted, the adjacent sections may be part of the same duct. In this context, we can state that with regard to granules polymorphism, the situation is more or less different not only from one granular duct to another but even along the length of the same duct. The fact that in mouse the granulations appear more polymorphic and with an obvious higher tendency to form large and very large conglomerates, they seem to be a species particularity. If this specific feature is a functional advantage or not, we can not say because the histological investigation does not provide information on this aspect.

Irrespective of the size and polymorphism of the granulations, they occupy most of the cell (at least ¾ of the cytoplasm) in mouse. They are only missing from the basal area, where the nucleus of the granular cells is situated.

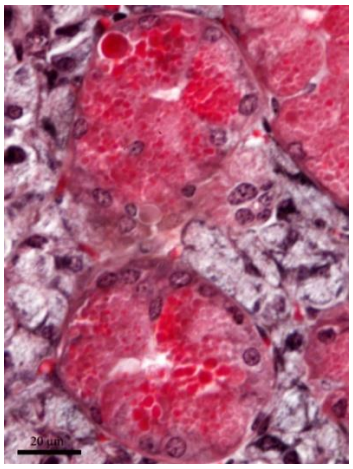

Fig. 1. Granules in white laboratory mouse granular ducts (Goldner's trichrome stain)

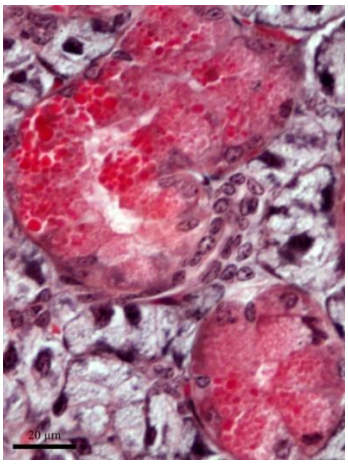

Fig. 2. Granules in white laboratory mouse granular ducts (Goldner's trichrome stain)

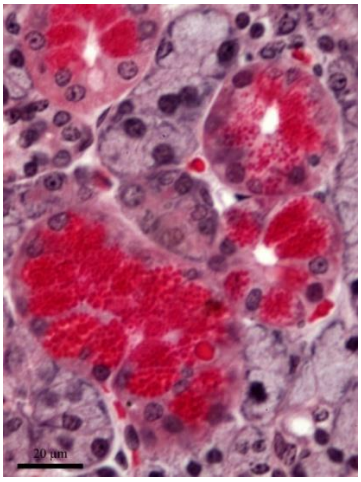

Fig. 3. Granules in Wistar rat granular ducts (Goldner's trichrome stain)

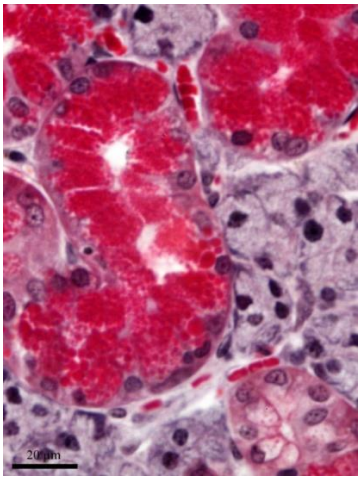

Fig. 4. Granules in Wistar rat granular ducts (Goldner's trichrome stain)

In Wistar rat, the mandibular gland resembles the one of white laboratory mouse as general arrangement, having long and contort granular ducts with cells containing numerous spherical granules in the cytoplasm. The granules in cell cytoplasm in Wistar rat mandibular gland are different from that of white laboratory mouse (Fig. 3). Intracytoplasmic granules appear with polymorphism, but significantly lower than in white laboratory mouse. On the sections of most granular tubes, the cells contain granules of relatively same size, framing from small to medium in size (Fig. 4). In other words, there is some polymorphism in each granular tube in Wistar rat, but it is relatively discreet in most sections. In a relatively small number of sections, some of the cells have medium size granules, either alone or together with a few large-scale granulations. Sometimes there is a small number of cells which contain aggregates formed by the fusion of smaller granules, but their number and dimensions are significantly lower than in mouse.

There are also differences between the granular ducts cells in white laboratory mouse (Fig. 5) and Wistar rat (Fig. 7) on PAS reaction. Although the cytoplasm of the mandibular gland cells of both species taken into study presented PAS+ materials, there are obvious quantitative differences. If in white laboratory mouse are present very small amounts (Fig. 6), in Wistar rat they

are slightly larger (Fig. 8). Note that only a part of the present granules in the granular ducts cells cytoplasm contain PAS+ materials for both species. The presence of PAS+ materials only in some of the granules in granular ducts cells cytoplasm clearly suggests that these substances do not represent the main secretion of these cells. This does not mean that they are not important for these animal species. As it is known, PAS reaction reveals several types of substances (polysaccharides, neutral mucopolysaccharides, acid mucopolysaccharides, mucoproteins, glycoproteins, glycolipids and lipids) (Mureşan *et al*., 1976; Suvarna *et al.*, 2013). In this situation, we can not know exactly whether the differences between mouse and rat in terms of intensity reaction are given by the synthesis of several types of substances in the rat compared to the mouse or the same substances are synthesized but in somewhat larger quantity.

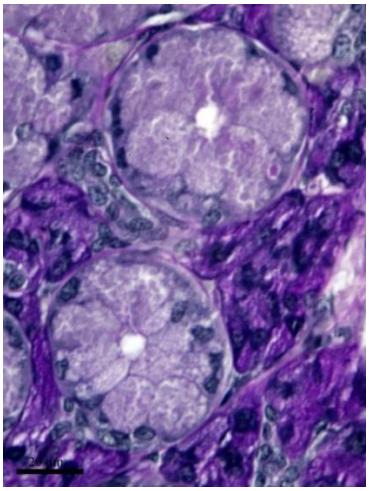

Fig. 5. Granular ducts in white laboratory mouse (PAS)

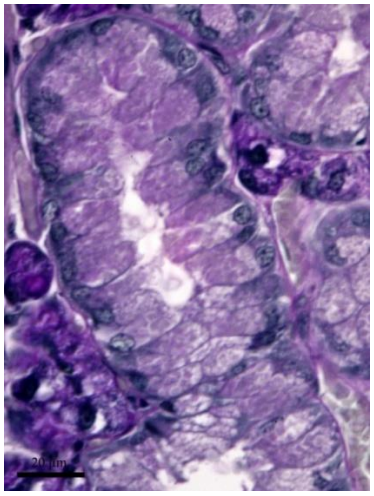

Fig. 6. Granular ducts in white laboratory mouse (PAS)

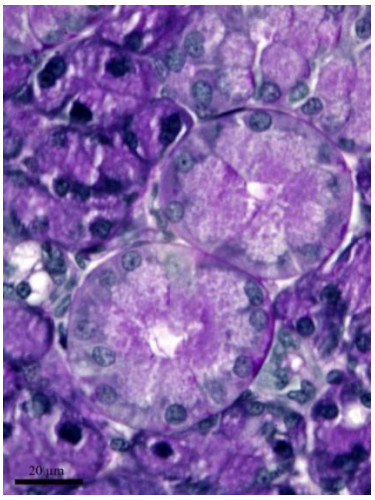

Fig. 7. Granular ducts in Wistar rat (PAS)

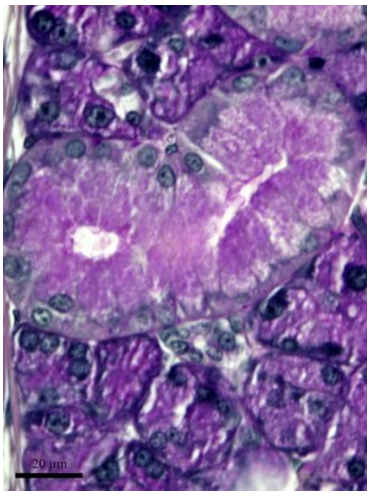

Fig. 8. Granular ducts in Wistar rat (PAS)

The results obtained by us are similar from many points of view to those found in special literature, but also different. Similarities refer mainly to the presence of granules and their shape, all of the authors state that they are round. In terms of size, the consulted authors consider that they

are between 0.2 - 2 μm in diameter, with the indication that in each cell there are some very small and rarely very large granules (> 5 μm in diameter). Small granules are in the range of 0.2 - 0.4 μm and the large ones between 1.0 - 1.3 μm in diameter, being a few granules of intermediate size (Gresik, 1994). In contrast to these assertions, we found a more pronounced polymorphism in granules diameter both in rat and mouse. Other authors affirm that the size of the granules varies with different strains of mice (Tom-Moy and Barka, 1981; Gresik, 1994). We also found different aspects regarding the cell surface occupied with secretion granules. The authors say that the granules occupy the apical half of the cell up to two-thirds (Cutler and Chaudhry, 1975; Gresik, 1994), and others claim that they only occupy the apical third of it (Gresik, 1994). We found a higher load of granules in both species studied.

Regarding the presence of PAS+ substances, our results are comparable to those in special literature in the sense that we have shown relatively small amounts present only in some cells, and other authors state that they may be present in the apical pole of some cells (Materazzi and Travaglini, 1960; Materazzi, 1969).

Conclusions

By comparing the granules of secretion from the granular ducts cells cytoplasm in the mandibular gland of the two studied species, we find that there are both similarities and differences. Similarities are due to the presence of large-scale numerous granules and the fact that they occupy in both species more than half the cytoplasm in most cells. Differences are due to the fact that the granules polymorphism appears to be more pronounced in white laboratory mouse compared to Wistar rat.

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Accidental fatal metaldehyde poisoning in a dog – a case report

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Abstract

Metaldehyde is a neurotoxic, frequently used molluscicide, that is implicated both in accidental or intentional poisonings. The majority of the commercial products are flavoured, so they have sweet taste, and are consumed voluntary by dogs. Here we report a complete description of the lesions found in a case of metaldehyde poisoning in a dog. A 1,5 year old German Sheperd dog was submitted for necropsy with a history of metaldehyde granules consumption. The animal was found dead by the owner, a few hours later. Complete necropsy and histopatological exam were undertaken. At post mortem examination, multisystemic congestive and haemorrhagic lesions, as well as multisystemic necrotic and degenerative modifications were observed. The most affected organs were the brain, liver, kidneys and the lungs. Metaldehyde produces primarily congestive and hemorrhagic lesions, with consecutive necrosis in the brain, lungs, liver and kidney, in high dose causing irreversible damage in these organs.

Introduction

Metaldehyde is a toxic product that is found usually in pesticides, alone or in combination with other substances such as carbamates or organophosphates. Chemically, metaldehyde is a cyclic polymer of acetaldehyde and it is used in snail baits and snail pellets (as a molluscicide) (Gupta, 2007).

In the market, this molluscicide can be found as powder, pellets, paste, granules or as a liquid (Richardson, 2003).

The majority of the commercial products are flavored to have a sweet taste. The sweet taste of the commercial molluscicide can attract dogs, the number of clinical cases being significant (Gupta, 2007).

Metaldehyde is a class II toxin (moderately hazardous pesticide) according to the World Health Organisation, while the United States Environmental Protection Agency classifies it as a slightly toxic chemical (acute oral toxicity class II) (Gupta, 2007).

Most of the clinical cases of poisonings are described in dogs, but the disease was also described in birds, and wild animals (Gupta, 2007;

Metaldehyde is primarly a neurotoxicant (EFSA,2017). The most important clinical signs associated with metaldehyde poisoning are ataxia, tremors and seizures (Gupta, 2007). Other target organs for metaldehyde are the lungs, the kidneys or even the liver.

The most important route of exposure is the oral one, both accidental and intentional poisonings being described.

Until this moment, only a few reports regarding pathological changes in dogs poisoned with metaldehyde were published, and the description is usually is limited to gross lesions or incomplete.

In the present paper, we describe post mortem and histopathological findings from a dog that died following accidental metaldehyde poisoning.

Materials and methods

A 1, 5 year old, male German shepherd dog was presented for necropsy at the Pathology department of the Faculty of Veterinary Medicine Cluj-Napoca.

According to the case history, the animal accidentally consumed approximatively 400 g of blue metaldehyde granules from the owner's warehouse and was found dead a few hours later. Near the dog a blueish, foamy liquid was identified.

A complete necropsy and histological examination were undertaken less than 12 h after death. For the histological examination, the samples were fixed in 10 % buffered neutral formalin, embedded in paraffin, and 5 µm thick sections were made with a Leica RM 2125 RT rotary microtome. Than the slides was stained using Hematoxylin– Eosin (H&E) method.

Results and discussion

Complete post mortem examination was performed. At a general inspection a blue coloration of the tongue and of the lips was observed.

In the gastric content a large quantity (approximatively 300g) of blue granular substance was observed (Fig.1)

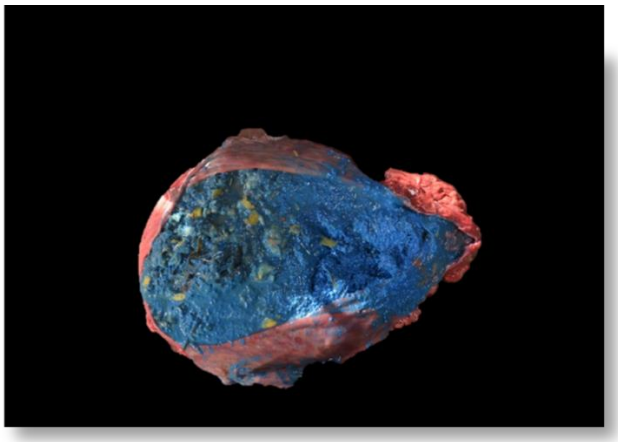

Fig.1 Macroscopical aspect of the gastric content; stomach full of blue granules.

This blue granular content was present also in the lower digestive tract.

The gross and histological examination of the brain and parenchymal organs revealed multisystemic congestive and haemorrhagic lesions, as well as multisystemic necrotic and degenerative modifications.

Acute, severe bilateral pulmonary congestion and petechial hemorrhages on the surface of the lungs were observed (Fig.3A). Microscopically, the alveolar capillaries were dilated and in the alveoli extravasation of numerous red blood cells was noticed (Fig.3B). In the liver, congestion of the sinusoids from the central areas (with consecutive hemosiderosis) and multiple foci of central necrosis were observed (Fig.2A). Mild central steatosis was also evident.

Kidneys were enlarged, and histologically vacuolization and necrosis of the renal epithelial cells were observed (Fig.2B).

The most important lesions were found in the brain. Grossly, severe diffuse congestion of the meninges was observed, while histologically congestion of the small and medium sized brain vessels and multiple areas of hemorrhage were evident (Fig.3A and 3B).

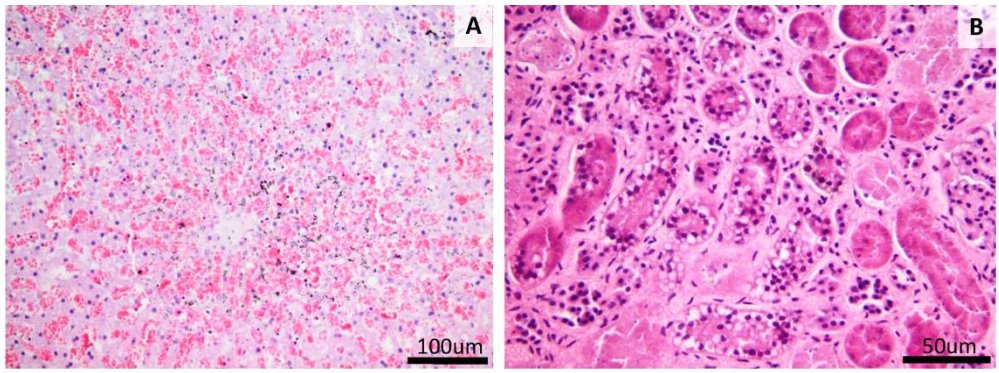

Fig.2. Microscopical aspects in metaldehyde poisoning. A) Foci of central necrosis, congestion of the sinusoids and hemosiderosis (HE stain, original magnification of 100X); Vacuolization of the renal epithelial cells (HE stain, original magnification of 200X).

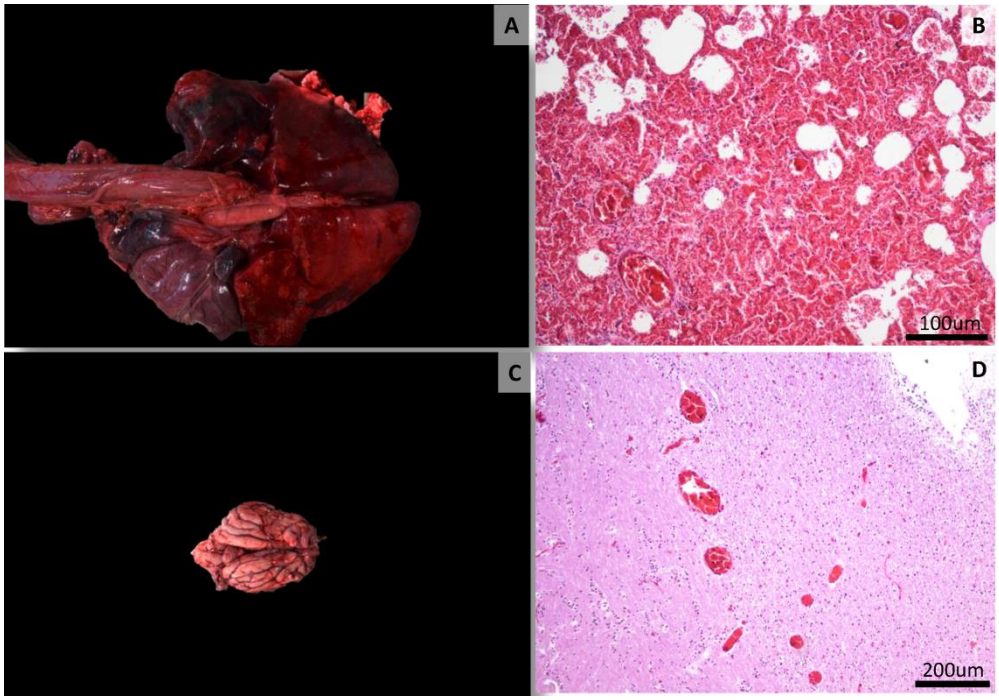

Fig.3. Gross and microscopic lesions in metaldehyde poisoning. A) Pulmonary congestion and petechial hemorrhages; B) Severe pulmonary congestion and diffuse alveolar hemorrhage (HE stain, original magnification of 200X); C) Acute diffuse meningeal congestion; D) Brain, congestion of the blood vessels and areas of hemorrhages (HE stain, original magnification of 100X).

The current paper describes a case of accidental fatal metaldehyde poisoning in a young German shepherd dog. Metaldehyde is a frequently used molluscicide, and is toxic to all animals. Intoxication can be primary or secondary, accidental or intentional (Gupta, 2007).

It is a moderately toxic substance with an oral LD50 of 100mg/kg (RED,2006; Gupta, 2007).

Metaldehyde it is primary a neurotoxicant, this neurotoxic effect it's due to decreased level of gamma aminobutyric acid, norepinephrine, and serotonin and increased monoamine oxidase levels (Gupta, 2007). Acetaldehyde, a metabolite of metaldehyde can also cause severe lesions in

the liver, kidneys or lungs. Our studies showed sever hemorrhagic and necrotic lesions in all these organs.

Most of the clinical cases are described in dogs, because they frequently consume the pesticide voluntary because of its sweet taste, so the most frequent route of exposure is the oral, but toxicoses can occur also after inhalation or dermal contact (Thompson, 1998).

Usually the diagnosis of metaldehyde intoxication is based on history and on the clinical signs presented by the patient (Gupta, 2007). The toxicant can be isolated from the gastrointestinal tract content or from the blood (Gupta, 2007).

There is no specific antidote in metaldehyde poisoning (Beasley, 1999;Yas Natan, 2007; Gupta, 2007).

The objectives of the treatment are decontamination, control of the seizures, restoring the ventilation and restoring of the fluid and electrolyte balance to correct metabolic acidosis that occurs in this disease (Gupta, 2007, Yas Natan, 2007).

Decontamination in asymptomatic patients can be realized by inducing the vomit, while in symptomatic patients by gastric lavage and administration of activated charcoal. Seizures can be controlled with diazepam or phenobarbital. Methocarbamol it's also beneficial in case of muscle twitching (Beasley, 1999;Yas Natan, 2007; Gupta, 2007).

With timely and appropriate treatment, clinical signs can be controlled, the survival rate being high. Without treatment the animal will die in a few hours due to respiratory failure (Gupta, 2007).

Conclusions

Metaldehyde produces primarily congestive and hemorrhagic lesions, with consecutive necrosis in the brain, lungs, liver and kidney, in high dose causing irreversible damage in these organs.

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Effectiveness of triple therapy with omeprazole, rifaximin and amoxicillin in experimental gastric infection with cagA$^+$/vacA$^+$ *Helicobacter pylori* in guinea pigs (*Cavia porcellus*)

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Abstract

The aim of this study was to evaluate the effectiveness of a triple combined therapy, including omeprazole, rifaximin and amoxicillin, in experimental gastritis induced by cagA$^+$/vacA$^+$ Helicobacter pylori (Hp) strains in guinea pigs. 20 guinea pigs (Cavia porcellus) were equally divided into 4 groups. The control group (I) consisted of 5 Hp$^-$ individuals, while group II, III and IV were composed of Hp$^+$ individuals; infection was confirmed in these groups by detection of Hp in stool specimens by polymerase chain reaction (PCR) analysis, 14 weeks after infection. Group II was left untreated; group III received 5 days of premedication with Lactobacillus casei, following 7 days of triple combined therapy, while group IV was treated with Lactobacillus casei in addition to triple therapy, for 7 days, without premedication. Complete blood cell count, serum biochemistry, microbiology, histopathology, rapid urease test, immunohistochemistry and PCR were performed in order to evaluate gastric lesions and treatment effectiveness. A significant decrease ($p<0.05$) in white blood cells was detected in group IV, in comparison with group II. A common side effect was related to amoxicillin administration, and consisted in severe thrombocytopenia ($p<0.05$). Concerning the severity of gastric lesions, there have been no significant differences between the experimental groups ($p>0.05$). The number of apoptotic cells and mitoses was higher in the infected groups in comparison with the control group ($p<0.05$). PCR was the most sensitive diagnostic assay for Hp in group II (4/5), while no amplified sequences were detected in groups III and IV, which showed the eradication of Hp infection following both of the proposed treatments. A combination of rifaximin, amoxicillin and omeprazole with probiotic premedication may increase the effectiveness of conventional therapy, being a valuable treatment option in future eradication of gastric Hp infections.

***Key words**: experimental infection, gastritis, H.pylori, probiotics, rifaximin, therapy.*

Introduction

Helicobacter pylori (*H. pylori*) infection is indisputably recognized as one of the major etiological agents currently involved in digestive tract disorders. Several reports have revealed that *H. pylori* infection is closely associated with the development of gastric disease, such as chronic antral atrophic gastritis (B type) [1], gastroduodenal ulcer [2], gastric adenocarcinoma [3] and mucosa-associated lymphoid tissue (MALT) lymphoma [4].

The guinea pig has proven to be an ideal experimental model for studying the significance of *H. pylori* in the development of gastric disease and the contributing factors to the inflammatory response towards *H. pylori* in the gastric mucosa [5]. Guinea pigs can also develop well-defined gastric adenocarcinoma following experimental *H.pylori* infection, mainly due to the fact that they cannot synthesize vitamin C, thus relying on dietary intake, like humans. Since *H. pylori* causes decreased concentrations of gastric juice ascorbic acid, known for its protective effect against the formation of reactive oxygen species and carcinogenic nitrosamines, the risk of developing gastric cancer is high and imminent [5,6]. The routes of transmission for *H. pylori* in both humans and animals are not fully known; still, the oral-oral and fecal-oral routes are currently accepted as the main transmission pathways [7].

Currently, standard treatment of *H. pylori* infection involves a triple therapy regimen, consisting of a proton pump inhibitor and two antibiotics [8]. Inadequate monitoring of treatment efficacy and antibiotic resistance are among the factors causing the persistence of *H. pylori* gastric infection and an increasing number of patients with gastric lesions [9]. Therefore, the present study aims to induce gastric infection in guinea pigs with cagA+ and vacA+ *H. pylori* strains, and to report on the changes of the histological, hematological and biochemical aspects that occur during eradication therapy, consisting of two antibiotics (rifaximin, amoxicillin), a proton pump inhibitor (omeprazole) and probiotics (*Lactobacillus casei*).

Materials and methods

Animals

The present study was conducted on a number of 20 guinea pigs (*Cavia porcellus*), aged 3 months (male n=10; female n=10), in the laboratory animal core facility of the Faculty of Veterinary Medicine Cluj-Napoca, after a period of acclimatization of 2 weeks. The study was approved by the Faculty of Veterinary Medicine Bioethics Committee concerning the animal experimentation. Housing conditions met the principles and standards established to maintain a suitable environment. Guinea pigs were fed with commercial pelleted diet, enriched with lucerne hay, white beets and carrots, without supplementing vitamin C.

Bacterial culture

H. pylori strains were obtained from 4 bacterial cultures, isolated from gastric biopsy specimens of human infected patients; subsequently, polymerase chain reaction (PCR) assay was performed to detect cagA and vacA virulence genes. Immediately afterwards, the colonies were suspended again in sterile phosphate-buffered saline (PBS) and centrifuged at 13,000 × g, for 20 min. Bacterial suspensions with a density of 10^8 colony-forming units (cfu)/ml were prepared for inoculation.

Experimental design

The $cagA^+/vacA^+$ *H.pylori* suspension (1 ml) was passed into the esophagus of 15 guinea pigs, through an esophageal tube, for 3 consecutive days. During this period, and one day after infection, 1 mg/kg omeprazole (Omeran 20 mg, Europharm) was administered; 6 hours prior to each administration, feed and water consumption was interrupted. After 14 weeks, fecal samples were collected from each infected individual and PCR was performed, by using specific primers designed to detect *H.pylori*.

Treatment regimen

Animals were equally divided into 4 groups of 5 each; group (I) consisted of Hp^- individuals, while group (II) was made up of Hp^+ guinea pigs, who received no treatment. Group (III) consisted of Hp^+ individuals and received the following treatment: 5 days of premedication with 3 g/group of *Lactobacillus casei* (Enterolactis 3g, Sofar), followed by individual administration of 0.4 mg of omeprazole (Omeran 20 mg, Europharm), 5.6 mg of rifaximin (Normix 200 mg, Alfawassermann) and 18 mg of amoxicillin (Amoxicilina, 250 mg, Europharm) starting with day 6, for a period of 7 days; probiotic supplementation was continued until the end of treatment (day 12). Group (IV) received the same treatment as group (III), but without 5 days of premedication with probiotics. Therefore, medication started on day 6, for a period of 7 days.

Assessment of hematology and plasma biochemistry

Hematological parameters such as: red blood cell count (RBC), white blood cell count (WBC), hemoglobin concentration (Hb), hematocrit (HCT) and platelet count were determined using the automated hematology analyzer Abacus Junior Vet (Diatron Messtechnik GmbH), after collecting venous blood samples from all of the individuals. Biochemical assays, including total cholesterol, calcium, blood urea nitrogen (BUN), glutamate oxaloacetate transaminase (GOT),

glutamic-pyruvic transaminase (GPT) and bilirubin (total, conjugated and unconjugated) were performed using the semi-automatic biochemical analyzer STAT-FAX 1904 Plus (Global Medical Instrumentation).

Necropsy and sampling

After 12 days of therapy, animals were sacrificed and post mortem examination was carried out. Subsequently, the stomach was sectioned along the minor curvature, extending between the cardia and the pyloric sphincter, and rinsed with 37°C PBS to remove gastric contents. Examination of the gastroduodenal mucosa and morphological assessment of the gastric lesions were performed, followed by examination of the other organs. Gastric mucosal biopsy samples were taken from the cardiac region, gastric greater curvature and pylorus (four mucosal biopsies from each gastric region). After its removal, the first biopsy was formalin-fixed and paraffin-embedded, for histological examination; the second one was used to perform the rapid urease test, while the third tissue sample was used for microbiological examination. Finally, the fourth biopsy was collected separately in order to perform the PCR analysis, using primers for typing of *H. pylori* cagA and vacA genes.

Histologic examination

Hematoxylin-eosin (H&E), Masson's trichrome (MT) and modified Giemsa stains were used for overall examination of the gastric mucosal samples. The histological slides were analyzed with an Olympus BX51 microscope and the microscopic images were captured with an Olympus SP350 digital camera.

Histological examination was focused mainly on the presence and distribution of acute and/ or chronic inflammatory cells, on the presence of lymphoid follicles and on the changes of the foveolar/ glandular epithelium (glandular atrophy, the presence of dysplastic and/ or metaplastic epithelial cells) and on the identification of *Helicobacter* organisms.

The essential histological features detected in the gastric mucosa allowed the following classification: absent gastritis (grade 0), characterized by the presence of reduced mononuclear inflammatory cells and intact epithelium of the gastric mucosa, without development of lymphoid aggregates; mild gastritis (grade 1), characterized by a number of 10-50 inflammatory cells/ 400x field and up to 2 lymphoid follicles/ 200x field, and normal gastric mucosal epithelium; moderate gastritis (grade 2) was described by the presence of 10-50 inflammatory cells/ 400x field, >2 lymphoid follicles/ 200x field and gastric mucosal damage; in severe gastritis (grade 3), more than 50 inflammatory cells/400x field were identified, together with significant lesions of the gastric lining mucosa and an increased number of lymphoid follicles/ 200x field. Gastric epithelial changes included: cell necrosis, epithelial desquamation and cystic glandular dilatation [10,11]. Mapping of the distribution and extent of atrophy, metaplasia and epithelial dysplasia was performed according to the Sydney grading system [12].

Immunohistochemistry

Serial sections from gastric mucosa were prepared for immunohistochemistry (IHC) analysis. Following dewaxing, heat-induced epitope retrieval pretreatment, and endogenous peroxidase inactivation (1% H_2O_2 in PBS), slides were incubated separately with 10% goat serum in PBS to reduce background staining. Sections were incubated at 4°C overnight, with a rabbit polyclonal antibody against *H. pylori* (B0471, Dako, Denmark), dilution 1:10, that exhibited consistent immunoreactivity towards a wide range of bacteria belonging to the *Helicobacter* genus.

Statistical analysis

Statistical data analysis was performed using the independent sample test (IST), to determine whether or not there are significant differences between two means. P values less than 0.05 were considered statistically significant.

Results and discussions

Detection of H.pylori

H. pylori was successfully inoculated in all of the animals, on the basis of positive PCR results obtained 14 weeks after infection, from the collected feces.

Hematological parameters

The infected guinea pigs demonstrated an elevated total white blood cell (WBC) count and lymphocytes, in comparison to the control group (I), but without statistical significance ($p>0.05$) (fig.1A and fig.1B). Following both treatment protocols, leukopenia was observed, with a significant difference in neutrophils ($p<0.05$), between group (II) and (III) (fig. 1C). A significantly lower number of platelets was also observed in treated guinea pigs (group III), compared to the untreated group (fig. 1D). No statistically significant differences between the two treated groups regarding this aspect were found ($p>0.05$).

Biochemical parameters

GOT and GPT elevations were detected in the infected and untreated group (II), compared to the control group (I), but only the GPT activities were found to be significantly increased ($p<0.05$) (fig.1F). Following treatment, GPT activity decreased in both treated groups (III and IV), reaching reference values; moreover, lower GPT values were found in the premedicated group (III) in comparison to group IV, but without statistical significance ($p<0.05$); however, GOT enzyme activity was found to be significantly increased in group IV compared to group III ($p<0.05$) (fig.1E).

Elevated mean alkaline phosphatase (ALP) levels were found in the infected group (II) in comparison to the control group ($p>0.05$). After therapy, ALP values decreased significantly ($p<0.05$) in both treatment groups, unlike in the *H.pylori* infected and untreated group. All other measured parameters did not suffer statistically significant changes among the studied groups.

Necropsy

All animals were subjected to pathological examination. There were no significant differences in terms of body weight between control and infected groups. In the infected and untreated group (II), multiple gastric erosions and foci of gastric mucosal hyperplasia were observed. The presence of gross lesions compatible with clostridial disease (*Clostridium difficile*) was seen in only one guinea pig from group IV.

Gastric colonization

Culture from gastric biopsy specimens allowed the isolation and detection of *H.pylori* colonies in 2 out of 5 animals from group II, after 7 days of incubation. Small sized (2-3/ 0.5 μm) *H. pylori* colonies, with a slightly curved rod-like appearance, and spiral-shaped gram-negative bacteria transforming into coccoid forms were identified on cytological examination. Bacterial colonies have not been isolated from treated animals (groups III and IV).

Urease activity was present in 3 out of 5 guinea pigs from group II, within 1-4 hours of starting rapid urease test. Turning of the yellow urea broth into magenta is an indicator of the urease activity of *H.pylori* bacteria, found in positive gastric biopsies. Positive rapid urease test results were found in the same individuals from which the colonies were isolated.

PCR analysis

H. pylori DNA was amplified in 4 out of 5 guinea pigs from group II. No PCR amplification products were seen in groups III and IV, which explains the eradication of *H.pylori* infection after treatment. Negative results were also found in the control group.

Histological evaluation of gastric biopsies

H.pylori was not identified in any individual by using H&E, Masson's trichrome and modified Giemsa stains.

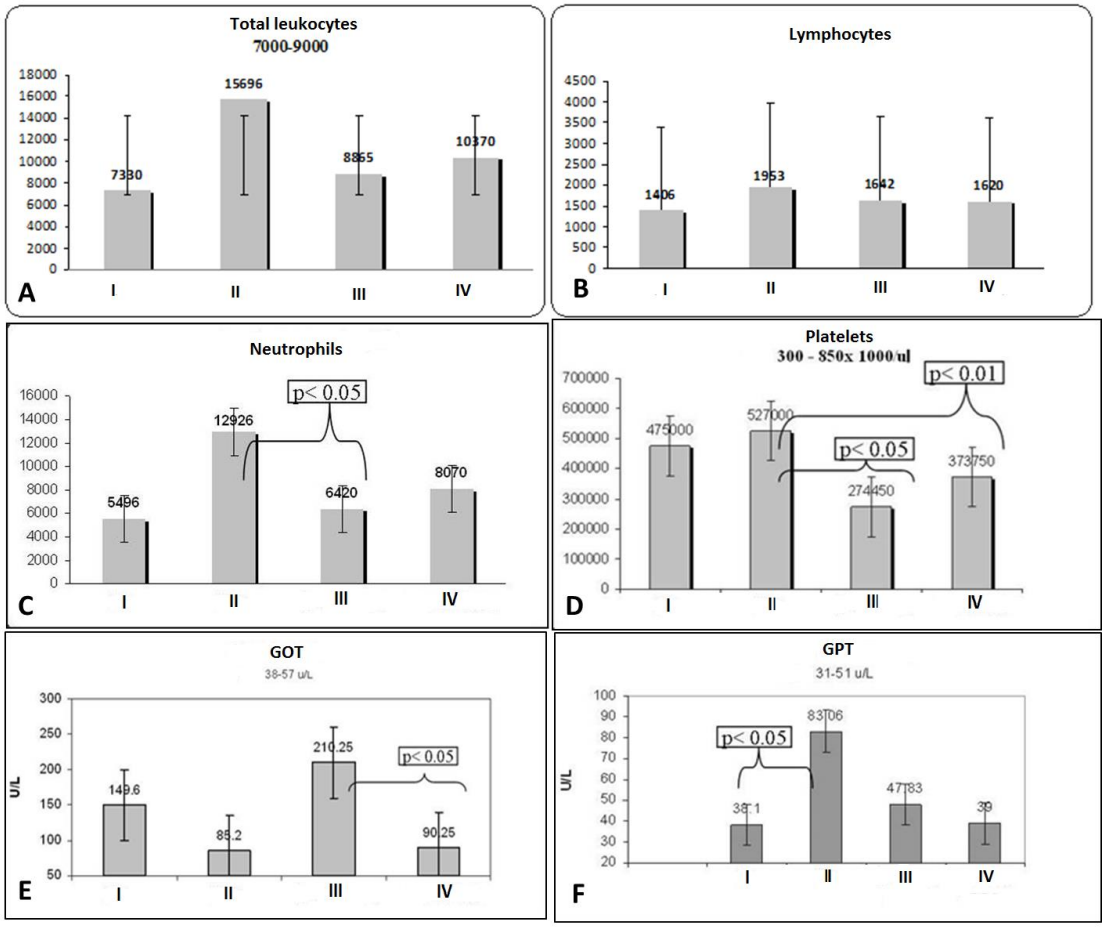

Fig. 1. Hematological and biochemical results. The mean: A) WBC count, B) total lymphocytes, C) neutrophils, D) platelets, E) GOT and F) GPT values in all of the groups, following treatment.

Histologically, no pathological changes related to the presence of inflammatory infiltrate and/or epithelial lesions were identified in the control group (I). Mild hyperplasia of the gastric glands was found in one animal from group II, while moderate inflammatory cell infiltrate composed of lymphocytes, plasma cells, macrophages, neutrophils and rare eosinophils was identified in 4 out of 5 animals from the same group (II). In 2 of these cases, the inflammatory cell infiltrate was present in the superficial area of the gastric mucosa and interspersed among the gastric glands. In another 2 cases, mixed inflammatory infiltrate was also present in the depth of gastric mucosa and in the submucosa, with lymphoid follicle-like structure formation. In one of the animals, neutrophilic infiltration of the lumen of gastric glands occurred (Fig. 2A, B, C). All of the cases exhibited epithelial alterations, including cell degeneration, necrosis and epithelial desquamation. Ulcers, fibroplasia in the lamina propria, glandular atrophy, intestinal metaplasia or epithelial dysplasia were not detectable in any case.

No signs of gastric inflammation were found in group IV, while group III showed mild gastric mucosal lesions, consisting of mixed inflammatory cellular infiltrates in the upper and middle third of the gastric glandular mucosa. In all of the animals, discrete infiltration of neutrophils and degenerative changes were present in the gastric epithelium and lamina propria, whereas in one individual, a decreased mononuclear inflammatory cell infiltrate was found in the

depth of the gastric mucosa. Statistically, no significant differences between the experimental groups in terms of gastric lesion severity were obtained (p>0.05). Because *H.pylori* gastric infection was histologically evidenced by IHC in only 2 guinea pigs, a correlation between the degree of gastric colonization and lesion severity was not achievable. Regarding the quantification of apoptosis and cell division, a significantly higher number was seen in the infected groups, compared to the control group (I). The only statistically significant difference in the amount of occurring apoptosis was identified between the control group and group II (p=0.028) (Fig. 2D, E, F).

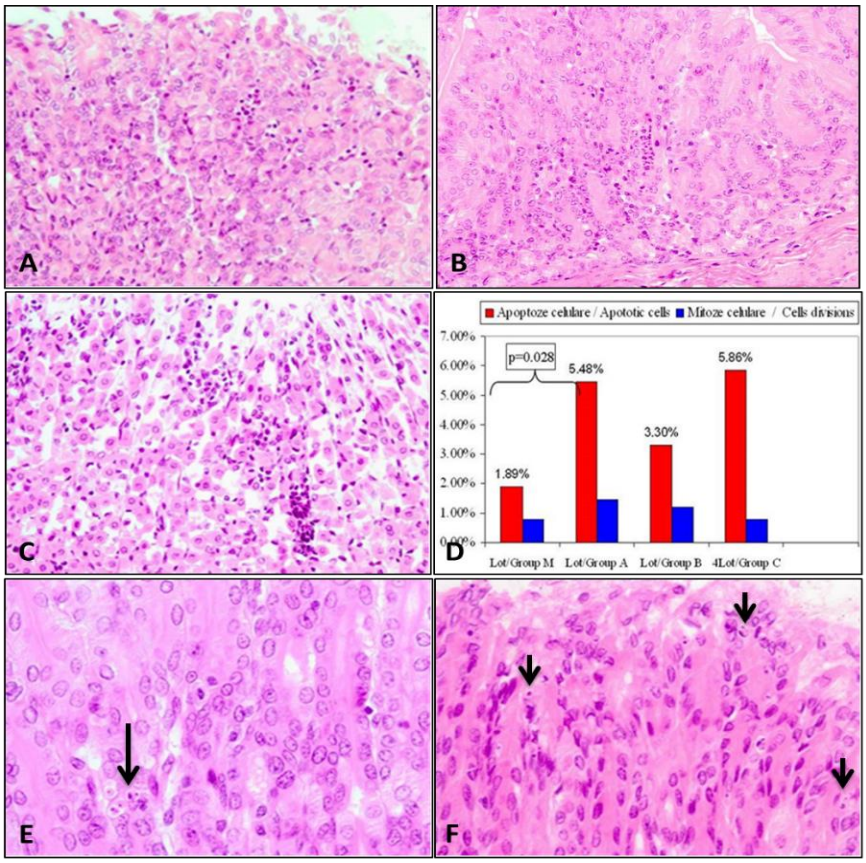

Fig. 2. Histological features of *H.pylori*-induced gastritis**: A/B/C)** Mild to moderate active chronic gastritis characterized by mixed inflammatory infiltrates composed of lymphocytes, plasma cells and neutrophils in the *lamina propria* in group II (H&E x 200).
D) The graphic showing the ratio of apoptotic/mitotic cells in the gastric mucosa of all groups of animals (M=group I; A=group II; B=group III; C=group IV). **E)** Nests of epithelial apoptotic cells of the gastric mucosa in group I and **F)** group II (black arrows).

Immunohistochemistry

The presence of *H.pylori* was detected by IHC in 2 animals from group II, at the gastric mucosal surface and in the lumen of the upper gastric glands. In the differentially treated groups (III and IV), IHC results were negative.

A better understanding of the connection between gastric colonization with *H. pylori* and certain pathologic conditions of the gastrointestinal tract requires the development of experimental animal models of *Helicobacter*-induced disease. Simulating human gastric disease allowed

researchers not only to study the pathogenicity of *H. pylori* infection and the host-bacterial interactions, but also to develop treatment guidelines for the management *of H. pylori*.

Therefore, use of experimental animal models, such as mice [13], gerbils [14,15], rats [16], ferrets [17], pigs [18] and guinea pigs [5] has allowed a thorough study of the pathogenesis of gastric inflammation. The guinea pig proved to be an ideal animal model for studying *H.pylori* infection, development of gastric lesions and modulation of the immune response by virulence factors [5].

The aim of this study was to induce gastric lesions associated with *H.pylori* infection in guinea pigs and to develop an effective eradication therapy. This was accomplished firstly by introducing a non-systemic, broad-spectrum antibiotic for use in gastrointestinal infections. Also, attempts have been made to atenuate various side effects of antibiotics, especially amoxicillin, by administering probiotics before and during antibiotherapy.

According to the literature, various side effects including pseudomembranous colitis and cholestatic hepatitis may be caused by amoxicillin [19]. When administered in addition to classical therapy, probiotics increased the rate of *H.pylori* eradication from 75% to 87%. However, probiotic therapy alone does not seem to determine eradication [19]. Administration of probiotics prior to classic therapy provides a greater gastric mucosal and hepatic protection than if administered during or after treatment [20].

In human medicine, various hematological, biochemical, histological and molecular markers, such as serum gastrin level, pepsinogen, hydrochloric acid secretion, ammonia, and hepatic enzymes are valuable paraclinical findings used for diagnosing *H.pylori* infection [21]. Numerous studies have shown elevated WBC and hepatic enzymes, especially AST, in *H. pylori* $CagA^+$ infected individuals [22,23]; similar results were obtained in the present study. Following treatment, leukopenia was detected.

In agreement with previous studies [19], a statistically significant decline in platelet count was identified in the differentially treated groups, unlike in group II. This was most likely caused by amoxicillin administration, which may result in transient myelotoxicity, with thrombocytopenia and leukopenia [24,25]. Administration of probiotics prior or during drug therapy did not seem to significantly influence these results.

Experimental data showed that gastric mucosa of *H.pylori* infected subjects may release increased GOT levels compared to *H.pylori*-uninfected individuals. Elevated GOT levels were also seen in amoxicillin-treated patients, most likely due to cholestatic hepatitis [26]. In our study, increased GOT, GPT and PAL levels were found in all of the *H.pylori*-infected animals, as well as in the amoxicillin-treated guinea pigs.

H.pylori infection causes chronic active gastritis; mononuclear (lymphoplasmacytic) and neutrophil infiltration of the gastric mucosa, along with the development of lymphoid aggregates and follicles, are typical features of *H.pylori* infection [5,27]. The results obtained in this study coincide with the aforementioned literature, in terms of histologically detected lesions, including: chronic active gastritis, glandular hyperplasia with cystic dilatation and moderate infiltration of mixed inflammatory cell population (lymphocytes, plasmocytes, macrophages, neutrophils and rare eosinophils).

Colonization of the stomach by *H.pylori* leads to an increased number of apoptotic epithelial cells in the gastric mucosa [28,29]. In our study, the guinea pigs were infected with $CagA^+/VacA^+$ strains, which resulted in an increased number of apoptosis and cell divisions in the infected groups (II, III, IV), in comparison to the control group (I). Eradication of *H.pylori* infection results in a decrease in the rate of apoptosis [30,31], as confirmed by the present findings.

The results of the present study demonstrated that infection in guinea pigs with *H.pylori* isolates from human gastric biopsies causes chronic active gastritis and induces cell proliferation

and apoptosis, which have been improved after eradication therapy. Also, through this research, a new protocol for treatment of *H.pylori* infection was developed, by introducing a new antibiotic combination therapy (rifaximin-amoxicillin) and probiotic premedication, leading to the reduction of antibiotic side effects.

Conclusions

The present research has identified an effective combination therapy with amoxicillin, rifaximin, omeprazole and probiotics for eradication of *H.pylori* gastric infection in guinea pigs. In addition, the previously mentioned therapy led to a decrease in gastric inflammatory infiltrate and diminished the apoptotic rates of gastric epithelial cells. Introduction of rifaximin into the classical eradication therapy and its association with probiotics may be a future choice in combating *H.pylori* infection both in animals and humans.

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Heavy metals in cat hair depending on keeping conditions

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Abstract

Many heavy metals serve no biological function and, given the appropriate dosage, they can be toxic, accumulating in keratin-rich tissues, like nails, hair, and skin. With hair analysis possessing non-invasive characteristics, the present study aimed to asses a possible relationship between heavy metals in cat hair and their living environment using ICP-MS. The study was conducted on hair samples taken from clinically healthy cats (n = 20), both males (n = 10) and females (n = 10), divided into groups of individuals living indoors or outdoors, and having ages below or above 5 years. Samples were assessed for levels of As, Cd, Cr, Hg, Ni, and Pb. Overall, cats above the age of five and cats living outdoors registered higher levels for most elements.

Keywords*: cat, hair, heavy metals, keeping condition*

Introduction

Industrial activity has redistributed many heavy metals from the Earth's crust into the environment, increasing the risk of animal exposure. Many heavy metals serve no biological function and, given the appropriate dosage, they can be toxic. (Chowdhury, 2011; Osweiler, 1996) Heavy metals accumulate in keratin-rich tissues, like nails, hair, and skin. (Poon, 2004; Ratnaike 2003) While hair analysis possesses non-invasive characteristics (Badea, 2016), it has been used in past years with various results. Results of heavy metal assessment from hair samples appear to be inconsistent because of lack of systematic methodological approach, while the absence of universally accepted and implemented reference ranges hinders the technique from becoming a useful and reliable method of assessment of heavy metal body burden and exposure of individuals. (Mikulewicz, 2013; Reis, 2010) Research and reviews show that a relationship between body burden, dosage, and exposure or toxicity is both reflected (Combs, 1982; Shamberger, 2002) and not reflected (Campbell, 2001) by heavy metal hair dosing. Researchers have tried to connect heavy metal levels in hair with the conditions cats are kept in using ICP-OES (Skibniewska, 2013) and AAS (Rzymski, 2015). Recently, ICP-MS has been extensively developed and has made significant advances in toxicological analysis. (Carter, 2016; Charlton, 2007; Evans, 2017; Goullé, 2005; Taylor, 2017) The present study aimed to asses a possible relationship between heavy metals in cat hair and their living environment using ICP-MS.

Materials and methods

The study was conducted on hair samples taken from clinically healthy cats (n = 20), both males (n = 10) and females (n = 10). The animals were divided into groups as presented in Table. 1. Five of the male cats were living indoors and five were living outdoors, and five of the female cats were living indoors and five were living outdoors. Of the male cats, five were below the age of five and five were above the age of five, and five of the female cats were below the age of five and five were above the age of five.

All hair samples were collected from the flank region, placed in disposable paper envelopes, labeled, and transported to the laboratory. The samples were degreased, washed, and rinsed. Each sample weighed roughly 0.5g, and was digested using 5 ml HNO3 and 1 ml HCl, then

diluted to 10 ml with ultrapure water and analysed by ICP-MS. Hair samples were assessed for the levels of As, Cd, Cr, Hg, Ni, and Pb.

Table 1. Numbers and categories of studied cats

Gender		Habitat		Age	
		Indoors	Outdoors	Below 5	Above 5
Male	10	5	5	5	5
Female	10	5	5	5	5
Total		20			

Results and discussions

Mean As levels depending on gender, habitat, and age are shown in Fig. 1.

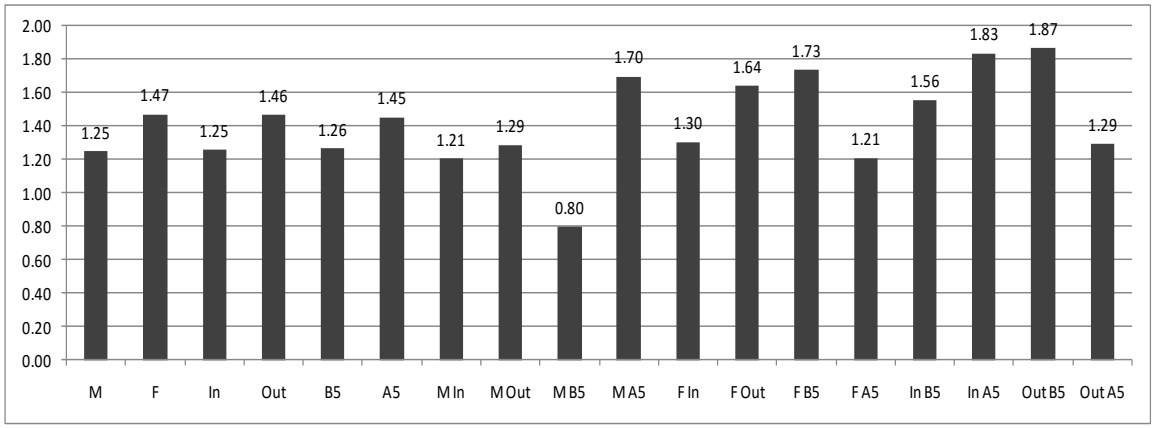

Fig. 1. Mean As levels depending on gender, habitat, and age (mg•kg^{-1}) (M – males; F – females; In – cats living indoors; Out – cats living outdoors; B5 – cats below 5 years of age; A5 – cats above 5 years of age; M In – males living indoors; M Out – males living outdoors; M B5 – males below 5 years of age; M A5 – males above 5 years of age; F In – females living indoors; F Out – females living outdoors; F B5 – females below 5 years of age; F A5 – females above 5 years of age; In B5 – cats living indoors below 5 years of age; In A5 – cats living indoors above 5 years of age; Out B5 – cats living outdoors below 5 years of age; Out A5 – cats living outdoors above 5 years of age)

Cats below the age of five and living outside registered the highest As level out of all the categories (1.87 mg•kg^{-1}). The lowest As levels were registered by males below the age of five (0.80 mg•kg^{-1}).

Higher As levels were registered in females compared to males, outdoor compared to indoor cats, and cats above the age of five compared to cats below the age of five.

Tomza-Marciniak et al. (2012) studied serum levels of As in pet dogs and found values of 0.49 mg•kg^{-1} in females and 0.64 mg•kg^{-1} in males.

Mean Cd levels depending on gender, habitat, and age are shown in Fig. 2.

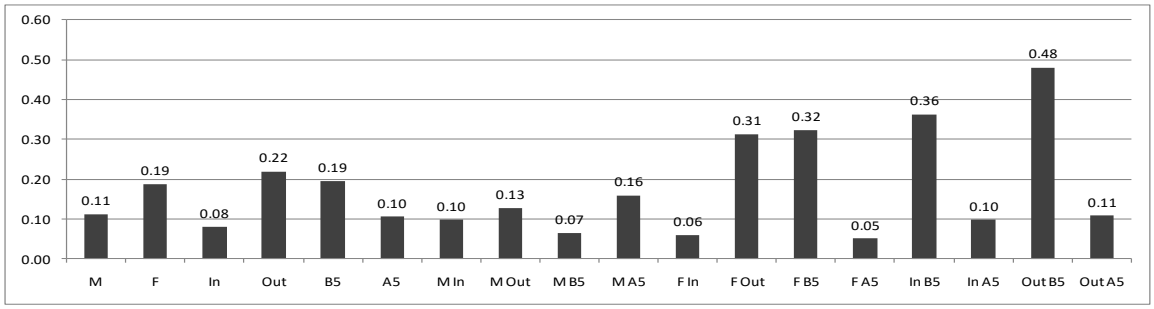

Fig. 2. Mean Cd levels depending on gender, habitat, and age (mg•kg^{-1}) (M – males; F – females; In – cats living indoors; Out – cats living outdoors; B5 – cats below 5 years of age; A5 – cats above 5 years of age; M In – males living indoors; M Out – males living outdoors; M B5 – males below 5 years of age; M A5 – males above 5 years of age; F In – females living indoors; F Out – females living outdoors; F B5 – females below 5 years of age; F A5 – females above 5 years of age; In B5 – cats living indoors below 5 years of age; In A5 – cats living indoors above 5 years of age; Out B5 – cats living outdoors below 5 years of age; Out A5 – cats living outdoors above 5 years of age)

Cats below the age of five and living outside registered the highest Cd level out of all the categories (0.48 mg•kg^{-1}). The lowest Cd levels were registered by females above the age of five (0.05 mg•kg^{-1}).

Higher Cd levels were registered in females compared to males, outdoor compared to indoor cats, and cats below the age of five compared to cats above the age of five.

Human hair samples were analyzed by Baran et al. (2013), and Cd concentrations were found to be 0.19 mg•kg^{-1} in females and 0.09 mg•kg^{-1} in males. Tomza-Marciniak et al. (2012) analyzed pet dogs serum and found 0.34 mg•kg^{-1} Cd in females and 0.30 Cd mg•kg^{-1} in males. Park et al. (2005) analyzed hair samples and found 0.05±0.09 Cd mg•kg^{-1} in female dogs and 0.09±0.18[2] Cd mg•kg^{-1} in male dogs.

Mean Cr levels depending on gender, habitat, and age are shown in Fig. 3.

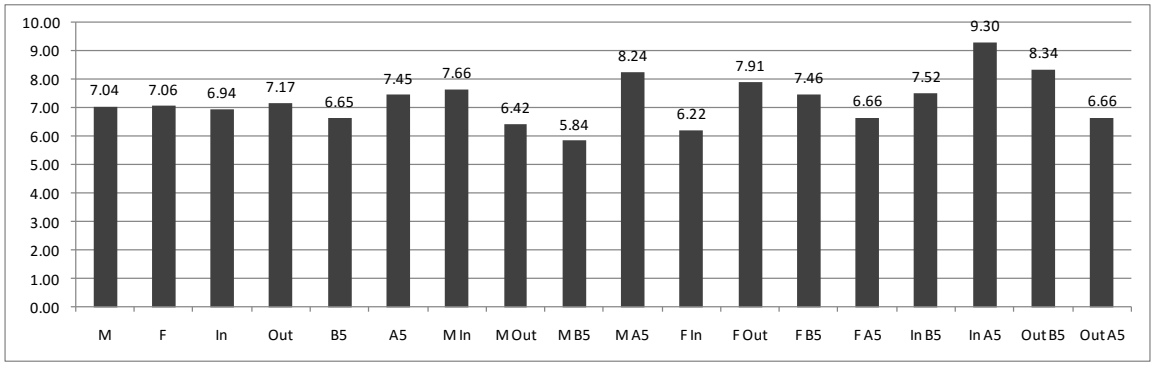

Fig. 3. Mean Cr levels depending on gender, habitat, and age (mg•kg^{-1}) (M – males; F – females; In – cats living indoors; Out – cats living outdoors; B5 – cats below 5 years of age; A5 – cats above 5 years of age; M In – males living indoors; M Out – males living outdoors; M B5 – males below 5 years of age; M A5 – males above 5 years of age; F In – females living indoors; F Out – females living outdoors; F B5 – females below 5 years of age; F A5 – females above 5 years of age; In B5 – cats living indoors below 5 years of age; In A5 – cats living indoors above 5 years of age; Out B5 – cats living outdoors below 5 years of age; Out A5 – cats living outdoors above 5 years of age)

Cats above the age of five and living inside registered the highest Cr level out of all the categories (9.30 mg•kg^{-1}). The lowest Cr levels were registered by males below the age of five (5.84 mg•kg^{-1}).

Cr levels were almost the same in males and females, and higher Cr levels were registered in outdoor compared to indoor cats, and cats above the age of five compared to cats below the age of five.

Filistowicz et al. (2011) found that Cr concentration in the hair of wild foxes was 0.26 and 0.20 in the hair of farm foxes.

Curi et al. (2012) studied the wild canids of the Brazilian Cerrado and found that Cr registered the following levels: 2.89±1.79 mg•kg^{-1} in maned wolves (*Chrysocyon brachyurus*), 2.95±1.5 mg•kg^{-1} in crab-eating foxes (*Cerdocyon thous*), and 3.55±1.77 mg•kg^{-1} in hoary foxes (*Lycalopex vetulus*)**.**

Tomza-Marciniak et al. (2012) analyzed Cr levels in serum of pet dogs and found a concentration of 0.26 mg•kg^{-1} in females and 0.24 mg•kg^{-1} in males.

Mean Hg levels depending on gender, habitat, and age are shown in Fig. 4.

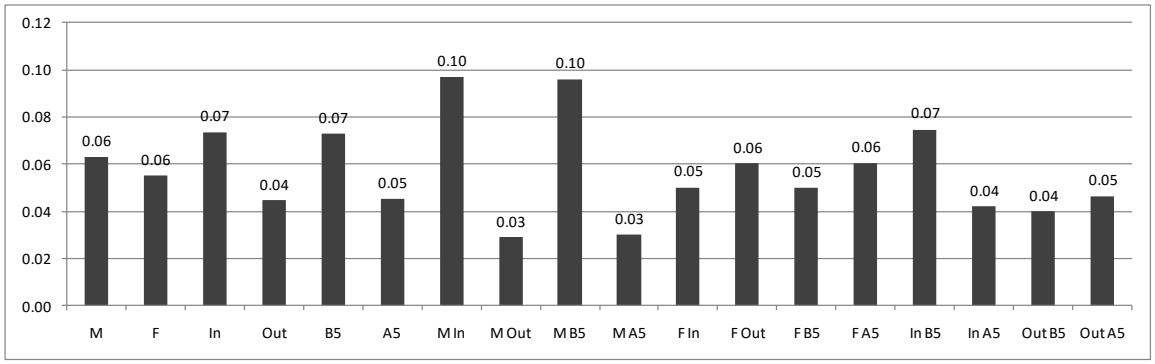

Fig. 4. Mean Hg levels depending on gender, habitat, and age (mg•kg^{-1}) (M – males; F – females; In – cats living indoors; Out – cats living outdoors; B5 – cats below 5 years of age; A5 – cats above 5 years of age; M In – males living indoors; M Out – males living outdoors; M B5 – males below 5 years of age; M A5 – males above 5 years of age; F In – females living indoors; F Out – females living outdoors; F B5 – females below 5 years of age; F A5 – females above 5 years of age; In B5 – cats living indoors below 5 years of age; In A5 – cats living indoors above 5 years of age; Out B5 – cats living outdoors below 5 years of age; Out A5 – cats living outdoors above 5 years of age)

The highest Hg level out of all the categories was registered by indoor male cats and male cats below the age of five. (0.10 mg•kg^{-1}). The lowest Hg level was registered by outdoor males and males above the age of five (0.03 mg•kg^{-1}).

Higher Hg levels were registered in males compared to females, indoor compared to outdoor cats, and cats below the age of five compared to cats above the age of five.

Sakai et al. (1995) analyzed Hg levels in cat hair and found 7.40 mg•kg^{-1} in males and 7.45 mg•kg^{-1} in females.

Park et al. (2005) analyzed hair samples and found 1.08±0.52 Cd mg•kg^{-1} in female dogs and 0.25±0.19 Cd mg•kg^{-1} in male dogs.

Mean Ni levels depending on gender, habitat, and age are shown in Fig. 5.

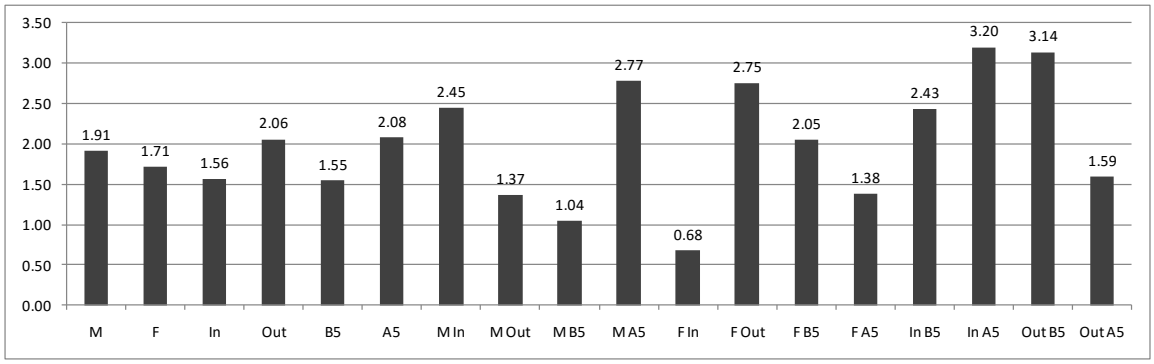

Fig. 5. Mean Ni levels depending on gender, habitat, and age (mg•kg^{-1}) (M – males; F – females; In – cats living indoors; Out – cats living outdoors; B5 – cats below 5 years of age; A5 – cats above 5 years of age; M In – males living indoors; M Out – males living outdoors; M B5 – males below 5 years of age; M A5 – males above 5 years of age; F In – females living indoors; F Out – females living outdoors; F B5 – females below 5 years of age; F A5 – females above 5 years of age; In B5 – cats living indoors below 5 years of age; In A5 – cats living indoors above 5 years of age; Out B5 – cats living outdoors below 5 years of age; Out A5 – cats living outdoors above 5 years of age)

The highest Ni level out of all the categories was registered by indoor cats above the age of five (3.20 mg•kg^{-1}). The lowest Ni level was registered by indoor females (0.68 mg•kg^{-1}).

Higher Ni levels were registered in males compared to females, outdoor compared to indoor cats, and cats above the age of five compared to cats below the age of five.

Filistowicz et al. (2011) found 0.29 Ni in the hair of wild foxes and 0.48 in the hair of farm foxes.

Ni levels in pet dogs serum were analyzed by Tomza-Marciniak et al. (2012), who found concentrations of 0.21 mg•kg^{-1} in both females and males.

Park et al. (2005) analyzed hair samples and found 0.05±0.09 mg•kg^{-1} in female dogs and 0.09±0.18^{2}mg•kg^{-1} in male dogs.

Mean Pb levels depending on gender, habitat, and age are shown in Fig. 6.

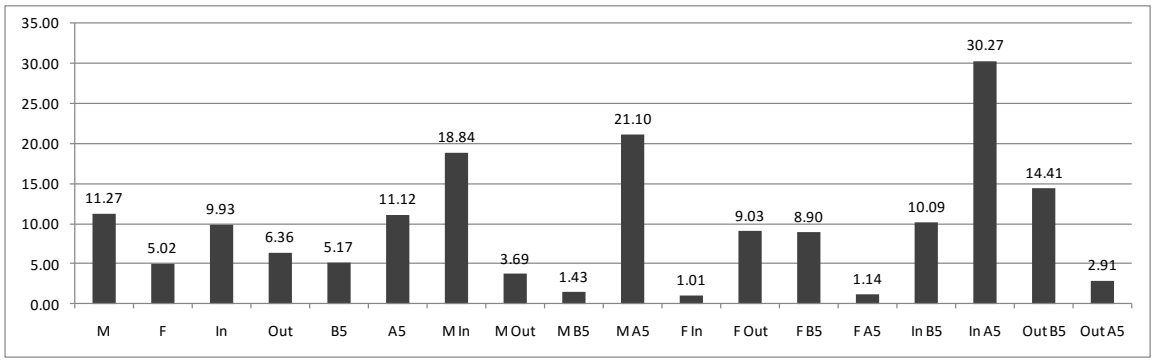

Fig. 6. Mean Pb levels depending on gender, habitat, and age (mg•kg^{-1}) (M – males; F – females; In – cats living indoors; Out – cats living outdoors; B5 – cats below 5 years of age; A5 – cats above 5 years of age; M In – males living indoors; M Out – males living outdoors; M B5 – males below 5 years of age; M A5 – males above 5 years of age; F In – females living indoors; F Out – females living outdoors; F B5 – females below 5 years of age; F A5 – females above 5 years of age; In B5 – cats living indoors below 5 years of age; In A5 – cats living indoors above 5 years of age; Out B5 – cats living outdoors below 5 years of age; Out A5 – cats living outdoors above 5 years of age)

The highest Pb level out of all the categories was registered by indoor cats above the age of five (30.27 mg•kg^{-1}). The lowest Pb level was registered by indoor females (1.01 mg•kg^{-1}).

Higher Hg levels were registered in males compared to females, indoor compared to outdoor cats, and cats above the age of five compared to cats below the age of five.

Pb was found to have 0.63 in the hair of wild foxes and 0.64 in the hair of farm foxes. (Filistowicz A, 2011)

Baran et al. (2013) studied human hair samples and found a concentration of Pb of 2.46 mg•kg^{-1} in females and 3.04 mg•kg^{-1} in males.

Skibniewski et al. (2013) conducted a study in which they analyzed Pb levels in hair samples from both feral and pet cats. They found that pet cats had levels of 1.00 mg•kg^{-1}, while feral cats had 2.89 mg•kg^{-1}. Pet males registered 1.02 mg•kg^{-1}, feral males 2.20 mg•kg^{-1}, pet females 0.98 mg•kg^{-1}, and feral females 3.58 mg•kg^{-1}.

Curi et al. (2012) studied the wild canids of the Brazilian Cerrado and concluded that Pb registered the following levels: 2.34±0.76 mg•kg^{-1} in maned wolves (*Chrysocyon brachyurus*), 2.45±1.22 mg•kg^{-1} in crab-eating foxes (*Cerdocyon thous*), and 1.50±0 mg•kg^{-1} in hoary foxes (*Lycalopex vetulus*).

Pb levels in serum of pet dogs were analyzed by Tomza-Marciniak et al. (2012), who found concentrations of 0.59 mg•kg^{-1} in females and 0.42 mg•kg^{-1} in males.

Combs et al. (1982) stated that environmental Pb exposure is positively correlated with concentrations of Pb in hair.

Conclusions

Males registered the highest levels of Hg, Ni, and Pb, compared to females, which registered the highest levels of As, Cd, and Cr.

Cats living outdoors registered the highest levels of As, Cd, Cr, and Ni, compared to cats living indoors, which registered the highest levels for Hg and Pb.

Cats below the age of 5 registered the highest levels of Cd and Hg, compared to cats above the age of five, which registered the highest levels of As, Cr, Ni, and Pb.

Overall, As and Cd were highest in outdoor cats below the age of five. Cr, Ni, and Pb were highest in indoor cats above the age of five. Hg was highest in indoor males and males below the age of five.

Overall, As and Cr were lowest in males below the age of five, and Cd was lowest in females above the age of five. Hg was lowest in outdoor males and males above the age of five. Ni and Pb were lowest in indoor females.

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Curcumin protects against the adverse effect of long term administration of lithium on cerebral and cerebellar cortices in rats "Histological and immunohistochemical study"

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Abstract

Administration of lithium, antidepressant and psychiatric medication, is always prolonged. This study was aimed to detect the adverse effects of long term administration of lithium on cerebral and cerebellar cortices in rats in addition to assess the possible protective effect of curcumin using histological and immunohistochemical methods. Rats were divided into 3 groups (10 for each); group I (control) given distilled water and DMSO orally, group II received lithium carbonate dissolved in distilled water (150 mg/ kg b. wt. / day / intragastric), and group III received curcumin dissolved in 50% DMSO (200 mg/ kg b. wt. / day/ intragastric) 1 hr before lithium carbonate administration for 6 weeks. We examined the cerebrum and cerebellum of rats for glial reactions and cell proliferation by using immunolabelling for glial fibrillary acidic protein (GFAP) and Ki67, respectively. In lithium treated group, both cerebral and cerebellar cortices showed an increased number of positive glial cells for GFAP that was decreased in curcumin treated group. For ki67, cerebral and cerebellar cortices of both lithium and curcumin treated groups showed an increased number of ki67 immunopositive cells. This study advises to administrate curcumin in concomitant with lithium therapy as it can protect against lithium neurotoxicity.

Introduction

Depression is a very common and worldwide disease (Aakhus et al., 2012). Although the efficacy of antidepressant drugs and decades of use, their side effects and how to avoid them is still under continuous investigations.

Lithium is a potent mood stabilizer (Zanni et al., 2017) therefore; it is one of the most widely used antidepressant and psychiatric medication (Sharma and Iqbal, 2005). In addition, it has an anti-suicide (Cerqueira et al., 2008) and anticonvulsant effects (Ahmed, 2013). Since the therapy is usually prolonged, it is unlikely to be without complications or side effects on the brain(Csutora et al., 2005) and other organs like kidney and heart (Sharma and Iqbal, 2005; Shah et al., 2015).

Medicines derived from plants play a pivotal role in the health care of many cultures. Curcumin is a yellow to gold colored spice that has been derived from the root turmeric plant Curcuma longa (Nabiuni et al., 2011). Curcumin is commonly known as antioxidant and anti-inflammatory (Aggarwal et al., 2007). Also, Curcumin has been described as a neuroprotectiveagent (Nabiuni et al.,2011) as well as its administration significantly control brain injury(Thiyagarajan and Sharma , 2004).

Since neurotoxicity has been assessed depending on classical histological observations(Gross and Kramer, 2003), the current study aimed to assess the possible protective effect of curcumin against lithium induced cerebral and cerebellar toxicity in adult rat using histological and immunohistochemical methods. Glial fibrillary acidic protein (GFAP), an intermediate filament protein of astrocyte, has been serving as a neurotoxicity biomarker (O'Callaghan and Sriram, 2005). The presence of ki-67 protein during all active phases of the cell cycle (G1, S, G2, and mitosis) makes it an excellent marker for cell proliferation (Scholzen and Gerdes, 2000).

Materials and methods

Material

Animals

Thirty adult male Albino rats (200-220 g b.wt) were purchased from lab animal house, Faculty of Veterinary Medicine, Benha University, Egypt to be used for this study. The rats were kept for 10 days before the experiment under good hygienic condition at room temperature, and were fed standard diet and watered ad libitum.

Chemicals

Lithium carbonate:

Tablets of Prianil CR (Nile Company for Pharmaceuticals and Chemical industries, Cairo, Egypt) were used as a source of lithium. Each tablet contains 400 mg of lithium carbonate.

Curcumin:

Curcumin powder was purchased from Sigma, Cairo, Egypt.

Experimental design:

The experiment followed the guidelines of Ethical Committee of Benha University. The rats were divided into 3 groups, each of 10 rats as follow:

Group I (control group): Rats were given the same amount of vehicle (distilled water and DMSO) orally for 6 weeks.

Group II: Rats received toxic dose of lithium carbonate dissolved in distilled water (150 mg/kg b.wt/ day/ intragastric according to Vijaimohan et al., 2010) for 6 weeks.

Group III: Rats in this group received curcumin dissolved in 50% DMSO (200 mg/kg b.wt/ day/ intragastric) according to Ahmed (2013) 1 hr before the administration of same dose of lithium carbonate as group II daily for 6 weeks.

Tissue collection and processing:

Twenty four hrs after the last dose, all rats were anaesthetized with ether inhalation and decapitated. Skull of each rat was opened and the brain was removed carefully. Mid sagittal section of the brain was obtained then immersed in 10% neutral buffered formalin for 48 hrs. Routine histological work was done to obtain paraffin blocks.

Histological examination

Five µm thickness paraffin sections were collected, deparaffinized and rehydrated using the standard techniques according to Bancroft and gamble (2007). Paraffin sections were stained with hematoxylin and eosin (H&E) for general structure and assessment of histological changes.

Immunohistochemical examination

Paraffin sections were collected into positive slides and processed for immunohistochemical examination using an avidin biotin peroxidase method according to Kiernan (2008). Deparaffinization and hydration were done before antigen retrieval which was performed by heating the slides in citrate buffer (pH 6.0) for 10 min in a steamer. To block endogenous peroxidase activity, slides were dipped in absolute methanol containing 3% (v/v) hydrogen peroxide for 10 min at RT. Sections were then incubated overnight at 4ºC with monoclonal mouse anti-ki67 (clone MM1, Novocastra Laboratories Ltd, UK) at 1:100 dilution and monoclonal anti-GFAP (AM020-5M Bio-genex) at 1:5000 dilution. Next, sections were exposed to biotinylated secondary antibody (Dako, USA) diluted 1:200 for 30 min at room temperature. Visualization was done using commercial peroxidase streptavidin complex (ABC; Dako, USA) for 30 min then 3,3´-diaminobenzidine tetrahydrocholoride (DAB; Dako, USA) for 2 min at room temperature. Finally, the sections were counterstained with hematoxylin. Negative control sections were incubated with normal goat or rabbit serum instead of the primary antibodies (Dako, USA).

Results

Group I (control group)

In H&E sections, normal histological appearance of both cerebrum and cerebellum was identified (Figs. 1A, 2A). Cerebral cortex of control group showed a clear pia mater, followed by a molecular layer then different pyramidal cells layers (Fig. 1A).The latter consists of nerve cells of various sizes and shapes and with vesicular nuclei (Fig. 1D).

Cerebellar cortex was formed of outer molecular, Purkinje cell layer and inner granular layers (Fig. 2A). The molecular layer is formed of scattered cells and nerve fibers. The Purkinje cell layer was the middle layer and consisted of large pyriform cells with clear vesicular nuclei arranged in one row along the upper margin of the granular layer. The granular layer was the inner most layer of the cerebellar cortex and composed of tightly packed small rounded cells with deeply stained nuclei (Fig. 2D).

Immunohistochemical staining of cerebral cortex for GFAP showed positive immunostaining in star shaped glial cells and their processes (Fig. 3A). Also, glial cells in molecular and granular layers of cerebellum showed positive immunostaining for GFAP (Fig. 3D). For ki67 immunostaining, some cells in cerebral cortex showed nuclear immunostaining for ki67 (Fig. 4A) in addition to, cerebellar cortex showed positive nuclear immunostaining in some cells of granular layer close to the molecular layer while Purkinje cells were not immunoreactive (Fig.4D).

Group II (lithium treated group)

Congestion and haemorrhage were observed in the blood vessels of the cerebral meninges (Fig. 1B). Marked degeneration of neurons with pyknotic nuclei, vacuolar spaces around the pyramidal cells and a pronounced interstitial edema were commonly detected (Fig.1E). Also, haemorrhagein the blood vessels of the cerebellar meninges could be seen (Fig. 2B). Purkinje cells appeared either degenerated with shrinkage of their cytoplasm and pyknotic or vacuolated nuclei (Fig. 2E).

There were an increased number of the positive GFAP immunostaining glial cells in cerebral cortex (Fig. 3 B) cerebellum (Fig. 3 E) and that appeared star shaped cells with increased branches of their cytoplasmic processes. The molecular layer of cerebellum showed an increased GFAP immunostaining in the Bergmann glia (modified astrocytes) which appeared perpendicular to the pial surface and parallel to each other (Fig. 3 E). For ki67 Immunostaining, cerebral cortex showed more positive cells and intense staining with mitotic figures (Fig. 4B). Also, cerebellar cortex revealed stronger immunostaining and more positive cells (Fig. 4E) in comparison to control.

Group III (protective group)

The cerebral cortex of the treated group showed an improvement in their histological structure with slight congestion of blood vessels (Fig. 1C). Although slight edema and vacuolation were still present (Fig. 1F). Cerebellum of this group showed slight congestion of meningeal blood vessels and medullary capillary (Fig. 2C). Most of Purkinje cell layer appeared same as normal cells (Fig. 2F).

Immunohistochemical staining of cerebral (Fig. 3C) and cerebellar (Fig. 3F) cortices showed fewer GFAP positive cells with their processes nearly similar to control group, especially those in granular layer. For ki67, both cerebral and cerebellar cortices showed nearly the same feature of lithtium treated group where more ki67 positive cells were seen (Figs. 4C and 4F, respectively) in comparison to control.

Discussion

The current work revealed normal histological structure of the cerebral and cerebellar cortices of the control rats as that stated by Mescher (2015). Histological observations of the cerebral cortex of lithium treated rats showed degenerated neurons with vacuolation indicating brain damage as reported by Young (2009)after prolonged lithium intoxication. Also, the cerebellar cortex of lithium treated rats showed distorted and degenerated Purkinje cells and vacuolation, in some areas indicating cell loss. This finding was in agreement with Cerqueira et al., (2008) and Bashandy (2013). Additionally, the observed haemorrhage of meningeal blood vessels was similar to Loghin et al., (1999) however Bashandy (2013) reported congestion of blood vessels.

Astrocytes, star-shaped glial cells in the central nervous system, have a major role in supporting neurons, scar formation and maintenance of the blood brain barrier (Mescher, 2015). Our immunohistochemical findings revealed positive GFAP immunostaining in the cytoplasm and processes of astrocytes in cerebrum and cerebellum of control rats that was in agreement with Bashandy (2013). In lithium treated group, an increase number of strongly GFAP immunostained glial cells (gliosis) and their processes was similar to Halliday et al. (1996) and Bashandy (2013). The increased number of glial cells may be a compensatory response to brain injury and neuronal degeneration caused by lithium. Ibrahim et al. (2015) demarcated that lithium induced noticeable degeneration of neurons and demylination of nerve fibers in cerebrum.

The increased GFAP staining in Bergmann glia of the cerebellar molecular layer was similar to the finding of Wagemann et al. (1995). The strong GFAP immunostaining with increased cell process in the astrocytes of granular layer was similar to Hashish (2014).

For ki67, the cells of both cerebral and cerebellar cortices of control group showed immunoreactivity. These cells increased in number and immunostaining intensity in lithium treated group. These finding was in agreement with Zanni et al. (2017), who owed this finding to the positive effects of lithium on neurogenesis; generation and integration of new neurons.

Histological examination of the curcumin protected group showed structural improvement of the cerebral and cerebellar cortices in comparison to lithium treated group, suggesting the neuroprotective property of curcumin(Nabiuni et al., 2011; Nasir and Jaffat, 2016). Immunohistochemical finding of the curcumin protected group showed decreased GFAP immunostaining compared to lithium treated group. This finding was in harmony with Parastoo et al. (2015), who detected significant decrease of GFAP in curcumin group following acute spinal cord injury, suggesting that curcumin can limit gilosis. On the other hand for ki67, curcumin protected group showed nearly the same feature of lithtium treated group where more ki67 positive cells were detected. This indicates that curcumin has stimulating effect on neurogenesis, likewise lithium (Xua et al., 2007; Attari et al., 2016).

Conclusion

The overdose and/or chronic therapy with lithium has neurotoxic effect on the cerebrum and cerebellum characterized by sever alteration in brain ultrastructure. This study advises to curcumin co-treatment with lithium therapy due to its ameliorating properties against drug neurotoxicity.

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Fig.1

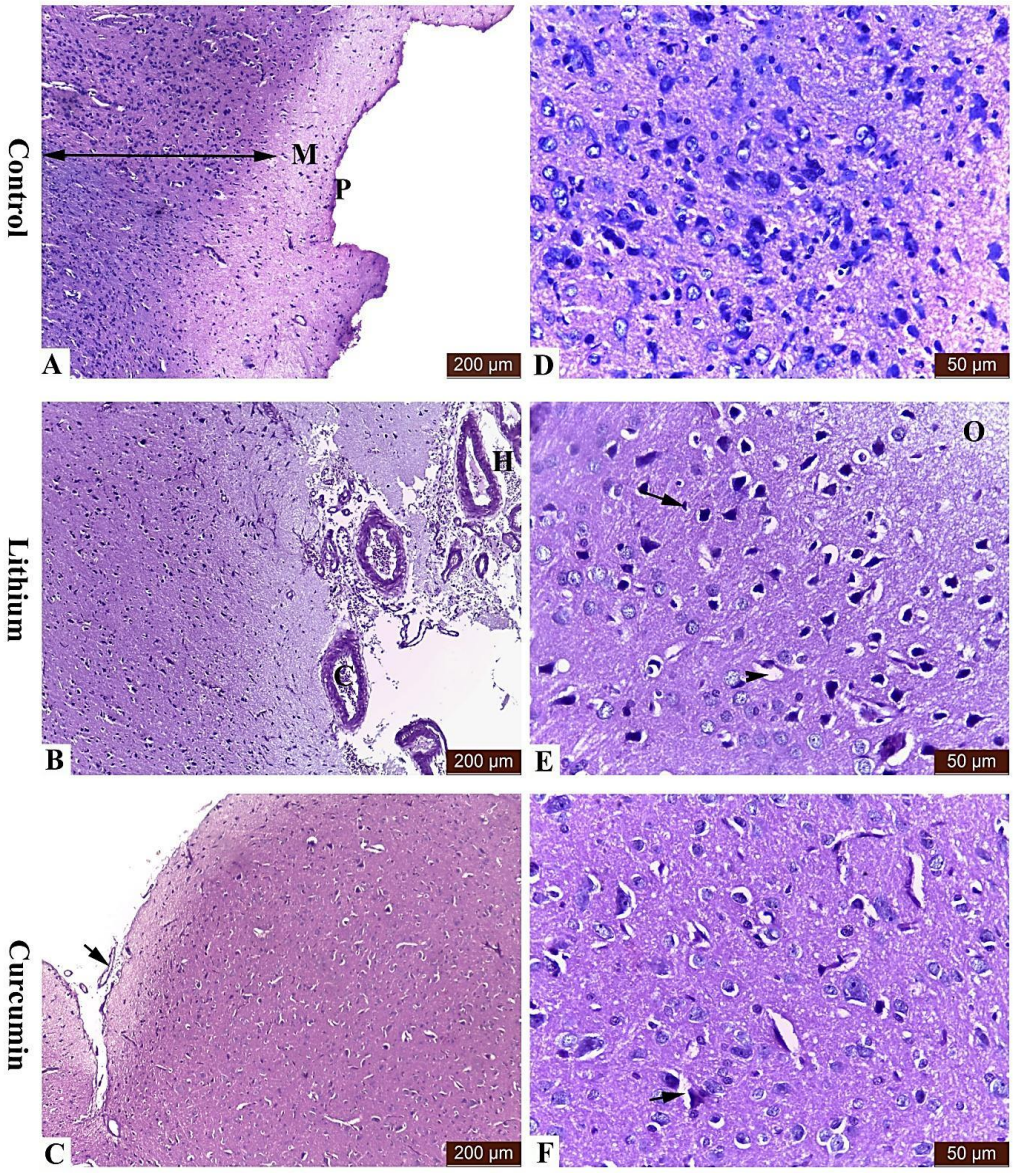

Fig.1. Photomicrograph of cerebral cortices of control (A, D), lithium treated (B, E) and curcumin treated (C, F) rats. A: Normal histological structure of cerebral cortex. P, pia mater; M, molecular layer;, pyramidal cells layers. B: Showing congestion (C) and haemorrhage (H) in the blood vessels of the meninges. C: Showing slight congestion of blood vessels (arrow). D: Showing different sizes and shapes of pyramidal cells with vesicular nuclei. E: Showing degenerated neurons with pyknotic nuclei (arrow), vacuolar spaces around the pyramidal cells (head arrow), and a pronounced interstitial oedema (O). F: Showing slight edema and vacuolation (arrow). Scale bars (A-C) = 200 µm and (D-F) = 50 µm.

Fig.2

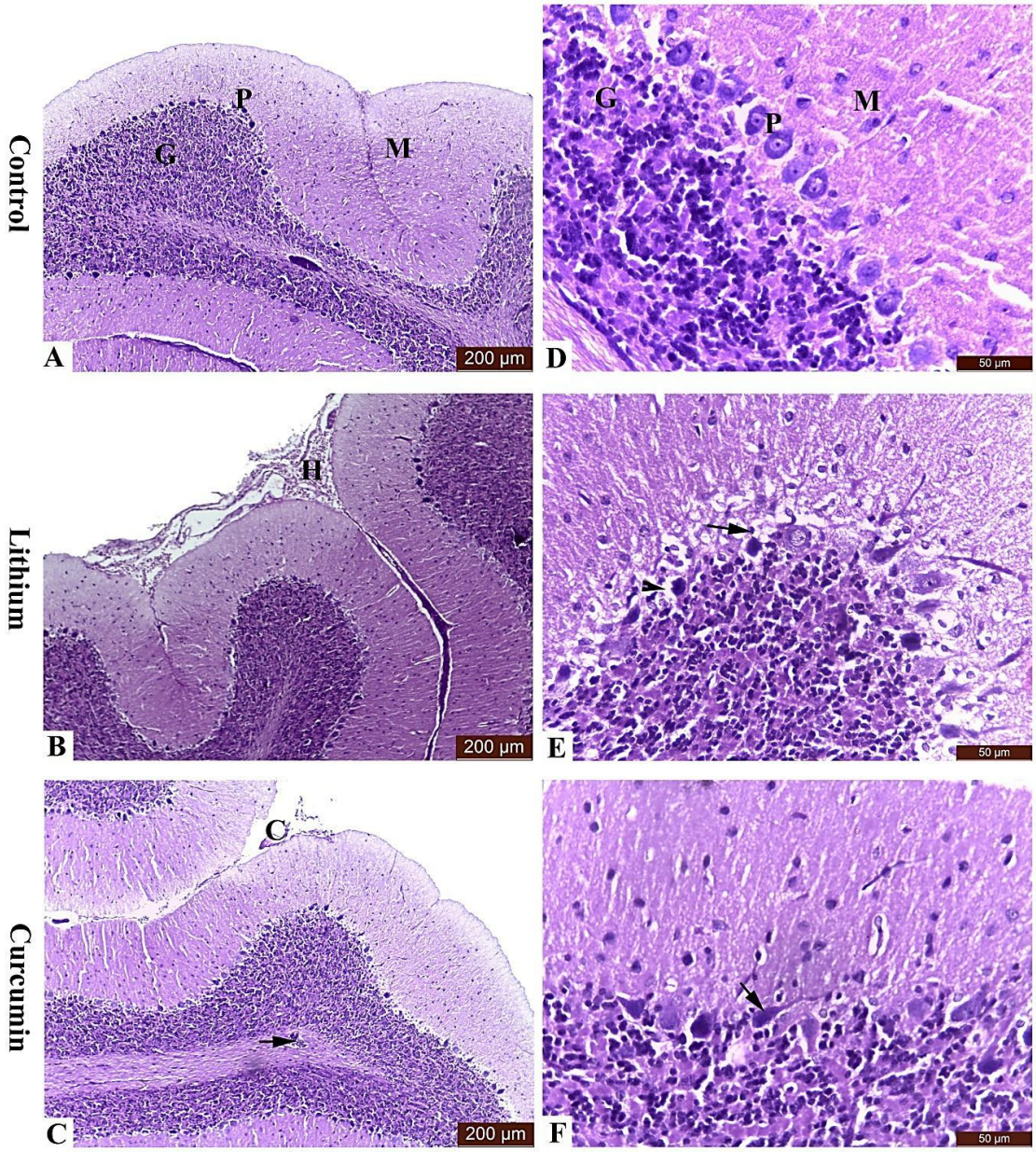

Fig.2. Photomicrograph of cerebellar cortices of control (A, D), lithium treated (B, E) and curcumin treated (C, F) rats. A: Normal histological structure of cerebellar cortex. M, molecular layer; P, Purkinje cells; G, granular layer. B: Showing haemorrhage at the cerebellar meninges (H). C: Showing slight congestion (C) of meningeal blood vessels with slightly congested medullary capillary (arrow). D: Higher magnification of A. M, molecular layer; P, Purkinje cells; G, granular layer. E: Showing Purkinje cells appeared either degenerated with shrinkage of their cytoplasm and pyknotic nuclei (arrow) or vacuolated indicating cell loss (head arrow). F: Showing normality of most of Purkinje cell layer (arrow). Scale bars (A-C) = 200 µm and (D-F) = 50 µm.

Fig.3

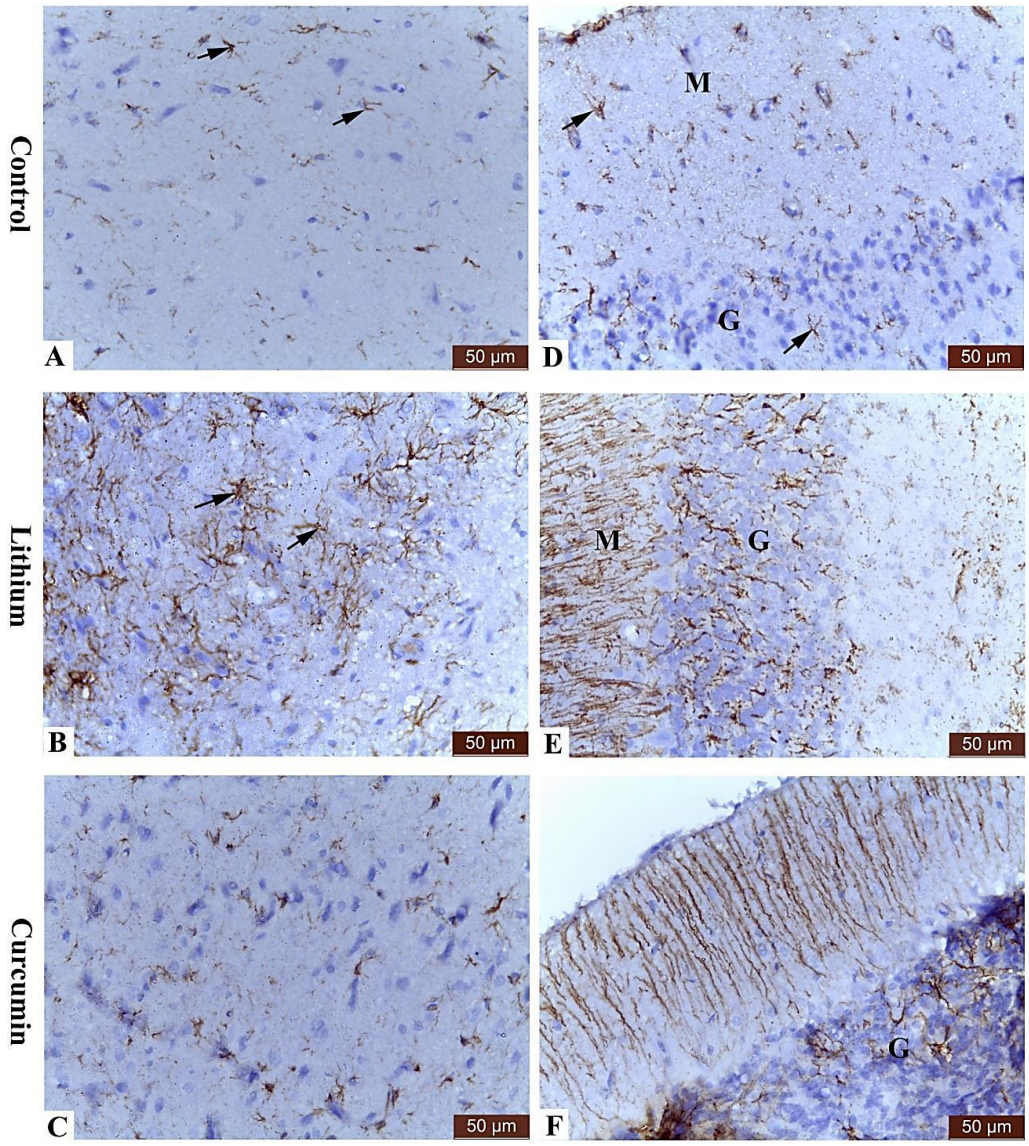

Fig.3. Photomicrograph of GFAP immunostainings in cerebral and cerebellar cortices of control (A, D), lithium treated (B, E) and curcumin treated (C, F) rats. A: Cerebral cortex showing positive immunostaining in star shaped glial cells and their processes (arrows). B: Cerebral cortex showed increased number of the positive glial cells with increased branches of their cytoplasmic processes (arrows). C: Cerebral cortex showed fewer positive cells. D: Cerebellar cortex showing positive immunostaining in glial cells (arrows) of molecular (M) and granular layers (G). E: Cerebellar cortex showed strong and increased immunopositive Bergmann glia of the molecular layer (M) and star shaped glial cells and their processes of the granular layer (G) than D. F: Cerebellar cortex showing fewer positive glial cells especially those in granular layer (G) than E. Scale bars = 50 µm.

Fig.4

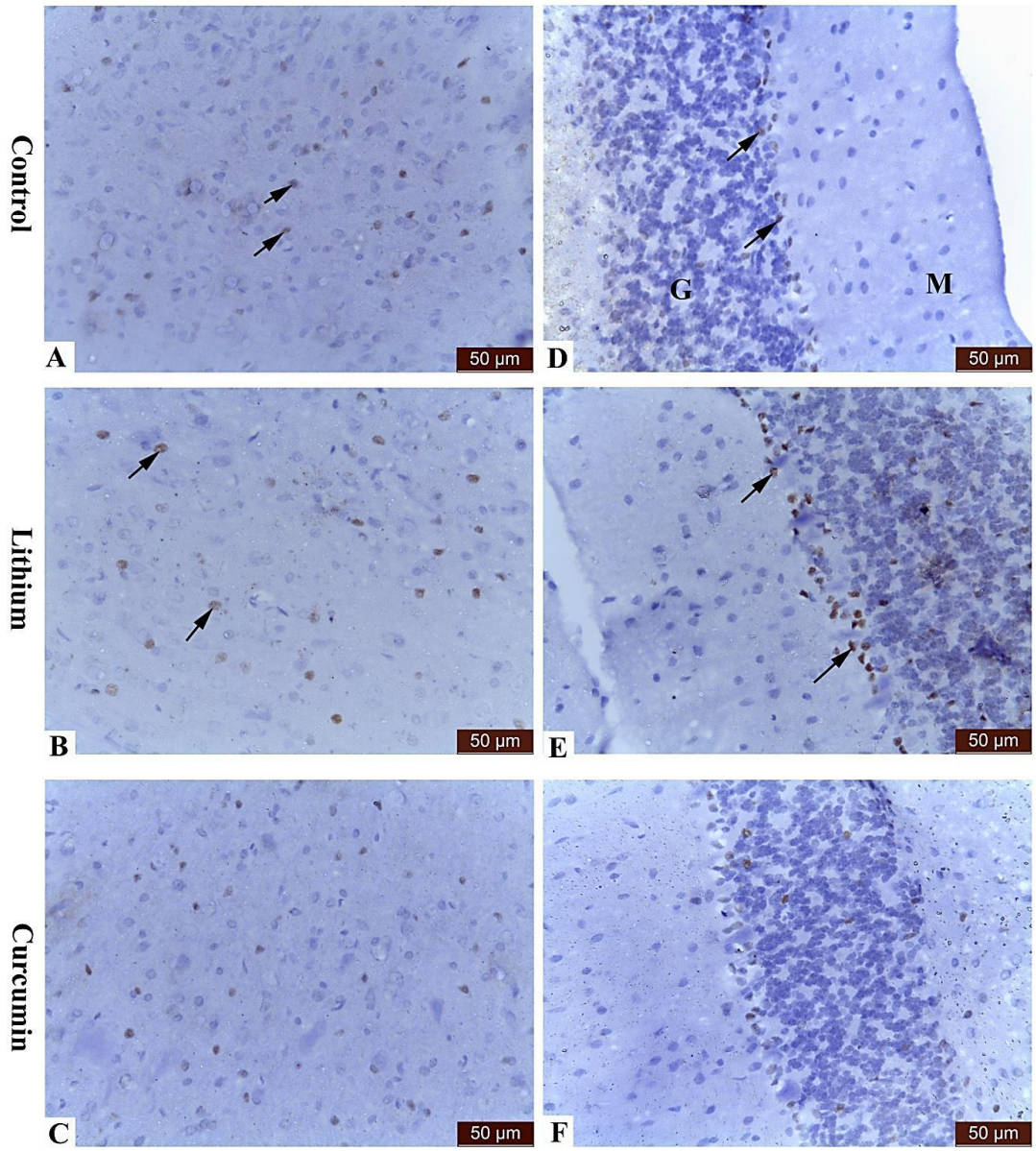

Fig.4. Photomicrograph of ki67 immunostainings in cerebral and cerebellar cortices of control (A, D), lithium treated (B, E) and curcumin treated (C, F) rats. A: Cerebral cortex showed some positive cells (arrows). B: Cerebral cortex showed more positive cells and increased staining intensity with mitotic figures (arrows). C: Cerebral cortex showed nearly the same feature of B. D: Cerebellar cortex showed some positive cells (arrows) of granular layer (G) close to the molecular layer (M). E: Cerebellar cortex showed strong immunostaining and more positive cells (arrows). F: Cerebellar cortex showed nearly the same feature of E. Scale bars = 50 µm.