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- این مجله به استناد نامه شماره ۳/۲۹۱۰/۵۴۵ مورخ ۸۴/۵/۸ کمیسیون بررسی نشریات وزارت علوم، تحقیقات و فناوری دارای **درجه علمی ـ پژوهشی** می‌باشد.
- این مجله در فهرست نشریات علمی پژوهشی معتبر **ISC** که دارای ضریب تأثیر (**IF**) می‌باشند، قرار دارد. این اعتبار از سوی وزارت علوم، تحقیقات و فناوری تعیین شده است و در آئین‌نامه ارتقای مرتبه علمی دانشگاه‌ها و موسسات آموزشی مورد بهره‌برداری قرار می‌گیرد.
- متن کامل مقالات در پایگاه استنادی علوم جهان اسلام (**ISC**) قابل دسترسی می‌باشد.

چاپ و صحافی
مرکز منطقه‌ای اطلاع‌رسانی علوم و فناوری و پایگاه استنادی جهان اسلام

بسم اللّه الرحمن الرحیم

مجله دامپزشکی ایران

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Effect of fishmeal replacement with poultry by-product meal on serum parameters and histomorphology of liver and kidney in Nile tilapia ((*Oreochromis niloticus*), Linnaeus, 1758)

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Abstract

Today, *Oreochromis niloticus* has become one of the most popular fish in the world. Due to the increasing prices of fishmeal and its limited availability, various protein sources, including poultry by-product meals (PBM) can substitute fishmeal in the aquatic diet. This study was thus intended to investigate the effect of replacing fishmeal with different ratios of PBM on the liver, and kidney tissue structure changes, growth performance, and some serum parameters of *O. niloticus*. To that end, 120 *O. niloticus* were randomly distributed into four groups: the control, 25, 50, and 100% PBM meal instead of fishmeal which was fed for 44 days. At the end of the treatment period, growth parameters, blood serum, liver enzymes, and histomorphology of the liver and kidney of all fish were evaluated. The results showed that the liver enzymes increased significantly in the groups with higher replacement (100% PBM) compared to the control group; and in the histological examinations of the liver, the liver tissue lost its normal structure and function with the increase in the amount of replacement diets and there were fat vacuoles accumulated in the cytoplasm of hepatocytes. The level of urea plasma also showed a significant difference with the increase in the amount of substitute diets among the groups with upward substitution compared to the control group. These changes were evident in the structure of tubules and glomeruli. Data suggests that 100% PBM meal is not recommended for fishmeal substitution in *O. niloticus* but PBMs up to 50% can replace fishmeal for *O. niloticus* diet without adversely affecting the growth performance and biochemical parameters of the fish.

Keywords: Fishmeal, Growth performance, Histomorphometric changes, *Oreochromis niloticus*, Poultry by-product (PBM)

Introduction

Aquaculture and fisheries play important roles in providing food and income for hundreds of millions of people around the world (FAO, 2018). *Oreochromis niloticus* (Linnaeus, 1758) belonging to the Cichlidae family, in the order Perciformes, is one of the most species-rich families of fish, particularly in tropical freshwater fisheries

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(Metwalli, 2013; Rahmati et al, 2022). It is also the second most farmed fish worldwide and its production has multiplied over the past decade because of its suitability for aquaculture, marketability and stable market prices. Given its reasonable price and high meat quality, *O. niloticus* can play a key role in providing the animal protein needed by the rapidly growing global population, especially low-income people in many developing countries. Among the important species of *O. niloticus* in the world, the culture of *O. niloticus* is now under practice almost everywhere (FAO, 2018). However, fish feed and nutrition management in aquaculture has been always one of the main challenges of breeders. Fishmeal is a commercial nutrient-rich feed ingredient that is mostly made from fish and is usually used to feed farm animals in agricultural settings (Zhou et al, 2020). Given the increasing prices of fishmeal and its limited availability (Irm et al, 2020), various food items have been thus considered to find alternative protein sources in diets for farmed *O. niloticus*, including sources of animal protein such as fish by-products, pet by-products such as feather powder, blood powder, bone meal, and PBM, as well as vegetable protein such as soy powder, sesame, and canola, etc (Ogunji, 2004; Panserat et al, 2009). Poultry by-product meal (PBM) is a by-product of the poultry processing industry that is made from waste parts of the poultry body such as blood, legs, head, and internal organs (Yu, 2023). Replacing fishmeal with other alternative protein sources such as PBM in aquafeed has been regarded as a way toward more sustainable development of universal aquaculture, given the high content of protein, amino acid, and phosphorus in PBM diets (Irm et al, 2020). Qiu et al (2023) reported that a diet containing PBM could well provide adequate protein for Chinese soft-shelled turtles. Likewise, the results of Irm et al (2020) showed that PBM can partially substitute the fishmeal without leaving any negative impact on growth performance and feed utilization of juvenile black sea bream. The fish liver and kidney are important target limbs that are specifically affected by dietary as well as biological and environmental parameters (Zhou et al, 2020). Nutritional imbalances in dietary components can indeed result in changes in the structure, metabolism, and morphology of fish liver and kidney (Roberts, 2003). Nutritional compounds can also have significant effects on the composition of blood parameters, making it possible to evaluate the effects of different food treatments on the fish body by measuring serum parameters, and histomorphology of the liver and kidney in fish (Nogales-Mérida et al, 2011).

Many studies have been done on different types of alternative foods in aquaculture. However, no studies have been reported on histomorphometric liver and kidney following consumption of PBM. Therefore, the current research is designed to investigate the effects of replacing feeding with PBM instead of fish meal in tilapia fish and its effects on the histology of the liver, kidney, and some blood parameters in farmed *O. niloticus* as an indicator of fish health.

Materials and Methods

Diets and experimental design

The studied fish were randomly divided into 4 groups each with 30 fish (Department of Aquatic Health and Diseases, at Shahid Chamran University of Ahvaz) (i.e., 10 fish in 3 repetitions for each group). In doing so, three treatment groups received diets containing approximately 40% protein, while the fish in the control group were fed a daily commercial diet containing 40% protein (Purely fishmeal) (PBM0). More precisely, the fish in the first treatment group received 25% PBM instead of fishmeal, and the second treatment group received 50% PBM rather than fishmeal, while the third group was, in turn, fed 100% PBM instead of fishmeal. The composition of the experimental diets (based on the dry

matter) was measured in one liter (weight) (Table. 1), and the fish diets were adjusted based on their standard nutritional needs so that all groups had the same diet in terms of all nutrients. Simultaneously with the addition of PBM to the groups' diets, two essential amino acids (namely, lysine and methionine) were also added to the diets for these groups so that they were similar in terms of amino acid levels (Zhou et al, 2020).

Table 1: Formulation and analyzed composition in different groups (dry-matter basis)

Ingredients	Dietary treatments			
	PBM0	PBM25	PBM50	PBM100
Ingredient Replacement of FM CP (%)	0.0	25	50	100
Fish meal (% dry matter)	52.5	39.4	24.2	0.0
Poultry by-product meal (PBM) (% dry matter)	0.0	13.7	25.9	52.8
Mineral premix (g. kg^{-1}) *	2	2	2	2
vitamin premix (g. kg^{-1}) **	2	2	2	2
Antifungal (g. kg^{-1})	0.1	0.1	0.1	0.1
Salt (g. kg^{-1})	5	5	5	5
Carbohydrates (%)	5.2	9.2	9.5	10.1
white flour (g. kg^{-1})	10	10	10	10
Chromium oxide (g. kg^{-1})	0.5	0.5	0.5	0.5
Analyzed composition				
Ash (%)	10.3	10.8	10.8	10.2
Moisture (%)	6.8	6.5	6.4	6.5
Dry matter (%)	91	90	91	91
Crude protein (%)	38.6	38.2	39.8	38.8
Crude lipid (%)	10.3	10.1	10.6	10.7

*Mineral premix was provided by Animal Feed Company Products (Mazandaran, Iran).
Mineral premix (g.kg^{-1}): choline chloride 1750 mg, copper 11 mg, iron 56 mg, zinc 92 mg, Magnesium 34 mg, cobalt 0.8 mg, iodine 3 mg, selenium 0.75.
** Vitamin premix was provided by Animal Feed Company Products (Mazandaran, Iran).
vitamin premix (g.kg^{-1}): D36000 IU, A9000 IU, K3 15 mg, E 600 mg, C 780 mg, Thiamine 45 mg, Riboflavin 75 mg, Inositol 350 mg, Cyanocobalamin 120 mg, Pantonic acid 135 mg, Niacin 450 mg, Folic acid 34 mg, Biotin 3 mg, Antioxidant 87 mg.

Dietary treatments belonging to different groups were indeed the same in terms of protein and energy. The average crude protein (41±5) % and energy (20±2) mJ were, in turn, measured based on the requirements of *O. niloticus* in the present weight range. To provide each treatment group with its respective diet, the researchers first weighted the required amounts of material for each diet using a digital scale that provided the weights nearest to one gram. Then, items that had smaller particles in the diet, including white flour, mineral supplements, vitamin supplements, antifungals, salt (used to enhance the taste of the diet) and chromium oxide (used to increase the digestibility of the diet) were well mixed and the resulting mixture was later mixed with items including coarser particles (i.e., fishmeal or PBM). Finally, water and a little soybean oil were added to the resulting mixture of 25-30% of the diet until a homogeneous dough was obtained. The resulting dough which was made into noodles was dried and finally made in the form of pellets considering the size of the fish mouth (less than 2 mm) (according to the guidelines of the Iranian Fisheries Organization). The prepared pellets were then kept at -20 °C till use.

Fish and experimental conditions

A hundred and twenty fish with an initial weight of about 40-60 g were prepared from the *O. niloticus* farm and transferred to 100-liter pre-disinfected aquariums in the Department of Aquatic Health and Diseases, at Shahid Chamran University of Ahvaz, Iran. At first, the fish were kept for

2 weeks and closely monitored to ensure their health and adaptation to their new environment in the laboratory. In the meantime, they were fed under natural photoperiod conditions of approximately 12 h light/12 h darkness. The physical and chemical factors of water were the same for all groups and were checked daily (Table 2). During storage and testing, aquarium water was siphoned off at a rate of 20% by volume every two days from the bottom of the aquarium floor to remove fish excrement. Fish were fed at 3% of weekly BW, twice a day (morning and afternoon) for 44 days with the prepared diets.

Table 2: List of physicochemical factors of water for all groups

Oxygen	Total pH	Total salinity	Ammonia	Nitrite	Nitrate	Hardness	Temperature
6-8 mg/L	8-8.4	1.2 ppt	0.1 mg/L	0.02 mg/L	5 mg/L	180 mg/L	24±2 ºc

Growth performance and feed utilization indices

At the beginning and end of the experiment, 5 fish (15 fish from each group) were taken from each aquarium and their weight and length were measured. To evaluate the growth rate of the fish in each treatment at the end of the final weight (FW) test period, the Total length (TL) of each of the studied fish was measured and recorded using a digital scale and ruler. Afterward, the fish growth indices including body weight gain index (BWI %), food conversion ratio (FCR), survival rate (SR), incidence cost (IC), and profit index (PI) of fish were examined. These indicators were calculated as BWI (%) =100 [final body weight(g)-initial body weight(g)]/initial body weight(g), FCR=dry feed intake(g.day-^{1}) /wet weight gain(g.day-^{1}), IC= cost per kilogram of feed consumed/per kilogram of fish produced PI=value per kilogram of fish product/per kilogram of feed consumed, and survival rate (%)=100 (final number of fish/ initial number of fish). These apparent digestibility coefficients (ADCs) of the experimental diets were calculated according to Qiu et al (2023).

Hematological and blood chemistry parameters

At the end of the experiment after a 24-hour fasting period (to minimize the possibility of error due to feed diet), the fish were anesthetized with clove oil solution (Giahine, Iran) at a dose of 75 mg/l. Then, blood samples taken from the caudal vein of the five fish in each replicate in all treatments were collected in Eppendorf tubes without anticoagulant and centrifuged at 3000 rpm for 15 minutes to separate their serum. The non-hemolysed serum was also collected and stored at -20°C until use. Levels of serum aspartate aminotransferase (AST), alkaline phosphatase (ALP), and alanine aminotransferase (ALT) were then determined and the samples were finally measured using an auto-analyzer (Hitachi, Japan) at a wavelength of 340 nm (laboratory kits of Pars Azmoun company, Iran). Serum albumin, urea (laboratory kits of Pars Azmoun company, Iran), Cholesterol, and triglyceride, were measured by the colorimetric enzyme procedure (Zist Shimi, Iran) (Basir and Abdi, 2015).

Liver and kidney histological evaluation

To prepare histological sections of the studied fish, liver and posterior kidney samples were taken from the fish immediately after blood sampling, and were placed in 10% neutral buffered formalin (Merck, German). Tissue samples that were dehydrated by a series of ethanol ascending solutions were then cleared in xylene and finally embedded in paraffin. Sections with 4-6 μm thickness were prepared from paraffin blocks with a rotary microtome machine (RM2245-LEICA, German), and then stained with hematoxylin and eosin (H&E) (Merck, German) using standard techniques (Moradkhani et al, 2020;

Nochalabadi et al, 2023). Histological changes were examined under a light microscope (Olympus BH-2, Tokyo, Japan) equipped with a Dino-lite lens with Dino capture2.0 software (Dino-lite, Taiwan). For grading histological changes, ten sections from each group and at least five fields in each section were analyzed microscopically at 400× magnification. In the histometric evaluation of liver tissue, the size of hepatocytes, the connective tissue between cells, and the presence of adipose tissue within liver cells in different groups were counted. According to Caballero et al., four degrees are considered for the vacuolation: Grade 0: No vacuolation within liver cells (zero vacuolation). Grade 1: Less than 1/3 cytoplasms of hepatocytes show vacuolation (low vacuolation). Grade 2: Between 1/3 and 2/3 cytoplasms of hepatocytes show vacuolation (moderate vacuolation). Grade 3: complete evacuation of the cytoplasm of liver cells (severe vacuolation) (Caballero et al, 2004). In the histometric evaluation of the kidney tissue, all changes in the diameter and epithelial cells of the urinary tubules, glomerular diameter, number of glomeruli, and the connective tissue between the urinary tubules in different groups were evaluated qualitatively according to Bernet et al., in three degrees: (-) without any visible histological changes in any microscopic field, (+) histological changes visible in each microscopic field were less than 20%, (++) The visible histological changes in each microscopic field were between 20-60%, (+++) Histological changes visible in each microscopic field were complete and more than 60% (Bernet et al, 1999). Slides were read in a 'blind' fashion to provide more accurate results**.**

Statistical analysis

The data were normalized using the Kolmogorov–Smirnov test. Then, to compare the means in different experimental groups with the control group, a one-way ANOVA test was followed by Tukey post hoc. Statistical analysis was performed using the Graph Pad Prism (V.5.03. San Diego, CA, USA). The results were presented as mean± SEM, and the level of significance was set at 0.05 for all the performed statistical tests.

Results

Growth performance and feed utilization indices

The effect of the experimental diets on various growth performances in the studied fish groups is shown in Table 3. All diets were well accepted by the fish and there were no significant differences in feed intake during the experiment period. Statistical analysis showed that increasing fishmeal replacement with PBM in *O. niloticus* diet caused significant changes in the bioassays of *O. niloticus* in different groups ($P<0.05$). As the replacement level of fishmeal with PBM increased, the level of BWI, IC, and survival rate decreased, while the amount of FCR and PI increased considerably, leading to significant changes among different groups ($P<0.05$). Also, these parameters were not significantly influenced by different fishmeal replacements (0%–25%) with PBM ($P>0.05$).

Table 3: Growth performance and feed utilization in different groups (mean± SEM, n = 5 of each tank in each repetition)

Growth performance	Dietary treatments			
	PBM0	PBM25	PBM50	PBM100
IBW[a]	58.4 ±8.10 [a]	58.2 ±7.52 [a]	58.1 ±9.50 [a]	58.1 ±7.26 [a]
BWI[b]	151.4 ±8.71 [a]	147.9 ±10.36 [a]	136.4 ±7.15 [ab]	124.9 ±4.21 [b]
FCR[c]	0.87 ±0.05 [a]	0.96 ±0.03 [a]	1.09 ±0.15 [ab]	1.37 ±0.08 [b]
IC[d]	2.30[a]	2.13[b]	1.58[c]	1.02[d]
PI[e]	0.37[a]	0.48[a]	0.71[ab]	1.25[b]
Survival	100	100	100	98.60

[a]IBW (%): initial body weight.
[b]BWI (%): body weight gain index =100[final body weight(g)-initial body weight(g)]/initial body weight(g).
[c]FCR: feed conversion ratio= dry feed intake (g.day-^{1}) /wet weight gain(g.day-^{1})
[d]IC: incidence Cost= cost per kilogram of feed consumed/per kilogram of fish produced
[e]PI: profit index= value per kilogram of fish product/per kilogram of feed consumed
Survival (%): survival rate=100 (final number of fish/ initial number of fish)
a, b, c, d. Different superscripts within each row indicate significant differences among groups(P< 0.05). Data are means ± SEM

Blood chemistry parameters

As the findings showed, the replacement level of fishmeal with PBM significantly (P<0.05) influenced plasma parameter contents (Figure 1). The plasma urea and albumin levels were significantly increased in the PBM100 group compared to the PBM0 group (P<0.05) and there was a significant difference between PBM50 and PBM100 groups (P<0.05). The plasma cholesterol and triglyceride levels were significantly decreased in the PBM100 group than in the PBM0 (P<0.05).

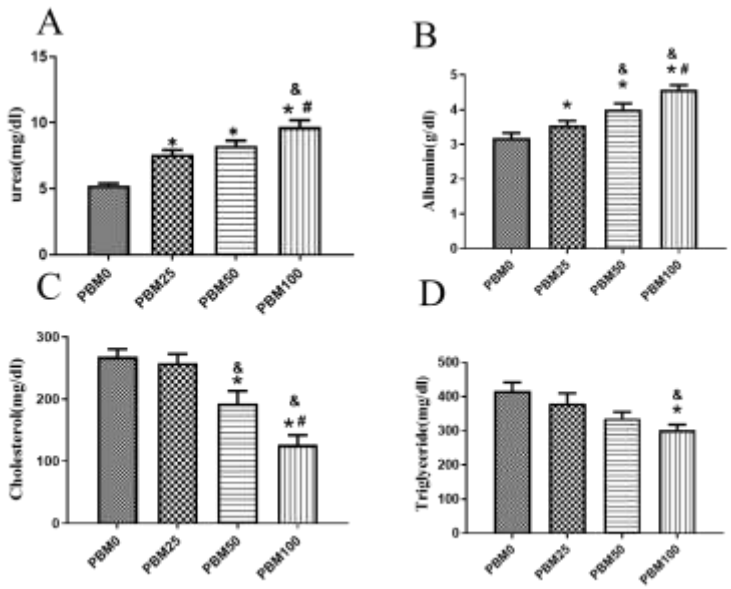

Figure 1: Blood chemistry parameters (Urea (A), Albumin (B), Cholesterol (A), Triglyceride (D)) in different groups determined up to 24h after the last meal (mean± SEM, n = 5 of each tank in each repetition).
*** Significant difference with PBM0 group (p< 0.05), #significant difference with PBM50 (p<0.05) and &significant difference with PBM25 group (p<0.05).**

The mean± SEM levels of liver enzymes in the fish groups are shown in Figure 2. Statistical analysis showed that increasing PBM levels in the diet of *O. niloticus* caused significant incremental changes in the level of liver enzymes in fish serum (P<0.05). There was also a significant difference between the PBM50 and PBM100 groups (P<0.05).

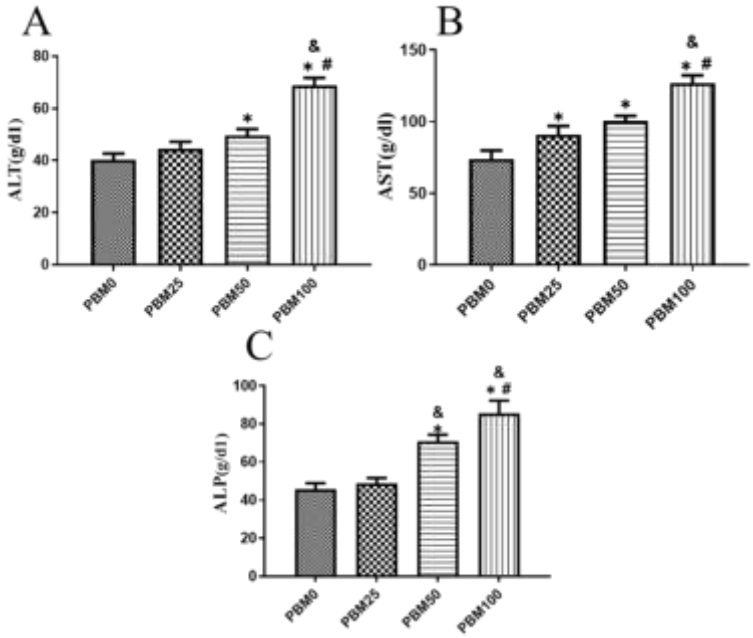

Figure 2: Liver enzymes (ALT (A), AST (B), ALP (C)) in different groups determined up to 24h after the last meal (mean± SEM, n = 5 of each tank in each repetition). * significant difference with PBM0 group (p<0.05), #significant difference with PBM50 (p<0.05) and &significant difference with PBM25 group (p<0.05).

Histological analysis

Macroscopically, *O. niloticus* liver was dark brown in the PBM0 group, while the liver with increasing PBM in the diet of PBM50 and PBM100 groups was turned brighter and whiter, which were, indicative of fatty liver. The liver histology of the fish fed with treatment diets is shown in Figure 3. In the control group (PBM0), normal hepatocytes are uniformly distributed and the integration of parenchymal tissues with a small number of lipid vacuoles is observed and the pancreatic acini are irregularly located in between them **(**3, A). Adding PBM rather than fishmeal to the fish diet in different proportions also caused fat to accumulate inside the hepatocytes, leading to fatty liver in the group receiving PBM 100% (3, D)**.** In effect, by increasing the amount of PBM in the fish diet, almost the entire cytoplasm of their liver cells became swollen with a lot of fat and changed to a vacuole-like appearance. This, in turn, compressed the sinusoids between them and reduced the interstitial tissue of the liver stroma. Likewise, in the PBM25 group, vacuolation is slightly detectable in hepatocyte cells (3, B). However, the hepatic vacuolization was found more in the group receiving PBM100 than PBM50.

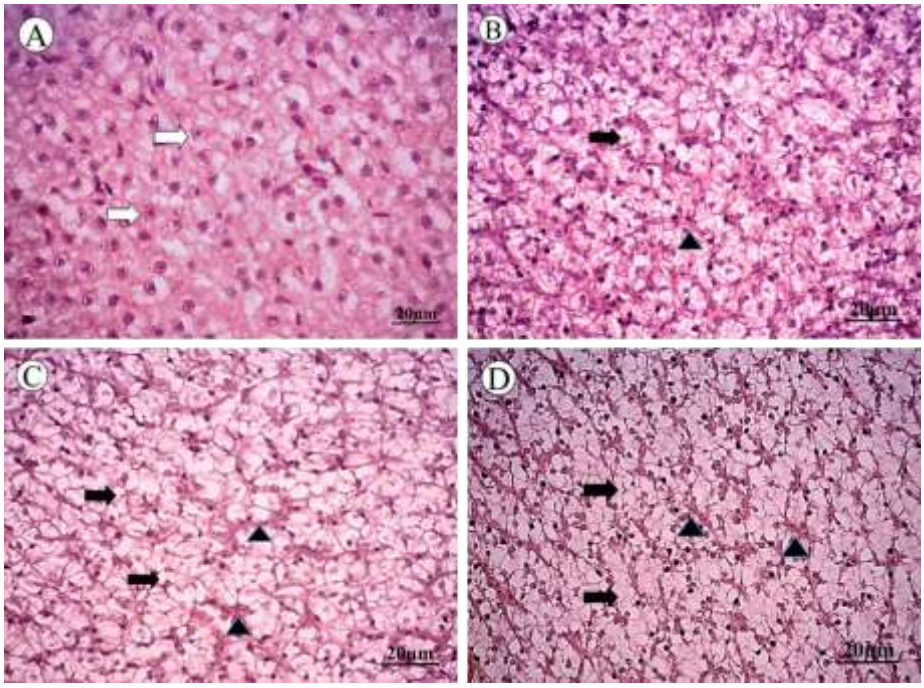

Figure 3: Sections of liver tissues in different groups (H&E, 400). (A) PBM0 group, normal liver morphology. (B) PBM25 group, hepatocytes, as well as sinusoids, are slightly degenerated. (C) PBM50 group, following the use of 50% replacing FM with PBM, liver tissue was a little more damaged. (D) PBM100 group, in response to high levels of replacing FM with PBM, severe damages to tissues including high degrees of vacuolation in the hepatocytes and compression of the sinusoids. White arrow indicates normal hepatocyte; a black arrow indicates swollen hepatocyte; arrowhead indicates compressed sinusoids

The swelling of hepatocytes and degree of vacuolation of liver tissue samples in different diets were initially measured by the magnification of 100 and then by the magnification of 400. The results are given in Table 4.

Table 4: The semiquantitative analysis of histological alteration assessment of hepatocyte size and degree of vacuolation in different groups (mean ± SEM, repetition of each tank in each n =5).

Liver changes	Dietary treatments			
	PBM0	PBM25	PBM50	PBM100
Hepatocyte size (µm)	8.83±0.42 [a]	9.64 ±0.52 [a]	11.65 ±0.19 [b]	14.76 ±1.29 [b]
Degree of vacuolation	0.40 ±0.24 [a]	1.0 ±0.20 [a]	2.20 ±0.20 [b]	2.80 ±0.20 [b]
a, b. Different superscripts within each row indicate significant differences amoung groups ($p<0.05$)				

The kidney of the *O. niloticus* is an elongated organ along the dorsal part of the body wall, below and along the spine, consisting of two parts, the anterior part of the kidney (head of the kidney), which includes mainly hematopoietic tissue, and the posterior part (posterior kidney), which often contains the tubes and glomeruli of the excretory system. The kidney histology of the fish in different groups is depicted in Figure 4. The gradual addition of different proportions of PBM rather than fishmeal to the fish diets causes the tubular cells to separate from the basement membrane, and increases the space between the basal part of these cells and the surrounding basement membrane, thus reducing the inner diameter of the tubule. Following that, the tubular cells are removed and fall into the space inside the tubule, causing epithelial cells to be seen inside the tubules. Following a gradual increase in the PBM ratio in the diets, the glomerulus inside of the Bowman's capsule condenses, and the diameter of the glomerule decreases, making the surrounding urinary space

inside the Bowman's capsule larger. Moreover, hemorrhage, congestion, and the accumulation of mononuclear cells were observed between the tubules (4, D).

Changes in kidney tissue in different diets were initially measured by the magnification of 100, and then by the magnification of 400, the results of which are shown in Table 5.

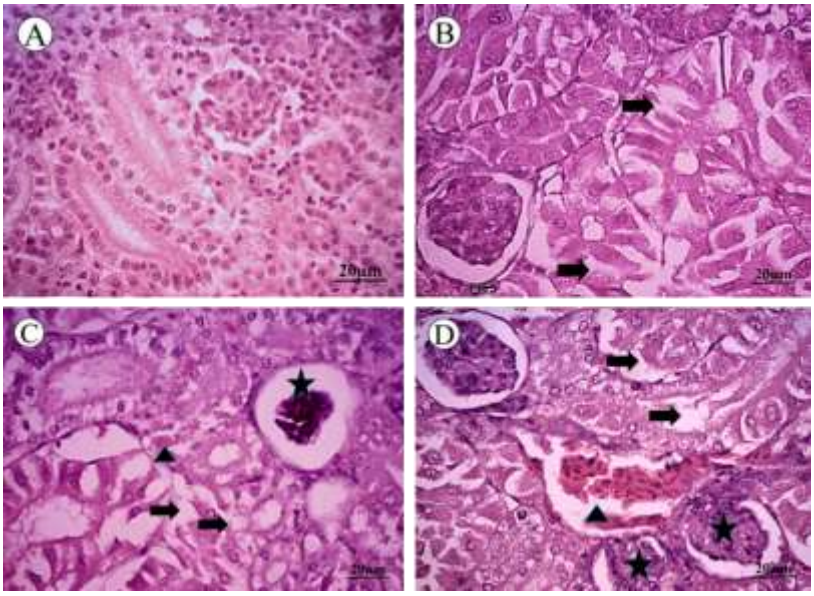

Figure 4: Sections of kidney tissues in different groups (H&E, 400). (A) PBM0 group, normal kidney morphology. (B) PBM25 group, kidney tissue slightly degenerates. (C) PBM50 group, following the use of 50% PBM, kidney tissue changes were partial. (D) PBM100 group, following replacement with %100, severe damages to tissues including glomerular compression, and degeneration of tubules. A star indicates glomerular compression; the black arrow indicates removal of tubule wall lining cells and the arrowhead indicates hemorrhage.

Table 5: Comparison of mean ± SEM of qualitative changes in kidney tissue in different groups (Repetition of each tank in each n =5)

Structures Kidney	Dietary treatments			
	PBM0	PBM25	PBM50	PBM100
Glomerular diameter (µm)	49.5 ±4.30 [a]	47.5 ±3.24 [a]	43.6 ±4.62 [ab]	34.7 ±2.70[b]
Bowman capsule diameter(µm)	58.7 ±6.15 [a]	56.9 ±5.20 [a]	57.8 ±4.20[a]	57.3±7.21 [a]
Internal diameter of the tubule(µm)	11.7 ±2.42 [a]	11.4 ±3.40 [a]	9.13 ±0.15 [b]	8.25 ±0.26 [b]

a, b. Different superscripts within each row indicate significant differences compared among groups **(**$p< 0.05$**)**

A comparison of qualitative changes in fish kidney tissues following the use of PBM in different proportions is presented in Table 6.

Table 6: Comparison of mean ± SEM of changes in kidney tissue in different groups (repetition of each tank in each. n =5)

Grading changes	Dietary treatments			
	PBM0	PBM25	PBM50	PBM100
Glomerular diameter (µm)	-	+	++	+++

Discussion

Aquatic animals are generally diverse in terms of food consumption, and *O. niloticus* as an omnivorous fish consumes a variety of food, making its proper nutrition one of the most important issues in aquaculture.

The results of the present study showed that by replacing PBM with fishmeal in *O. niloticus* diet between the groups of 100% PBM and the control group, the ratio of the final weight gain of the fish compared to their initial weight and specific growth rate index decreased, but the feed conversion ratio increased significantly. However, several studies have reported fish weight loss due to the replacement of PBM with fishmeal in the *O. niloticus* diet (Parés-Sierra et al, 2014; Irm et al, 2020; Qiu et al, 2023). In this research, after a 44-day feeding trial, the results showed that the growth performance of *O. niloticus* in the PBM25 group was similar to that in the PBM0 group.

Zhou et al, (2020) found that African catfish could tolerate up to 53.5% of PBM in their diet without significant changes in their performance and biometric parameters. In another study, Parés-Sierra et al, (2014) showed that PBM could be used up to 44% in diets for juvenile rainbow trout as a source of dietary energy. Nogales-Mérida et al, (2011) showed that substituting PBM up to 75% for fishmeal resulted in no change in biomarkers of European Cass.

Based on the findings of the present study, no significant variations in values of BWI%, FCR, PI as well as survival rate were observed in groups PBM0 and PBM25, indicating that up to 50% fishmeal could be replaced by PBM without negatively affecting the growth performance and feed utilization of *O. niloticus*. The findings of the present study are similar to those of Yones and Metwalli's in 2015 on juvenile Nile Tilapia (Yones and Metwalli, 2015). Many researchers attribute the fish weight loss and reduction in its growth index resulting from the replacement of PBM with fishmeal to a variety of reasons, including the presence of some compounds in PBM such as feathers as a horny tissue, connective tissue, as well as mixed skin and blood, or due to high temperature used during processing of PBM and its storage environmental conditions. In effect, these factors reduce digestibility and ultimately food consumption by fish (Parés-Sierra et al, 2014). In the present experiment, the lowest growth indices and the highest changes were observed in the treatment of PBM100% compared to the control group, indicating the inefficiency of PBM at higher levels in providing the energy required for daily fish consumption.

Plasma biochemical indicators are known as indicators of general fish health status (Liang et al, 2018). The results of the present study showed that serum albumin levels in the diet containing 50% and 100% PBM increased as compared to those in the control group. Albumin is a protein produced by the liver. Albumin helps to maintain the level of fluids in the blood so that they do not leak to other tissues, and the level of albumin is also a criterion for determining health and nutritional status. High serum albumin levels can be due to a diet containing too much protein or problems with the liver or kidneys (Lim et al, 2023). This finding is in line with the results of previous studies on *O. niloticus* (Metwalli, 2013), on Sturgeon and Shiny bass (Zhu et al, 2011), and on juvenile bighead carp (Liang et al, 2018). Plasma urea content in aquatic animals is the second most important nitrogen excretion product after ammonia whose changes are often used as an important parameter to evaluate the digestion of amino acids, proteins, and kidney function (Liang et al, 2018). In this study, the amount of plasma urea increased significantly in groups with 50 and 100% substitution of PBM compared to that in the control group. However, more studies are needed to confirm renal dysfunction, given that only a slight change in plasma urea

level is not sufficient to conclude the presence of renal lesions (Rahmati et al, 2022). Therefore, the histomorphology of the fish kidney was considered in the present study to provide more reliable results. The results of this study showed that the amounts of total cholesterol and triglyceride in the groups with 50 and 100% PBM substitution significantly reduced compared to those in the control group, which is consistent with the results on fish parrots (Lim et al, 2023), on rainbow trout (Panserat et al, 2009), and on gilthead seabream (Sabbagh et al, 2019). Sabbagh et al, (2019) and Mata-Sotres et al, (2018) concluded that Fish are universally considered to have a low level of cholesterol and the Σn-3/Σn-6 ratio decreases significantly with the increase of vegetable oil or animal substitutes in the diet. This may be related to the low content of Σn-3 fatty acids in PBM and this imbalance of Σn-3 fatty acids and Σn-6 in the liver can lead to fat deposits in the liver. The liver secretes specific enzymes for its function. The results of this study revealed that the amount of liver enzymes of AST, ALP, and ALT increased significantly with the replacement of 50% and 100% PBM with fishmeal compared to those in the group control, which confirms the findings of Lin and Luo (2011). An increase in liver enzymes in the serum can be a sign of inflammation or destruction of cells in the liver. Inflamed or damaged liver cells release higher amounts of certain chemicals, including liver enzymes, into the bloodstream, which can lead to elevated liver enzymes in blood tests. One of the reasons for the increase of these enzymes is the accumulation of fat in the liver and fatty liver disease. As here, following histological investigations, fat vacuoles were seen more in the cytoplasm of hepatocytes in the groups with higher substitution (Bruslé and Anadon, 2017). To further investigate the findings, the histomorphology of the fish liver was also considered in this study. The alkaline phosphatase enzyme which is found in all tissues of the fish body (Soltan et al, 2011) has an important role in the transmission of material between cell membranes It is also highly active in the kidneys and intestines of aquatic animals (Zahran et al, 2024). Several studies have suggested that an increase or decrease in fish liver enzymes is likely attributable to decreased production, increased excretion, or a change in their half-life due to dietary changes, suggesting that these enzymes act as a link between carbohydrates and protein metabolism; moreover and when liver cells are damaged for any reason, the levels of ALT and AST enzymes will change in the fish body (Soltan et al, 2011; Nochalabadi et al, 2023). For instance, by reducing the level of liver enzymes, transaminations are inactivated and amino acid catabolism is reduced(Soltan et al, 2011). Both of these aminotransferases (namely, ALT and AST) can, indeed, function as an interface between carbohydrates and protein metabolism. As such, in other organisms under the pressure of adverse conditions leading to internal lesions in the kidney and liver tissues, mitochondria of damaged cells release their aminotransferase contents into the bloodstream, causing an increase in the levels of these enzymes in blood serum (Rahmati et al, 2022).

The liver is one of the most important metabolic organs which regulates nitrogen and other metabolites in the body (Peyghan et al, 2023). The findings of the present study showed that increasing PBM proportions in the fish diet led to the accumulation of dietary fat inside fish hepatocytes, and also increased vacuolation within hepatocytes which ultimately resulted in fatty liver in the fish. Histological alterations in the liver, which are likely to affect fish health, were thus found in the treatment groups rather than the control groups. Zhou et al, (2020) reported that the use of a diet with over 53.5% PBM meal protein and soybean meal protein increased the vacuolation of hepatocytes

and the fat storage profile in fish muscle tissue, leading to the fatty liver as compared with the control group. As such, large vacuoles existed more in group PBM50 and PBM100 than PBM0 and PBM25 due to the direct relationship between unabsorbed and non-metabolizable fat at high levels of PBM use. The level and amount of alternative protein sources for different fish species have been already considered in several studies (Campos et al, 2019; Monteiro et al, 2018). There are different results here, In the study of Aydín et al, (2015), PBM inclusion in the diet of Nile tilapia (*Oreochromis niloticus*) up to 100% did not reveal any effect on the histological examination of the liver tissue and livers. Contrarily, Hu et al, (2013) demonstrated a negative effect of FM replacement by animal protein blend (a mixture of 40% PBM, 35% meat, and bone meal, 20% spray-dried blood meal, and 5% hydrolyzed feather meal) in Japanese seabass (*Lateolabrax japonicus*) livers that were characterized by enlarged hepatocytes and apparent hepatic steatosis with intense vacuoles in the hepatocytes resemble lipids. As in our results, the hepatocytes were abnormally shaped. The kidney in fish is mesonephric in shape and its posterior or excretory part consists of glomeruli of variable size and a long folded tubule leading from a glomerulus to an excretory duct (Gholami et al, 2018; Koohkan et al, 2024). The results of this study showed that adding different proportions of PBM rather than fishmeal to the fish diet increased the plasma urea level, thus affecting the tissue of urinary tubules and glomeruli. This way, changes in fish kidney tissue are detectable in the PBM50 and PBM100 groups compared to the PBM0 and PBM25 groups and are in line with results on other similar species (Yones and Metwalli, 2015).

According to the present study, the substitution of PBM instead of fishmeal up to 50% could not change the biological indicators of *O. niloticus*. Its liver enzymes increased significantly compared to the control group, and in the histological studies on the liver, the liver tissue had lost its normal structure and function with the increase in the amount of alternative diets. The amount of urea plasma also showed a significant difference between the different groups with the increase in the amount of alternative diets compared to the control group, and these changes are also evident in the histological examinations of the kidneys. Therefore, based on the obtained results, the use of PBM in the diet of *O. niloticus* can be replaced up to a maximum of 50% without any change in the growth and health of these fish. But again, to investigate the replacement rate more accurately, up to 50% replacement, alternative diets with shorter intervals and more tests should be tried.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Original Article

Molecular identification of *Fusobacterium spp.* in dogs with or without gingivitis/ periodontitis in Ahvaz and Tehran districts

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Abstract

Periodontal disease is one of the most common disorders seen in small animal practices. Oral bacteria play an important role in periodontitis. *Fusobacterium spp.* is one of the important bacterial agents in the progression of periodontitis. The aim of the present study was to investigate the association between the presence of *fad*A and *leukotoxin* genes in *Fusobacterium spp.* isolated in dogs with or without gingivitis/ periodontitis in districts of Tehran and Ahvaz. One hundred and fifty samples (75 dogs from Tehran and 75 from Ahvaz district), between 2 to 11 years old, 78 males and 72 females, were studied during ten months. The studied major breeds included White Terrier, Poodle, Pomeranian, Shih Tzu, Yorkshire terrier, Pug, Spitz, Maltese and the rest were other breeds. They were fed with dry, homemade or mixed food. Twenty samples had healthy gums (13.33 %), 32 cases periodontitis grade 1 (21.33%), 47 other cases periodontitis grade 2 (31.33%) and 51 samples periodontitis grade 3 (34%). Twenty-seven out of 150 samples were infected with *Fusobacterium* (18.0%; 95% CI: 11.8%-24.1%). The percentages of the relative frequency of these bacteria were 21.3% (95% CI: 12.0%-30.6%) and 14.6% (95% CI: 6.6%-22.6%) in Tehran and Ahvaz, respectively. Survey of *leukotoxin* gene in 18 samples of *Fusobacterium necrophorum* showed that 11 samples (61.11%) (9 cases from Tehran and 2 other cases from Ahvaz) had this gene; the observed difference in the presence of this gene, was not statistically significant (p-value=0.43; df=1; X^2=0.62). Nine out of 26 samples (34.61%) had *fad*A virulence gene and the relationship between the presence of *fad*A gene and periodontitis grades was not statistically significant (p-value=0.41; df=1; X^2=0.68). Multivariable logistic regression showed that age, gender, breed, periodontitis, district, and type of food explained 97.6% of the infection and only gender and periodontitis had a significant effect on infection. The presence of *fadA* gene in *Fusobacterium nucleatum* isolated from dental plaques of dogs suffering from periodontitis and *leukotoxin* gene in *Fusobacterium necrophorum* subspecies *necrophorum* were not significant in periodontitis in related to two different districts of Tehran and Ahvaz. In conclusion, the prevalence of *Fusobacterium* was 16% and 2% in periodontitis grade 3 and healthy gums, respectively.

Key words: Periodontal diseases, *Fusobacterium*, *Leukotoxin*, *Fad*A, Gingivitis, Dog

Introduction

Periodontal disease (PD) is one of the most common diseases seen in small animal practices. It is reported that 80% or more of dogs over five years of age have PD. This disease occurs as a result of infection and chronic inflammation of the gums, bone,

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dental cement, and surrounding tissues that support the tooth (Jamshidi et al, ۲۰۰۵; Riggio et al, 2011; Robinson et al, 2016; Stella et al, 2018; Wallis et al, 2020). Periodontitis is a collective term used to describe two conditions: gingivitis and periodontitis. Gingivitis is where the gingiva becomes erythematous and inflamed as a result of dental plaque bacterial activity and is reversible. Periodontitis is where the supporting structures of the tooth are destroyed as a result of the host's inflammatory response to the dental plaque and can be resulted in odontogenic infection and, ultimately, tooth loss. Periodontitis is irreversible without surgical intervention, but is often controllable. The occurrence of PD depends on many factors, such as the breed and size of the dog, increasing of age, weight loss, changing behavior, hygiene and dental care (Niemiec, 2013; Lobprise et al, 2019; Wallis et al, 2020).

The pathogenesis of periodontitis, from early stages of gingivitis to advanced periodontitis, is that plaque formed first on the outer surface of the tooth. Salivary glycoproteins that have adhesive properties stick to the tooth surface, then the bacteria in the oral cavity are added to this composition and finally, plaque is formed. This plaque can be mineralized and become calculus (tartar). Undoubtedly, bacteria play a major role in the oral cavity in the process of periodontitis. The diagnosis of PD is based on taking history, clinical examination, and radiological evaluation (Niemiec, 2008; Gorrel, 2013). According to the American College of Veterinary Dentistry classification, periodontitis can be classified or staged into four stages based on clinical signs and severity of lesions. Non-surgical periodontal therapy is always the first line of treatment and involves scaling plaque and calculus removal from the tooth crown, gingival sulcus, and root surfaces is essential for the prevention and control of PD (Niemiec, 2008; Lobprise, 2019).

Fusobacteria is one of the most common bacteria found in the gums of humans and dogs (Conrads et al, 2004). This bacterium belongs to the *Bacteriodaceae* family and is a dominant microorganism in the periodontal tissue. *Fusobacteria* are obligate anaerobic bacteria, gram-negative, non-spore-forming, non-motile, pleomorphic rod-shaped bacilli (Signat et al, 2011). *F. necrophorum* is classified into four biotypes A, B, AB, and C according to biochemical characteristics. Based on the 16S ribosomal RNA sequence, it is closely related to both A and B biotypes (biotype A of *Fusobacterium necrophorum* subspecies *necrophorum*, and biotype B of *F. necrophorum* subspecies *funduliforme*) (Nagaraja et al, 2005; Wright, 2016). The main virulence factor in *F. necrophorum* is *leukotoxin*, and variation in this gene enables *Fusobacterium* cause different diseases in multiple hosts (Bennett et al, 2011). The *F. necrophorum* subspecies *necrophorum* has been isolated from infections more than the *F. necrophorum* subspecies *funduliforme* while the *F. necrophorum* subspecies *funduliforme* is usually isolated from infections as a secondary agent and in a non-specific manner (Nagaraja et al, 2005). These bacteria are a part of the normal flora of the oral cavity, but *F. nucleatum* is also considered as a pathogenic organism in periodontitis. These bacteria can accumulate with other pathogens and be on the enamel surface as a bridge to connect new bacteria to the older group of plaque pathogens. They are the main focal point in the physical interactions between gram-positive and gram-negative species (Machuca et al, 2010; Signat et al, 2011). Adhesion gene *fad*A has been identified, which makes *F. nucleatum* capable of high adhesion to host cells (Liu et al, 2014).

The prevalence of *Fusobacterium* was reported in healthy gingiva 7%, gingivitis 25% and periodontitis 10-40% (Hennet et al, 1991). It was announced that the frequency rates of gingivitis and

periodontitis were 24% and 12%, respectively in the referred dogs to the small animal hospital of Tehran University (Jamshidi et al, 2005). Also, it was sated that *F. canifelinum* was the most predominant flora in sub-gingival plaques of dogs (Dahlen et al., 2012). The prevalence of *F. nucleatum* was estimated up to 52% with periodontitis and 24% without periodontitis (Senhorinho et al, 2012). According to the previous studies and the investigation of virulence genes of different subspecies in different districts, determining the existing genetic factors is crucial at the molecular level. Considering the prominent role of *Fusobacterium* in the creating of periodontitis, the present study was conducted for the first time in Ahvaz district and also a comparative evaluation between Ahvaz and Tehran districts.

Materials and methods

Sample collection

In the present survey, after the determining of the periodontitis/gingivitis levels, dental plaque samples were taken from 75 small breed dogs referred to the Veterinary Faculty and clinics in Ahvaz district (Iranian Vet Clinic and Royal Vet Clinic), as well as 75 small breed dogs from the Veterinary clinics in Tehran (Oxygen Pet Hospital and Nella Pet Clinic) during ten months from January to November 2022. Characteristics of dogs such as age, gender, breed, and type of feed (dry, homemade, or mixed), dental hygiene and administration of immunosuppress drugs or antibiotics were recorded. All the studied dogs were in the age range of 2 to 11 years. Seventy-two out of 150 dogs were female and 78 male. Among the studied dogs in each district, at least 30 cases had periodontitis or gingivitis. Dogs that did not allow oral examination were sampled under sedation drugs with ketamine (10 mg/kg IM) and acepromazine (0.05mg/kg IM). Silness-Loe index was used to detect grading, diagnosis and severity of periodontitis. The normal depth of the gum pocket is 1 to 3 mm in dogs using the probe, and depths greater than these amounts were considered as periodontitis. The probe was entering the gum pocket vertically, and different parts of the tooth were examined around the tooth. The measurement had been done from the free edge of the gum to the end of the gum pocket based on references (Gorrel, 2013). In the following, sampling was done by scrubbing plaque with scaler, and calculus forceps. Calculus and plaque were collected from the surface of the tooth in cases of periodontitis and in gingivitis and healthy gums, from the border between the gum and the tooth by sterile cotton swab and placed in a sterile 1.5 ml and finally stored at -20 °C until the DNA extraction.

DNA extraction

Following the protocol indicated by the manufacturer (DENAzist-Iran), 10 mg of plaque tissue was combined with 450 µl AT1 solution in a 2 ml microtube. The sample was mixed and adjusted to the desired volume before adding the correct solution. Glass beads were added to plaque solutions and vortexed until tissue dissolved. 5 µL proteinase K added and incubated at 60°C for 20 minutes. 450 µl of AT2 buffer was mixed with the solution and incubated for 5 minutes at 60°C. The solution was then centrifuged and the supernatant was moved to a new tube. 10 µL RNAse A was added and incubated for 30 minutes at 37°C. 700 µl of lysate was transferred to a column, centrifuged, and discarded. The remaining solution was transferred into the column and the process was repeated. Finally, 700 µL AT3 solution was centrifuged at 10,000 rpm for 1 minute. 100 µl of AT4 solution was added to the column and incubated for 5 minutes. The column and 1.5 ml tube were centrifuged at 10,000 rpm for 1 minute. The collected liquid was returned to the column, centrifuged at 13000 rpm for 3 minutes, and the DNA was stored at -20 °C.

Detection and confirmation of 16S rRNA-based PCR for *Fusobacterium spp*. in the obtained samples and virulence genes

After DNA extraction, the presence of *Fusobacterium spp.* was investigated first in all 150 samples by PCR. The primers were designed to detect the *Fusobacterium* genus, including the $Fuso_1$ (F) and $Fuso_2$ (R) primers. DNA extracts from samples using *F. nucleatum* primers of $F.N_1$ (F) and $F.N_2$ (R) to amplify a 360-bp region of the 16S rRNA gene (Figures 1-5). They were also used to identify the *fad*A virulence gene and differentiate *F. necrophorum* subspecies. Specific primers were utilized for the *lekotoxin* virulence gene. Detailed gene sequences, primers, and fragment lengths are provided in Tables 1 and 2.

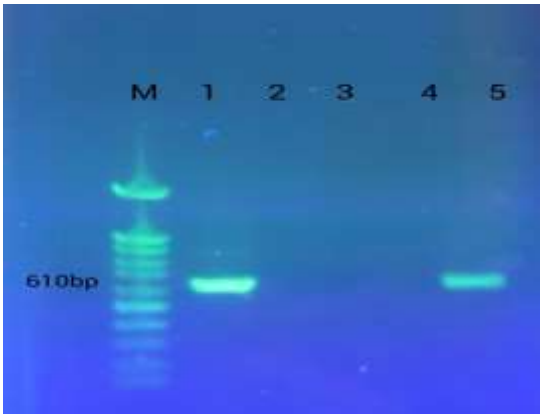

Figure 1: PCR detection of *Fusobacterium* genus in 610bp. M: Molecular ladder, No. 1: Positive control, No. 5: Positive sample, No. 2, 3, 4: Negative samples.

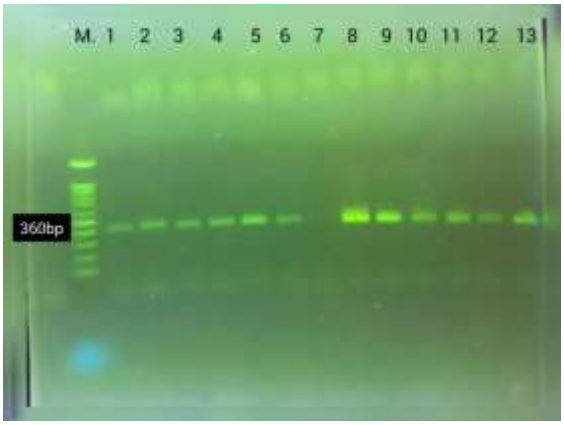

Figure 2: PCR detection of *F. nucleatum* by primers of 16S rRNA-F and 16S rRNA-R to amplify a 360-bp region of the 16S rRNA gene. M: Molecular ladder, No. 1-6 and 9-13: Positive samples, No. 8: Positive control, No. 7: Negative control.

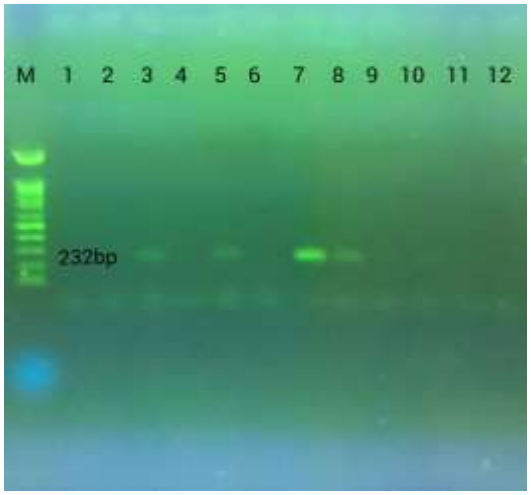

Figure 3: PCR detection of the virulence gene of *F. nucleatum* using the *fad*A primers of *fad*A-F and *fad*A-R to amplify a 232-bp region of the *Fad*A gene from positive samples of *F.nucleatum* with a band of 232 bp. M: Molecular ladder, No. 3, 5, 8, 10 positive samples, No. 7: Positive control, No. 12: Negative control, No. 1, 2, 4, 6, 9, 11: Negative samples.

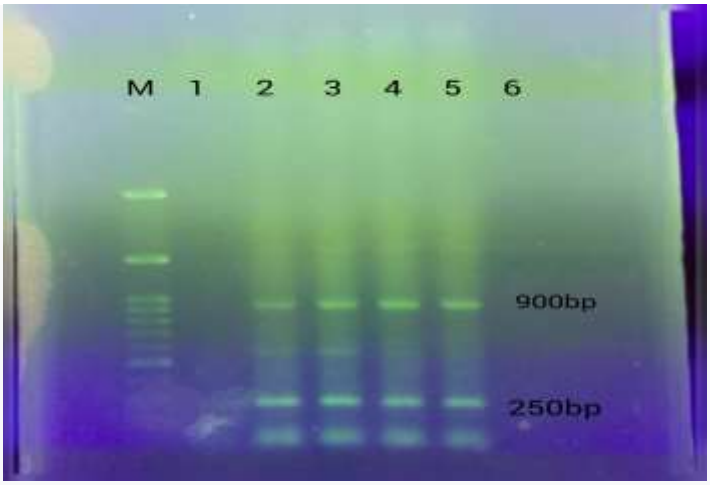

Figure 4: PCR detection of *F. necrophorum necrophorum* and *F. fonduliform necrophorum* species in bands of 250 and 900. M: Molecular ladder, No. 4, 5: Positive *F.* n*ecrophorum necrophorum*, No. 3: Positive *F.* n*ecrophorum fonduliform*. No. 2: Positive control, No. 6: Negative control, No. 1: Negative sample.

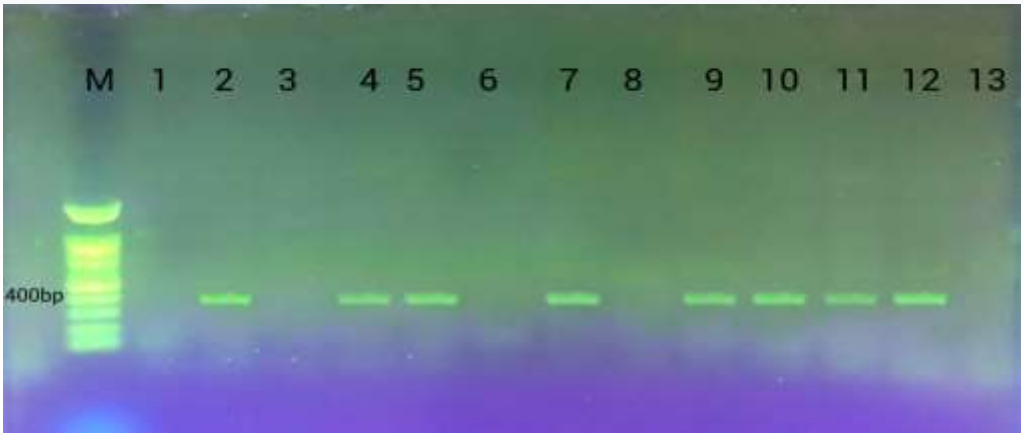

Figure 5: One-step duplex PCR which used to enable the detection and differentiation of *F.necrophorum* subspecies in a single reaction. Primer combinations of TP1–TP2 and WLF2–WLR1 were used to determine of *leukotoxin* virulence gene with 400 bp band. M: 400 bp indicator, No. 2, 4, 5, 7, 9, 10, 11 *F. necrophorum* subspecies that have *leukotoxin* virulence gene Positive *F. nucleatum* DNA, No. 1, 3, 6, 8: Negative samples No. 12: Positive control, No. 13: Negative control.

Table 1: Primer sets and PCR target regions, used in the studied dogs in Ahvaz and Tehran districts

Primers	Primer sequence (5-3)	Bacterial detection/ differentiation	Target (gene fragment)	Size of amplicon	Reference
Fuso$_1$	5'-GAG AGA GCT TTG CGT CC-3'	*Fusobacterium* genus	16S rDNA (position 212e821 in *F. nucleatum*	610	(Nagano et al., 2007)
Fuso$_2$	5'-TGG GCG CTG AGG TTCGAC -3'	*Fusobacterium* genus	16S rDNA (position 212e821 in *F. nucleatum*	610	(Nagano et al., 2007)
F.N$_1$(F)	5`-AGA GTT TGATCC TGG CTC AG -3`	*F. nucleatum*	*F. nucleatum*	360	(Sallum et al., 2004)
F.N$_2$(R)	5`-GTC ATC GTG CAC ACA GAA TTG CTG-3`	*F. nucleatum*	*F. nucleatum*	360	(Sallum et al., 2004)
*fad*A-F	5′-CAC AAG CTG ACG CTG CTA GA -3	*F. nucleatum*	*fadA*	232	(Sallum et al., 2004)
*fad*A-R	5′-TTA CCA GCT CTT AAA GCT TG -3′	*F. nucleatum*	*fadA*	232	(Sallum et al., 2004)
*lkt*A1	5`-AATCGGAGTAGTAGGTTCTG-3`	*Leukotoxin* gene from *F. necrophorum*	*Leukotoxin*	401	(Zhou et al., 2009)
*lkt*A2	5`-CTTTGGTAACTGCCACTGC-30	*Leukotoxin* gene from *F. necrophorum*	*Leukotoxin*	401	(Zhou et al., 2009)

Table 2: Specific primer, used in the present study to detection of *F. necrophorum* subspecies

Primer	DNA sequence (5`–3`)	position	Size of amplicon	Target (gene fragment)	Reference
TP_1 (F)	TCTACGTATGCCTCACGGAT	173–192	900	*rpoB*	(Narongwanichgarn et al., 2003)
TP_2 (R)	AGGAATATGAGGATGAGGAT	1075–1094	900	*rpoB*	(Narongwanichgarn et al., 2003)
WLF_2 (F)	AGGTGCTTCTTCCACAGC	94–111	250	hemagglutinin-related protein gene of *F. n. necrophorum*	(Narongwanichgarn et al., 2003)
WLR_1 (R)	GCACCATTTTGAGCGCGT	323–340	250	hemagglutinin-related protein gene of *F. n. necrophorum*	(Narongwanichgarn et al., 2003)

PCR primers and amplification

16s rRNA-based PCR for the detection of *Fusobacterium* genus

The used primers for 16S rRNA-based PCR were based on the primer designed by Nagano et al, 2007 (Table 1). The primer pair $FUSO_1$ (Forward primer) and $FUSO_2$ (reverse primer) were used to target conserved regions of the 16S rDNA gene for *Fusobacterium* species. PCR reaction system contained 12.5 µl PCR Master Mix, 1 µl (10 pmol/µ) Forward and 1 µl (10 pmol/µ). Reverse primers were included, 1 µl $MgCl_2$, 4 µl DNA template, and 5.5 µl H_2O. Following a 'hot start', the reaction mixtures were subjected to the empirically optimized thermal cycling parameters 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, followed by a final extension at 72°C during 7 minutes.

16S rRNA-based PCR for the detection of *F. nucleatum*

The 16S rRNA-based PCR was used to determine the prevalence of *F. nucleatum*. The PCR was performed on DNA extracts from sub-gingival biofilm samples by using *F. nucleatum* primers of 16S rRNA-F and 16S rRNA-R to amplify a 360-bp region of the 16S rRNA gene (Sallum et al, 2004). For the detection of *F. nucleatum* amplification reaction, it was run in a 20 µl reaction mixture containing 10 µl master, 1 µl $MgCl_2$, 1 µl of each primer (Forward and Reverse primers), 3 µl of Extracted DNA from sub-gingival biofilm samples, and 4 µl H_2O. The 16S rRNA PCR of *F. nucleatum* was carried out for 5 minutes at 94˚C and 30 cycles, with each cycle consisting of denaturation at 94˚C for 30 seconds, annealing at 58˚C for 30 seconds, extension at 72˚C for 1 minute, and final extension for 10 minutes. The amplified products were then electrophoresed on 1.5% agarose gel in Tris-acetate buffer (40 mM Tris-acetate, 1 mm EDTA, pH 8.0).

The 16S rRNA-based PCR and *fad*A specific PCR

The *fad*A primers of *fad*A-F and *fad*A-R were used to amplify a 232-bp region of the *fad*A gene from positive samples of *F. nucleatum* (Liu et al., 2014). Amplification reaction was performed for the detection of *fad*A in a reaction mixture of 25 µl containing 12.5 µl master, 1 µl of each primer 1 µl (10 pmol) Forward and 1 µl (10 pmol) Reverse primers, 3 µl of DNA extracted, 1 µl $MgCl_2$, and 6.5 µl H_2O. The PCR of *fad*A was carried out for 4 minutes at 94˚C and 30 cycles, with each cycle consisting of denaturation at 94˚C for 30 seconds, annealing at 55.8˚C for 30 seconds, extension at 72˚C for 40 seconds, and final extension for 6 minutes.

Determination of *F. necrophorum* species and *necrophorum* and *fundoliform* subspecies

PCR amplification of *F. necrophorum* strains was carried out with 10–23 mer-length random primers. A primer used in the present study was based on Narongwanichgarn et al. (2003). TP_1–TP_2 and WLF_2–WLR_1 were designed based on the nucleotide sequences of each unique band generated with respective random primers D11344 and W1L2. Specific PCR

was performed.to confirm the specificity of the primers to *F. necrophorum*. The PCR was subjected to 30 cycles, with each cycle consisting of 94˚C for 1 minute, 55˚C for 1 minute, and 72˚C for 1 minute. After the final cycle, samples were then heated at 72˚C for 10 minutes for the final extension reaction.

Duplex PCR

One-step duplex PCR was used to enable the detection and differentiation of *F. necrophorum* subspecies in a single reaction. Primer combinations of TP_1–TP_2 and WLF_2–WLR_1 were used in the present survey. PCR was performed in a reaction mixture of 25 µl containing 3 µl of template DNA from *F. necrophorum* tested strains, 4.5 µl H_2O, 1 µl MgL_2, 1 µl primer (WLF_2), 1µl primer (WLR_1), 1µl primer (TP_1), and 1µl primer (TP_2).

Analysis of virulence gene *lkt*A

The primers of *lkt*A1 and *Ikt*A2 were designed based on the study of Zhou et al, (2009). Amplification was performed in a 20 µl reaction containing 3 µl of extracted DNA, 1 µl of each primer 1 µl (10 pmol) Forward and 1 µl (10 pmol) Reverse primers, 1 µl $Mgcl_2$, and 4 µl H_2O. Amplification was carried out in a Master cycler EP thermocycler, and the thermal profile was consisted of denaturation at 94˚C for 2 minutes, followed by 35 cycles of 94˚C for 30 seconds, 60 ˚C for 30 seconds and 72˚C for 40 seconds, with a final extension step at 72˚C for 5 minutes (Tables 1, 2).

Statistical analysis

Obtained data were analyzed by SPSS software version 21 (IBM corporation, USA). Differences were assessed using Independent-T test. A *p* value of less than 0.5 was considered significant. Chi-square test and Univariate logistic regression methods were used for data analysis.

Results

Twenty out of 150 samples (13.33%) had healthy gums, 32 cases (21.33%) periodontitis grade 1, 47 samples (31.33%) periodontitis grade 2, and 51 other cases (34%) periodontitis grade 3. From 150 dental plaque samples that were collected from small breed dogs in Ahvaz and Tehran (75 samples from each district), 27 cases (18%; 95% CI: 11.85-24.15%) (17 cases from Tehran and 10 cases from Ahvaz) were positive in terms of the presence of *Fusobacterium* genus. The percentages of the relative frequency of these bacteria in Tehran and Ahvaz was 21.33 (95% CI: 12.0%-30.6%) and 14.67 (95% CI: 6.6%-22.6%), respectively. The Chi-square test showed that this difference was not statistically significant (P=0.4; df=1; X^2=0.72). Out of 27 samples tested positive for *Fusobacterium* genus, *F. nucleatum* was present in 26 samples (96.3%). Analysis of the *fad*A virulence gene in *F. nucleatum* revealed that out of the 26 samples, 9 tested positive for the *fad*A gene (34%). However, the presence of the *fad*A gene was not statistically significant in relation to periodontitis. (P=0.41; df=1; X^2=0.68). Also, the analysis of these 27 samples in terms of *F. necrophorum* showed that *F. necrophorum* subspecies were present in 17 samples (62.96 %) (14 samples from Tehran and 3 samples from Ahvaz, respectively) and one sample (3%) infected with the subspecies of *F. necrophorum funduliforme* (from Ahvaz). The detection of the *leukotoxin* gene in 18 samples of *F. necrophorum* showed that 11 samples (61.11%) (9 samples from Tehran and 2 samples from Ahvaz) had this gene, and the observed difference, in the presence of this gene, was not statistically significant (P=0.43; df=1; X^2=0.62).

Table 3: Distribution of *Fusobacterium* and its subspecies and virulence genes in relation to different degrees of periodontitis in dogs of Ahvaz and Tehran districts

Bacteria and virulence genes	Healthy gingiva	PD G1	PD G2	PD G3
Fusobacterium genus	3	0	0	24
F. nucleatum	3	0	0	23
F. n. subspecies *necrophorum*	1	0	0	16
F. n. subspecies *fonduliforme*	0	0	0	1
*fad*A	2	0	0	7
leukotoxin	0	0	0	11

Table 4 presents the frequency of *Fusobacterium* infection in both male and female dogs. The survey of *Fusobacterium*-positive samples in Tehran showed that 14 samples were related to male dogs (82.35%) and 3 samples (17.65%) in females and in the Ahvaz, all of the positive samples were in males. The obtained results showed that the relative frequency of positive cases was higher in males than females and this difference was significant ($P<0.001$; df=1; $X^2=16.19$). Univariate logistic regression showed that the chance of infection in male dogs was 10.22 times higher than females (95% confidence interval, 2.92-35.75) ($P<0.001$) and the gender explained 20.6% of the changes for infection by this bacterium (Table 4).

Table 4: Distribution of absolute and relative frequency of *Fusobacterium* infection in dogs of Tehran and Ahvaz districts based on gender

Gender	Total		Positive		Negative	
	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute
F[b]	48	72	4.2	3	95.8	69
M[a]	52	78	30.8	24	69.2	54
Tot	100	150	18	27	82	123

Different lowercase letters indicate significant differences between different gender groups

Table 5 presents the frequency of *Fusobacterium* infection in two different age groups. All of the positive samples in Tehran were above 2 years old and in Ahvaz above 3 years old. The chi-square test showed that there was no significant relationship, between age and infection ($P=0.56$; df=1; $X^2=0.33$). Single-variable logistic regression showed that the chance of infection in dogs 5 years old and older was 1.4 (95% confidence interval, 0.61 - 3.24) and in dogs less than 5 years was 0.7% of changes ($P=0.43$).

Table 5: Distribution of absolute and relative frequency of *Fusobacterium* infection in dogs of Tehran and Ahvaz districts, according to age

Age (Year)	Total		Positive		Negative	
	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute
5[a]>	58.7	88	15.9	14	84.1	74
≥5[a]	41.3	62	21.0	13	79.0	49
Total	100	150	18	27	82	123

Same lowercase letters indicate no significant different between age groups

A survey of the distribution of positive samples for breed in Tehran district showed that three samples (17.65%) were miniature poodles (all females), 7 cases (41.18%) Shih Tzu, 5 samples (29.41%) Yorkshire Terriers and 2 cases (11/76) Pomeranian and in Ahvaz district, three cases (30%) were miniature poodles, 3 items (30%) Yorkshire terriers, 2 samples (20%) Spitz and 2 other samples (20%) white terrier. Table 6 shows the frequency of *Fusobacterium* infection based on the breed. Relative prevalence varied from zero to 57.1%, which was statistically significant (P=0.002). Univariate logistic regression showed that breed explained 36.8% of changes for infection with this bacterium.

Table 6: Distribution of absolute and relative frequency of *Fusobacterium* infection in referred dogs of Tehran and Ahvaz districts, based on breed

Breed	Total		Positive		Negative	
	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute
Poodle	15.3	23	26.1	6	73.9	17
Dachshund	1.3	2	0.0	0	100	2
Shih Tzu	10.0	15	46.7	7	53.3	8
Pomeranian	14.7	22	9.1	2	90.9	20
Yorkshire	9.3	14	57.1	8	42.9	6
Maltese	5.3	8	0.0	0	100	8
Beagle	4.0	6	0.0	0	100	6
Chihuahua	2.0	3	0.0	0	100	3
Cavalier	3.3	5	0.0	0	100	5
Pug	6.0	9	0.0	0	100	9
White Terrier	19.3	29	6.9	2	93.1	27
Jack Russell	1.3	2	0.0	0	100	2
Spitz	5.3	8	25.0	2	75	6
Lhasa Apso	2.0	3	0.0	0	100	3
Basenji	0.7	1	0.0	0	100	1
total	100	150	18	27	82	123

Table 7 shows the frequency of *Fusobacterium* infection based on the periodontitis. All positive samples in Tehran had periodontitis grade 2 and in Ahvaz district, Poodles had gingivitis and white terriers, Yorkshire terriers, and Spitz had periodontitis grade 3. The general assessment of this table showed that the relative frequency of positive cases in dogs with periodontitis grade 3 was higher than grades 1 and 2 and this difference was statistically significant (P<0.001; df=3; X^2 =64/46). The frequency of *Fusobacterium* in dogs with periodontitis grade 3 was significantly higher than periodontitis grades 1 and 2 (P<0.001) or without periodontitis (P=0.02). Univariate logistic regression showed that the type of consumed food explained 49.5% of the changes related to infection with this bacterium. In the studied dogs of Tehran, 11 cases had healthy gums (14.66%), 20 cases gingivitis (26.66%) and 44 cases some degree of periodontitis (58.66%). In dogs of Ahvaz district, 9 cases had healthy gums (12.0%), 12 cases gingivitis (16.0%) and 54 cases some degree of periodontitis (72.0%).

Table 7: Distribution of absolute and relative frequency of *Fusobacterium* infection in referred dogs of Tehran and Ahvaz districts, according to periodontitis

Periodontitis	Total		Positive		Negative	
	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute
Healthy gingiva	13.3	20	15.0	3	85.0	17
grade I	21.3	32	0.0	0	100	32
grade II	31.3	47	0.0	0	100	47
grade III	34.0	51	47.1	24	52.9	27
total	100	150	18	27	82	123

During a study on dog nutrition in Tehran, it was observed that all the dogs included in the research were nourished with either commercially available dry food or homemade meals. These observations revealed indications of poor oral and dental hygiene among the subjects. In Ahvaz district, positive samples were found in dogs that were fed homemade food, and their oral hygiene was very poor. Table 8 shows the frequency of *Fusobacterium* infection based on food consumption. The table revealed that the relative abundance of positive cases in dogs fed with homemade food was significantly higher than in those fed with dry food (P<0.001). The frequency of *Fusobacterium* in dogs fed with homemade food and the combination of dry and homemade was significantly higher than dry (P<0.001). Univariate logistic regression showed that the type of consumed food explained 27.6% of the changes for infection with this bacterium.

Table 8: Distribution of absolute and relative frequency of *Fusobacterium* infection in referred dogs of Tehran and Ahvaz districts, according to the type of food

Food	Total		Positive		Negative	
	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute
dry[b]	34	51	0.0	0	100	61
homemade[a]	32.7	49	20.4	10	79.6	39
both	33.3	50	34.0	17	66.0	33
total	100	150	18	27	82	123

Different lowercase letters indicate the significance between different groups based on nutrition

Multivariable logistic regression showed that age, gender, breed, periodontitis, district, and type of food explained 97.6% of the changes for infection and only gender and periodontitis had a significant effect on infection (P<0.001). Based on the history and blood sample, none of the cases had a history of underlying disease or the administration of corticosteroid drugs.

Discussion

The obtained results of the present study showed that twenty-seven out of 150 samples were infected with *Fusobacterium*. The percentages of the relative frequency of these bacteria were

21.3% and 14.6% in Tehran and Ahvaz, respectively. Survey of *leukotoxin* gene in 18 samples of *F. necrophrum* showed that 11 samples had this gene. The presence of *fadA* gene in *F. nucleatum* isolated from dental plaques of dogs suffering from periodontitis and *leukotoxin* gene in *F. necrophorum* subspecies *necrophorum* were not significant in creating of periodontitis related to two different districts of Tehran and Ahvaz. Most of the dog's populations had gingivitis, which can

progress to periodontitis with increasing of age.

The development of periodontitis is influenced by factors such as age, breed, diet, and oral hygiene. It is important to study the bacteria involved in this disease and the presence of virulence genes for prevention, control, and treatment. *Fusobacterium spp*. plays an important role in causing of periodontitis. Several studies have shown the presence of different bacteria of the sub-gingival samples from dogs with gingivitis or periodontitis (Jamshidi et al, 2005; Stella et al, 2018). In the current study, all positive samples for the presence of the *Fusobacterium* genus were found in dogs with periodontitis. One hundred and thirty out of 150 samples, had some degree of periodontitis, and the prevalence of *Fusobacterium* genus in these was 20%. In this sense, it is consistent with the results of some researchers (Senhorinho et al, 2012). In the present study, the prevalence of *F. nucleatum* was higher than *F. necrophrum*, which confirms the importance of *F. nucleatum* in periodontitis and it is aligned with the results of Zhou et al, (2009); and the lower prevalence rate of *F. necrophorum* and *F. funduliforme* subspecies is consistent with the results of some other researchers indicating that this subspecies is not very pathogenic and it is less important in periodontitis (Antiabong et al, 2013). The presence of *Fusobacterium* genus and its species and even virulence factors were not significant in two districts; probably the geographical factors had no effect on the presence of these bacteria.

According to a survey by Liu et al, (2014), it was reported that the prevalence of *F. nucleatum/fad*A was observed significantly higher in human patients with periodontitis; but in the present study in dogs with periodontitis, this relationship was not significant. This is probably due to the limited number of samples and species differences. The *F. nucleatum* carrying *fad*A may have a higher pathogenicity and can lead to a classification of these strains, which is more closely related to the development of high grade of periodontitis rather than gingivitis.

In the present study, 11 out of 18 tested samples were positive for *F. necrophorum* and carried the *leukotoxin* virulence gene. However, there was no significant correlation between the presence of this gene and the prevalence of periodontitis in these samples. This lack of significance may be due to the specificity of the cytotoxicity of this gene for other species(Nagaraja et al, 2005).

Considering the relationship between the prevalence of *Fusobacterium* infection in gender, according to Table 4, the infection rate was higher in males; so, the gender played an important role in the infection rate with this bacterium. Regarding the relationship between gender and periodontitis and infection with *Fusobacterium*, there was no comprehensive and accurate information available. Some studies had reported that there was no relationship between them, such as the results of some researchers who reported that there was no relationship between periodontitis disease and gender (Carreira et al, 2015; Stella et al, 2018). The previous results have shown that the prevalence of *Enterococci* and *Yersinia* were 30% and 1% respectively, in dogs of Ahvaz district by PCR (Mosallanejad et al, 2023; Mosallanejad et al, 2024). In the present study, the degree of infection had a significant relationship with gender and it was more in males. In old or spayed female dogs, this was probably due to a decrease in estrogen levels and osteoporosis and a decrease in the density of the bone tissue of the mandible and maxilla was observed (Carreira et al, 2015).

In the present study, no significant relationship was observed between the levels of infection in two different districts. We aimed to investigate the influence of geographical and cultural conditions on hygiene and dental care in Ahvaz and Tehran, and it was found that the district had

no significant effect on the prevalence of infection. In Iran, a few studies have been done in this regard. In a study on 300 dogs, referred to the small animal hospital of the Faculty of Veterinary Medicine of the University of Tehran, it was reported that the prevalence of gingivitis and periodontitis was 24% and 12%, respectively (Jamshidi et al, 2005). Unlike the recent results, the obtained data of the present study showed that the prevalence of periodontitis in both districts was higher than gingivitis. This issue is probably due to the fact that dogs over two years of age were selected in both districts and dogs over two years of age are more likely to have periodontitis than gingivitis. It seems that the occurrence of periodontitis disease, the presence of *Fusobacterium spp.* and its virulence genes are independent of the geographical conditions.

As previously reported in companion dogs, increasing of age was associated with increased risk of periodontitis. It has been hypothesized that this can be due to a decline in immune function with getting older (Wallis et al, 2020). Additional studies conducted from 1968–1987 showed an association between increasing of prevalence of periodontitis and increasing of age (Harvey et al, 1993). In the present study, it was also found that the prevalence of periodontitis increased with age and the level of infection with *Fusobacterium* was higher at the age of 5 years and older. It was reported that the prevalence of *Fusobacterium* increases with age and the progression of periodontitis is more in higher grades, and this is probably due to the weakness of the immune system in the elderly due to the production of interleukin I (IL-1) by the lipopolysaccharide (LPS) of periodontopathogenic pathogens such as *Fusobacterium* (Hennet et al, 1991). It also increases the production of lymphokines, including T cell growth factor (IL-2) and osteoclast activating factor (Jewett et al, 2000). According to the present study, the rate of infection with *Fusobacterium* was the highest in periodontitis grade 3. The higher prevalence of *Fusobacterium* in dental plaques in periodontitis grade 3 may be due to the sampling method. Sampling of more severe dental plaques is easier than gum pockets or teeth without plaque, but with gingivitis. Another reason can be that the prevalence of *Fusobacteria* without the *fad*A gene is lower, in more severe periodontitis.

Breed is one of the most influential factors in the prevalence of periodontitis. The previous studies had proven that small breed dogs were more prone to this disease (Stella et al, 2018; Dos Santos et al, 2019; Wallis et al, 2020).

According to Table 6, the most breeds with *Fusobacterium* infection were White Terrier, Poodle, Pomeranian and Shih Tzu. Probably, the prevalence of *Fusobacterium* in these breeds is higher, which makes them prone to periodontitis. In small breed dogs, it may be associated with the challenges related to brushing the teeth of very small dogs, greater reluctance in smaller dogs to accept dental chews and a reputation for fussy eating habits (Mateo et al, 2020). In another survey, on 22,300 dogs, 18 breeds were very susceptible to periodontitis, one of which is poodles (O'Neill et al, 2021). In the obtained results from our study, periodontitis in poodles and the presence of *Fusobacterium* in them was more, which is in agreement with the above results. According to the characteristics of the poodle breed, which is very energetic, it is very difficult for the owners to brush their teeth, and this is one of the reasons for this breed's susceptibility to periodontitis. Perhaps it is better to compare the oral flora of these breeds in the condition of healthy gums and periodontitis in the future studies. In this sense, our study was consistent with some researchers (Wallis et al, 2020). Brachycephalic breeds have 1.25 times the odds of periodontal disease compared with mesocephalic breeds and Spaniel types have 1.63 times the odds compared with non-spaniel types (Wallis et al, 2020). In the

present study, no such results were obtained, due to the low number of this breed.

Nutritional factors have the potential to affect oral tissues and therefore may play an important role in the development of periodontal disease (Wallis et al, 2020). It has been reported that feeding pets with commercial food reduces the incidence of periodontitis (Gorrel, 1998). Daily use of a toothbrush and dental chews is essential for preventing periodontitis in dogs. Homemade food may lack important minerals and vitamins such as zinc, calcium, vitamin C, and B complex, which can also contribute to periodontal disease in dogs (Mateo et al, 2020; Wallis et al, 2020). The present study revealed that dogs fed with homemade food had a significantly higher incidence of *Fusobacterium* infection and periodontitis compared to dogs with commercial diets. Additionally, these dogs exhibited poor oral and dental hygiene, which is a known factor contributing to the increased risk of periodontitis. In conclusion, the presence of *fad*A gene in *F. nucleatum* isolated from dental plaques of dogs suffering from periodontitis and *leukotoxin* gene in *F. necrophrum* subspecies *necrophrum* in two different districts of Tehran and Ahvaz were not significant for periodontitis. In the present study, age, gender, breed, periodontitis, district and type of food explained 97.6% of the changes in *Fusobacterium* infection and only gender and periodontitis had a significant effect on infection. Future work should focus on the relationship between the presences of *Fusobacterium* virulence genes in different degrees of periodontitis and in a larger number of samples. It is also possible to correlate the presence of *Fusobacterium* and *fad*A genes in the dental plaques of dogs and their owners. As dogs age, the probability of periodontitis increases and the owner's emotional dependence on the animal increases also; so, this issue becomes more important for the presence of other zoonotic and pathogenic bacteria between human and dogs.

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Conflict of Interest

The authors declare that they have no known conflict of interest.

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Original Article

Hematologic and electrocardiographic findings in sub-acute experimental monensin toxicosis in goats

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Abstract

Toxic effects of monensin, a polyether antibiotic mainly used as coccidiostat, have been described in a wide range of animals. The present study aimed to investigate the hematologic and electrocardiographic features of sub-acute monensin toxicosis in goats. For this purpose, seven adult goats were administered sodium monensin, 13 mg/kg, daily for 5 consecutive days via gastric gavage. Hematologic parameters including PCV, hemoglobin (Hb), total white blood cell (WBC) and differential count, total protein of plasma (TPP) and fibrinogen, were determined in baseline and daily blood samples for 10 days. Significant elevation of Hb at day 1, WBC at day 7, neutrophils percent at days 5 and 8, lymphocytes percent at days 5, 8 and 9, monocytes percent at days 1 and 3, absolute numbers of monocytes at days 1, 3, 6 and TPP at day 1, were observed in monensin exposed goats. At electrocardiography, sinus tachycardia, sinus bradycardia, S-T segment depression, and ventricular premature complexes and ventricular tachycardia were the most prominent findings. These findings suggest that sub-acute monensin toxicosis in goats, alters some hematologic parameters and causes a numbers of electrocardiographic abnormalities related to toxic cardiomyopathy in exposed goats.

Key words: Monensin, Ionophores antibiotic, Goats, Electrocardiography, Arrhythmia

Introduction

Monensin is an antibiotic produced as a byproduct of fermentation by *Streptomyces cinnamonensis* which belongs to a family of drugs known as polyether antibiotics or ionophores. It was discovered in 1967 by Agtarap et al. as a metabolite formed in a biosynthesis of aforementioned bacteria. Monensin was the first antibiotic that showed an effect at practical concentrations for incorporation in feed as an anticoccidial agent (Chapman et al, 2010). However, in the recent years it has widely been used as a feed additive to improve performance in livestock production systems (Duffield et al, 2012). Monensin has also minor additional uses in the treatment of ketosis, lactic acidosis, bloat and acute pulmonary edema and emphysema (Constable et al, 2017).

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Monensin has a low therapeutic index and may be fatal in certain species when used in excessive doses. Accidental monensin toxicosis, usually occurs after mixing errors that result in its inclusion in feeds of non-target species or in excessive concentration in the diets of target species (Novilla, 2007). Monensin toxicosis has been reported in cattle (Basaraba et al, 1999; Gonzalez et al, 2005), water buffaloes (Garcia et al, 2020; Silva et al, 2022), sheep (Jones, 2002; Mendes et al., 2003), horses (Peek et al., 2004; Gy et al 2020), swine (Miskimins and Neiger, 1996), chickens (Zavala et al, 2011), ostriches (Dedoussi et al, 2007), deer (Glover and Webeser, 1983), camels (Mu) and dogs (Vitello and Good 2023). Cases of accidental monensin intoxication have also been recorded in humans (Zhang et al, 2018). Monensin is approved for use in non-lactating goats (Smith and Sherman, 2009). However, field cases of monensin toxicosis rarely reported in goats (Anios et al, 2023). Experimental data has indicated that the LD_{50} for monensin in goats is 26.4 mg/kg body weight (Beasley, 1999).

Susceptibility to monensin toxicity varies considerably between species. Horses are usually sensitive to monensin and other ionophores intoxication and fish are the most tolerant to high levels of ionophores. The LD_{50} of monensin in horses is as low as 1.4 mg/kg, while its LD_{50} for chicken, the least sensitive species, is 214 mg/kg (Novilla, 2007). Monensin toxicity may also be potentiated by the concurrent use of various antibiotics, including tiamulin, oleandomycin, chloramphenicol, macrolides, and sulfa drugs (Barsaraba et al, 1999; Novilla, 2007). Electrocardiographic changes have not been reported in monensin intoxicated goats.

In this study, hematological and electrocardiographic features of sub-acute monensin intoxication were demonstrated in goats.

Materials and Methods

Animals and treatments

Seven clinically normal local-breed, female, non-lactating and non-pregnant goats that weighed 35-40 kg and aged 2-4 years, were purchased from a local market for use in the study. Prior to commencement of the experiment, goats were dewormed by subcutaneous injection of ivermectin and oral administration of rafoxanide (at the dose of 0.22 and 7.5mg/kg, respectively) and fed for 14 days to ensure proper acclimation. Monensin powder (Monensin 10%) was obtained from Razak Co., Iran. Fresh water was available all the time. Monensin was administered orally via orogastric tube at the dose of 13 mg/kg body weight daily for 5 days.

Blood collection and serum biochemistry

Venous blood samples were collected in the tubes containing anticoagulant (EDTA) for hematology and determination of plasma total protein (TPP) and fibrinogen, on day before monensin administration and daily until day 10. Hemoglobin (Hb) and total white blood cells (WBC) was measured by an automated cell counter (Nihon kohden, MEK 64500). Packed cell volume (PCV) was determined by using microhematocrit method. Differential count of white blood cells (WBC) were undertaken by using the manual method. The total protein was determined by refractometry and the fibrinogen concentration was measured by the heat-precipitation and refractometry method.

Electrocardiography

The baseline of the electrocardiogram (ECG) was recorded using base-apex leads (Constable et al, 2017). Electrocardiography was done using an electrocardiograph (Kenz ECG 110, Suzuken co- Ltd, Japan) on the day before starting the experiment and daily until the day 10.

Statistical analysis

Statistical analysis was conducted using SPSS for windows (release 16, SPSS Inc, Chicago, USA). Based on Kolmogorov–Smirnov normality test, non- parametric Friedman and Wilcoxon Signed tests were used to investigate significant differencees within group for measured parameters. For all comparisons, P≤0.05 was considered as significant.

Results

Hematology

Friedman test showed a significant difference in Hb and TPP levels during the experiment. Wilcoxon signed test showed the following results when the data was compared with the baseline levels: Hb was significantly increased (P<0.05) at day 1. Elevation of WBC at day 7 was significant (P<0.05) (Table 1). There was a significant elevation of neutrophils (P<0.05) at days 5 and 8. Lymphocytes was significantly increased (P<0.05) at days 5, 8 and 9 (Table 2). Monocytes was significantly increased (P<0.05) at days 1 and 3. Also, absolute numbers of monocytes were significantly increased (P<0.05) at days 1, 3 and 6. There was a noticeable increase in total protein of plasma at day 1 (P<0.05) (Table 3). Monensin exposure did not cause any significant changes in eosinophil counts and plasma fibrinogen concentration (Tables 4 & 5).

Table 1: Levels of PCV, Hb and WBC in goats with sub-acute monensin intoxication

Day	N	PCV (%)			P ★ value	Hgb (gram/deciliter)			P ★ value	WBC (per/microliter)			P ★ value
		25th	50th	75th		25th	50th	75th		25th	50th	75th	
0	7	32	35	37	-	8.2	8.5	9.2	-	12000	12600	13100	-
1	7	36	38	44	0.063	9.3	9.7	10.9	0.018	12400	12600	13000	0.499
2	7	31	36	40	0.344	7.7	8.9	10.2	0.600	12000	13200	15400	0.075
3	7	31	36	43	0.611	7.2	8.5	10.1	0.600	12300	13000	13800	0.612
4	7	27	30	35	0.093	7.5	8.5	10.2	0.500	11000	12000	14700	0.735
5	6	28	32	35	0.225	7.725	8.7	9.55	0.674	12500	13600	14500	0.116
6	6	28.75	31.5	32.75	0.080	8.075	8.55	9.15	0.400	12100	13200	14300	0.279
7	6	30.75	32	33.75	0.104	8.25	8.85	9.1	0.528	12200	13250	14000	0.046
8	6	32	34.5	36.5	0.916	8.35	9.3	9.9	0.686	13000	14150	14900	0.116
9	6	31.5	35	37.5	0.915	8.4	9.25	9.725	0.917	12600	13550	14600	0.115
10	6	32.25	35.5	38.25	0.686	8.25	9.5	10.225	0.465	12100	13850	14200	0.249
P value ★ ★		0				0.051				0.193			

Table 2: Neutrophils and Lymphocytes in goats with sub-acute monensin intoxication

Day	N	Neutrophils (%)				Neut (per/Microliter)				Lymphocytes (%)			
		25th	50th	75th	P ★ value	25th	50th	75th	P ★ value	25th	50th	75th	P ★ value
0	7	27	37	38	-	3406	4560	4736	-	59	61	71	-
1	7	30	35	36	0.611	3458	4030	4464	0.735	57	61	68	1
2	7	29	35	38	1.000	3696	4576	4837	0.237	59	64	68	0.833
3	7	28	35	36	0.233	3861	4428	4550	0.866	60	62	70	0.715
4	7	27	29	40	0.498	3300	3822	4635	0.735	59	66	70	0.463
5	6	41.025	48.89	53.8	0.028	4102.5	4889.5	5380	0.463	78.995	86.08	90.5	0.028
6	6	30.25	35.5	38.5	0.768	3516	4631.5	5370.5	0.753	57.75	61.5	68	0.752
7	6	27.75	31.5	35.5	0.207	3689.3	4035	4661.5	0.917	62.25	64.5	68.5	0.249
8	6	24.75	30.5	35	0.043	3298	4319.5	4856.3	0.917	61.75	66	72.75	0.043
9	6	28.5	30.5	33.75	0.116	3913.5	4041.5	4547.3	0.917	81.705	88.685	94.618	0.028
10	6	28.75	31	35.5	0.207	3672.3	4352	4875.5	0.463	67	67	68.5	0.072
P value ★ ★		0.385				0.854				0.658			

Table 3: Monocytes and TPP in goats with monensin intoxication

Day	N	Monocytes (%)				Monocytes (per/microliter)				TPP (gram/deciliter)			
		25th	50th	75th	P ★ value	25th	50th	75th	P ★ value	25th	50th	75th	P ★ value
0	7	1	1	1	-	125	128	133	-	7.3	7.5	7.7	-
1	7	1	2	3	0.039	130	248	378	0.028	7.9	8.4	8.9	0.018
2	7	0	1.31	4.8	0.176	0	131	480	0.310	7.4	7.7	8.6	0.207
3	7	1	3	5	0.041	123	390	585	0.028	7.1	7.7	8.1	0.553
4	7	1	1	4	0.102	118	153	441	0.128	7	7.5	7.9	0.734
5	6	1	2	2.25	0.059	88.5	267	330.75	0.116	7.2	7.4	8.125	1
6	6	1	1.5	3.25	0.109	125	227	425.25	0.028	7.2	7.6	8	0.590
7	6	0	2	3	0.330	0	252	406.5	0.173	7.275	7.75	8.35	0.340
8	6	0.75	2	2.25	0.157	123	247	321	0.116	7.325	7.9	8.45	0.144
9	6	0	0.5	2	0.783	0	63	282.5	0.917	7.15	7.3	7.825	0.595
10	6	0	2	3	0.336	0	50.5	425.25	0.686	7.05	7.5	8.075	0.917
P value★★		0.626				0.627				0.002			

Table 4: Lymphocyte and Eosinophil in goats with monensin intoxication

Day	N	Lymph (per/Microliter)				Eosinophil (%)				Eosinophil (per/Microliter)			
		25th	50th	75th	P ★ value	25th	50th	75th	P ★ value	25th	50th	75th	P ★ value
0	7	7320	7560	9301	-	0	1	4	-	0	126	304	-
1	7	7182	7625	8844	0.735	0	3	4	0.680	0	273	496	0.753
2	7	6720	9372	9856	0.237	0	1	1	0.713	0	120	132	0.686
3	7	7020	7930	9660	0.612	0	0	1	0.144	0	0	130	0.225
4	7	7150	7920	9996	0.398	0	1	3	0.680	0	110	360	0.600
5	6	7899.5	8608	9050	0.249	0	0.5	1.25	0.168	0	66	179.5	0.249
6	6	7312.3	8396.5	9184.5	0.345	0	0	1.25	0.131	0	0	163.75	0.225
7	6	7944.8	8565	9282.5	0.075	0	1.5	2.75	1	0	189.5	381.25	0.917
8	6	7939.5	9156	10154	0.075	0	2	3.5	1	0	281.5	540.25	0.463
9	6	8170.5	8868.5	9461.8	0.116	0	0	3.5	0.414	0	0	458.5	0.500
10	6	7527.5	8817.5	9508	0.249	0	0.5	1.75	0.197	0	70	249	0.463
P value★★		0.114				0.452				0.427			

Table 5: Fibrinogen in goats with monensin intoxication

Day	N	Fibrinogen (milligram/deciliter)			P value★
		25th	50th	75th	
0	7	300	300	400	-
1	7	300	400	400	0.157
2	7	300	400	400	0.180
3	7	400	400	400	0.059
4	7	400	400	400	0.157
5	6	375	400	400	0.317
6	6	375	400	400	0.157
7	6	375	400	400	0.157
8	6	300	400	425	0.157
9	6	375	400	400	0.157
10	6	300	400	400	0.655
P value★★		0.899			

Electrocardiography

At electrocardiography, sinus arrhythmias, sinus tachycardia, sinus bradycardia, ventricular tachycardia, ventricular premature complexes, S-T segment depression and increase in T wave amplitude were the most prominent findings. Sinus arrhythmias and sinus tachycardia were seen in 6 goats (Figure 1-1). Sinus bradycardia in one goat (Figure1-2), ventricular premature complexes in two goats (Figure 1-3), ventricular tachycardia in one goat (Figure 1-4) and S-T segment depression were observed in three goats (Figure 1-3).

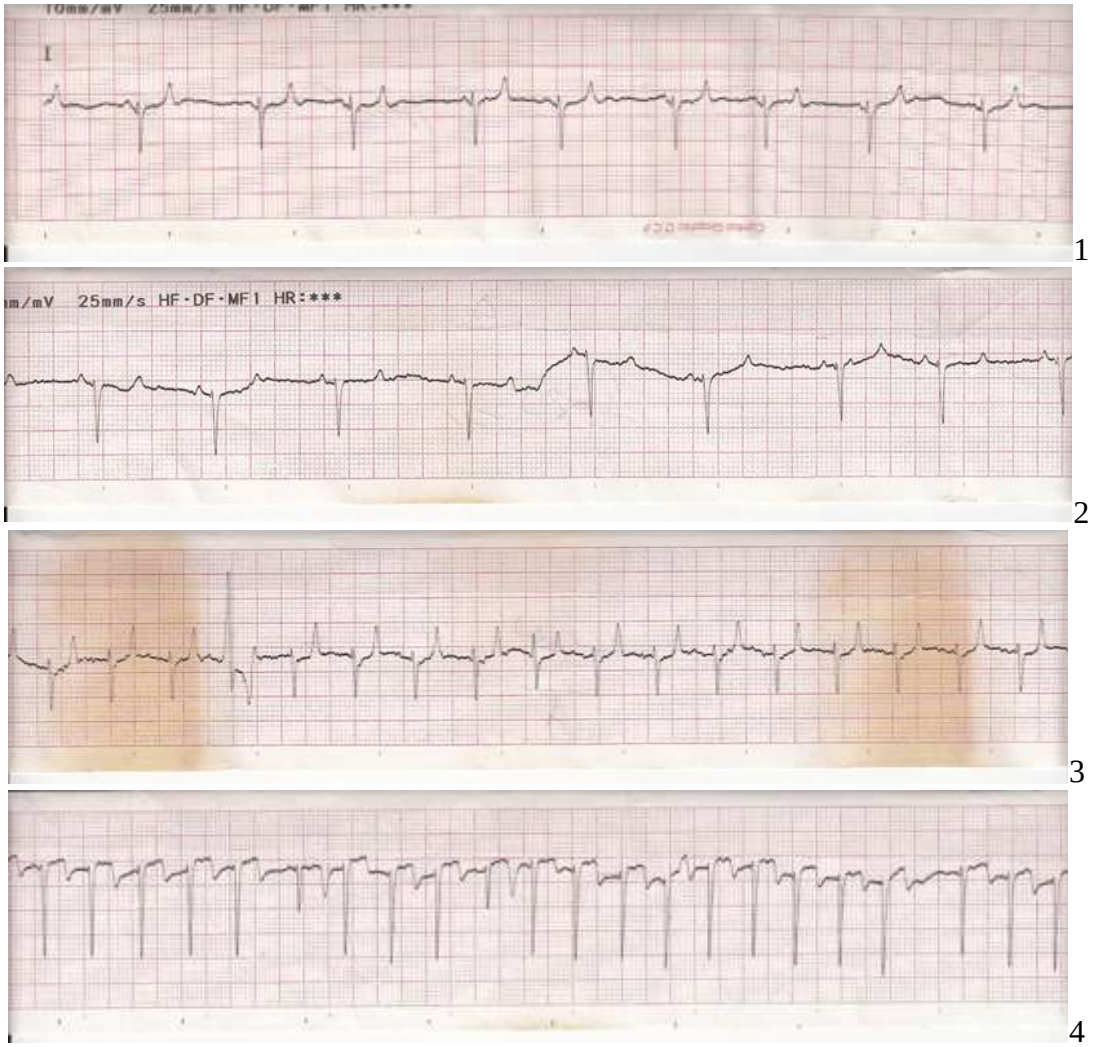

Figure 1: Electrocardiograms of goats intoxicated with monensin. 1. Sinus arrhythmia, 2. Sinus bradycardia, 3. Ventricular premature complex, 4. Ventricular tachycardia (non-sustained)

Discussion

During the past decades, it has been an increasing data of monensin beneficial and toxic effects in cattle and sheep (Bourqe et al, 1986; Duffield et al, 2012; Gonzalez et al, 2005; Mendes et al, 2003; Wang et al, 1990). However, while monensin has been approved for using in goats (Smith and Sherman, 2009), similar data for this animal are rather limited and to our knowledge, this study is the first report regarding the hematologic and electrocardiographic features in monensin exposed goats.

In this study, monensin exposure of goats did not result in noticeable changes in hematologic parameters. It is in accordance with Bouque et al, (1987) and Gonzalez et al, (2005) reports in which monensin intoxication in sheep had no effect on CBC. In this study, elevation of neutrophil percent at days 5 and 8 was significant. Elevation of blood neutrophils in these goats may be related to pain due to myopathy and associated stress Mild neutrophilia has been reported in cases of acute monensin intoxication in horses (Peek and et al, 2004). Janzen et al, (1981) also observed a mild neutrophilia in a group of monensin intoxicated bulls.

At tissue levels, necrotic myopathy and cardiomyopathy are the main features of monensin and other ionophers intoxications in different species of animals as well as in humans (Omidi et al, 2010; Mousas and El-Hamamsy, 2013; Zhang et al, 2018;

Pavarini et al, 2018; Pistán et al, 2020). Monensin, an ionopher antibiotic, has been approved to be used in domestic ruminants and widely mentioned as coccidiostat and growth promotant. However, because it has a narrow safety index in target and non-target species, intoxication occurs as a result of inappropriate use or accidental contaminations of ration (Hall, 2004). All ionophores including monensin facilitate transmembrane ion fluxes and dissipation of ion gradients, which are exaggerated at toxic levels. Cells respond to the metabolic insult by expending energy to maintain homeostasis. When homeostatic mechanisms are exceeded, toxicity ensues from excessive influxes of sodium and calcium ions leading to degeneration and necrosis of cardiac and skeletal muscle cells. Monensin also causes release of catecholamines from cultured adrenal chromaffin cells. Catecholamines and toxic oxidation products have been implicated in myocardial necrosis through greater influx of Ca^{++} and formation of free radicals (Novilla, 2012). Although cardiac arrhythmia has been mentioned in cases of monensin intoxication (Peek et al, 2004; Anjos et al, 2023), there are no details about the type of those disorders.

Monensin exposure of goats in the present study resulted in sinus tachycardia in 6 goats. This finding has also been reported in accidental monensin intoxication in goats (Anjos et al, 2023). Pain due to monensin intoxication induced sinus tachycardia. Sinus bradycardia or simple bradycardia is used to describe a decrease in heart rate due to a decreased rate of discharge from the sino-atrial node. In this study, sinus bradycardia was observed in one goat and it was accompanied by sinus arrhythmia which may be due to anorexia and hypoglycemia. Sinus arrhythmia is usually present in animals with sinus bradycardia (Constable et al, 2017).

Ventricular premature complexes may arise from an irritable process anywhere within the ventricular myocardium and are often seen in association with structural heart disease (Cha et al, 2012). In this situation, normal rhythm is interrupted by a beat that occurs earlier than expected but the initial rhythm is established following a compensatory pause. On the electrocardiogram, ventricular premature complexes are characterized by bizarre QRS morphology (Constable et al, 2017). In this study, two of monensin intoxicated goats showed ventricular premature complexes in the electrocardiograms. Premature complexes of all site origins are indicative of myocardial disease (Cha et al, 2012; Constable et al, 2017). Ventricular premature beats have also been reported in experimentally induced salinomycin, an ionophore antibiotic, toxicosis in sheep (Hesseini et al, 2013).

Ventricular tachycardia may produce either a regular heart rate or an irregular heart rate or rhythm. When the discharge rate of the irritant focus exceeds that of the sino-atrial pacemaker, the ectopic focus will take over completely as the pacemaker of the heart (Constable et al, 2017). Monensin exposure of goats resulted in ventricular tachycardia in one goat. Ventricular tachycardia is an evidence of severe cardiac disease and is usually accompanied by signs of acute heart failure (Reef and McGuirk, 2015). Myocarditis, nutritional cardiomyopathy and myocardial neoplasia could primarily cause ventricular tachycardia. Ventricular tachycardia is often a life-threating arrhythmias (Delesalle et al, 2002).

In conclusion, this study demonstrated that sub-acute monensin intoxication caused particular electrocardiographic features. However, further investigations are needed to clarify the relationship between those changes and doses of monensin in goats.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Original Article

Effects of L-tryptophan on diencephalic tryptophan hydroxylase gene expression in heat-stressed broilers

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Abstract

The brain monoaminergic system is changed during heat stress condition. Research has shown that commercial poultry are very sensitive animals to heat stress. Hence, this study investigated the effects of L-tryptophan on diencephalic tryptophan hydroxylase type 1 and 2 (TH1 and TH2) gene expression in heat-stressed broiler chicks as animal model. Forty eight, seven-day old broiler chicks were divided into three groups. Chicks were intraperitoneally injected L- tryptophan (25 and 50 mg/Kg) and normal saline. Then they were exposed to the heat stress (39ºC) for 5 hours. After 5 hours of treatment, the birds were anesthetized with isoflurane before being euthanized. The brain samples were taken for gene expression evaluation. The data showed declined diencephalic gene expression of TH1 and TH2 in heat stress condition. Tryptophan administration at dose of 50 mg/kg significantly increased the expression levels of TH1 and TH2 in heat stress exposed chicks. It can be concluded that diencephalic serotonergic pathway may have an important role in tryptophan ameliorating effect during heat stress condition.

Key words: Broiler, Diencephalon, High ambient temperature, Tryptophan hydroxylase

Introduction

Physical and psychological stressors disrupt homeostasis and can lead to mental and physical illness. The hypothalamic-pituitary-adrenal (HPA) axis and adrenergic pathways are the primary systems involved in processing stress, leading to elevated plasma corticosteroid levels, depletion of bodily reserves, and impaired immune function. Thermal stressors such as heat stress stimulate neuroendocrine responses including activation of the HPA axis, and decline of brain serotonin content. Hence, there exists an interaction between the HPA (Hypothalamic-Pituitary-Adrenal) axis and monoaminergic neurotransmitter systems in the regulation of stressful conditions (de Lima et al, 2017; Nakagawa et al, 2016).

Serotoninergic system is a monoaminergic pathway and hypothalamic content of serotonin is important agent for coping with stress clues. Serotonin is synthesized from amino acid tryptophan by tryptophan hydroxylase (TH) enzyme. Two isoforms of tryptophan hydroxylase enzyme, tryptophan hydroxylase 1 (TH1) and tryptophan hydroxylase 2 (TH2) have been known until now. TH1 is mainly synthesized in entrochromaphin cells of gut

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and pineal gland while TH2 is commonly expressed in central nervous system (Walther et al, 2003).

Serotonin modulates many physiological functions including cardiovascular, respiratory and immune function, and energy balance as well as body temperature. The balance and levels of serotonin in the brain, compared to norepinephrine and dopamine, have demonstrated delayed recovery in rats exposed to both short and long periods of heat. This suggests that brain serotonin levels are more susceptible to damage during heat stress conditions (Nakagawa et al, 2016). In addition, serotonin and dopamine concentration are increased in dorsal and posterior hypothalamic areas during exposure to cold weather condition (Ishiwata et al, 2018). Serotonin-mimicking drugs or tryptophan treatment during both normal and high temperatures reduce body temperature and improve heat stress-induced hyperthermia (Kumar et al, 2018; Mota et al, 2020), increase blood cortisol level and telencephalic 5-hydroxytryptamine concentration (Höglund et al, 2017). Furthermore, heat stress exerts a deleterious effect on brain electrical activity by alteration in brain neurotransmitters levels and administration of para-chlorophenylalanine before heat stress exposure significantly elevates body temperature in rats (Sinha, 2008).

It has been demonstrated that Brain-Derived Neurotrophic Factor (BDNF) plays key roles in the development and function of the central nervous system, and stressful conditions have a vulnerable effect on BDNF expression (Li et al, 2016; Tang et al, 2016).

Serotonin is a trophic agent for brain BDNF production and BDNF expression will be reduced by brain serotonin depletion (Zhou et al, 2008). BDNF expression and synthesis is influenced by environmental temperature. So that, 20 minutes head-out water (with 42 °C temperature) immersion had a significant increasing and decreasing effect on plasma BDNF and cortisol, respectively (Kojima et al, 2018). It has been shown that exercise at high room temperature elevates body temperature and leads to higher BDNF level than exercise at low room temperature (Goekint et al, 2011). It also has been investigated that exposure to high ambient temperature increases body temperature and plasma glucocorticoids concentration and reduces brain BDNF expression in chicks (Tanizawa et al, 2014).

The aim of the present study was to investigate the potential effect of L-tryptophan treatment during acute heat stress on levels of tryptophan hydroxylase 1 and 2 expression in neonate broiler chickens.

Material and Methods

A total of forty eight one-day-old broiler chicks (Gallus gallus domesticus) were prepared from a commercial hatchery (Mahan poultry Farming-Kerman, Kerman Province-Iran). The chicks were kept in a flock (30±1 ◦C and 50±2% relative humidity (RH)) for 2 days. After 48 hours, they were transferred into individual plexi-glass cages (14×24×21 cm) (width× length× height) for acclimatizating to the individual cages. They had free access to water and commercial starter diet (2850 Kcal/Kg metabolizable energy, 23 % crude protein) and lighting. As the heat stress group, seven days old chicks (N=36) were exposed to high temperature for 5 hours (39±1 °C and 50±2 % RH) and the control group were kept in normal temperature (N=12) (30±1 °C and 50±2% RH).

All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals by the National Institute of Health (USA) and the current laws of Iranian Government for Animal Care (IR.UK.VETMED.REC.1398.037).

L-tryptophan was purchased from Bio-Basic Inc. (20 Konrad Cres, Markham Ontario, Canada). The white crystalline powder was dissolved in 0.9% saline and

0.1 N hydrochloric acid (9:1), 1 h before the intraperitoneal injection. Tryptophan was injected based on BW of chicken. Chicks average BW was 75 g. Thus, at the dose of 25 mg Trp/kg BW, about 1.88 mg for each chick was dissolved in 0.25 ml (volume of intraperitoneal injection) HCl solution (for 24 chicks, 24 × 1.88 mg in 24 × 0.25 ml HCl solution). Finally, in primary stock, each 0.25 ml of solution contained 1.88 mg Trp. At the dose of 50 mg Trp/kg BW two fold Trp concentration, according to above instruction primary stock was prepared.

The heat stressed chicks were randomly divided into three equal groups (N=12). Different doses of L-tryptophan (25 and 50 mg/Kg body weight) and normal saline were intraperitonealy injected. The control temperature group received normal saline. All injections were in a volume of 0.25 ml. Immediately the chickens were exposed to the heat stress or control temperature. Rectal temperature was recorded after four hours of heat stress induction. Rectal temperature was measured by inserting thermistor probe (Contact-type digital thermometer-TES-1310-Taiwan) in the cloaca to a depth of 2 cm. At the end of the experiment, all chicks were properly anesthetized with isoflurane (Baxter international Inc, AErrane-isoflurane-USP) before being euthanized. The blood was immediately collected from the jugular vein into tube and centrifuged at 4000 g for 15 minutes to collect the serum. The brain sample (diencephalon) was collected following blood collection and snap frozen using liquid nitrogen. All the collected samples were stored at −80°C until further analysis.

Total RNA was extracted from each brain tissue sample by RNX-Plus solution (SinaClon BioScience Co). The final RNA pellets were re-suspended in 30 µl diethyl-pyrocarbonate-treated water (DEPC-treated water). The amount of the purified RNA (A260⁄A280 ratio was ≥1.9) was determined by Nanodrop and the integrity of RNA samples was analyzed on a 1.5% agarose gel (Sigma).

Briefly, the reaction was performed using Oligo-dT primer and M-MuLV reverse transcriptase (Thermo Fisher Scientific, Germany) based on the manufacturer's protocol. The reproducibility of single results was determined with two strategies: two-time measurement of cDNA aliquots; analysis of two different cDNA prepared from the same RNA extract.

Quantification of relative RNA expression was followed by an established method using qPCR with the SYBR green reporter dye and protocol. The 2X universal master mix (Biofact-DQ383-40H without ROX, South Korea) was used in the PCR reactions. Thermal cycling utilized LightCycler® 96 Instrument (Roche-Germany).

A final melting curve of fluorescence versus temperature was generated to screen the primer dimers and to document a single product formation. Primer sequences, RT-PCR fragment lengths and NCBI accession numbers are reported in Table 1.

Table 1: Primer sequences

Primer name	length	sequence	NCBI code
BDNF	109	F:GAAGGCTGCAGGGGCATAGA	NM_001031616.1
		R: ACCGCCAGCCAACTCTCTTT	
TH2	200	F:GACCTCCGCAGTGATCTAAACA	NM_001001301.1
		R: CACAATGACACAAGCCGCAG	
TH1	180	F: AAGAGCATTGCCAGTGTGGT	NM_204956.1
		R:AGTCCTGCATACAGACGTTACA	
GAPDH	162	F:TGACCACTGTCCATGCCATC	NM_204305.1
		R:TAAGCTTCCCATTCAGCTCAGG	

All primer pairs produced a single band on agarose gel electrophoresis corresponding to the predicted size. The amount of PCR products were normalized with housekeeping (GAPDH) primers in separate reactions. All samples were assayed in triplicate. The relative mRNA levels were calculated by the expression $2^{-\Delta\Delta CT}$ equation.

Serum corticosterone level was measured by using a solid phase sandwich ELISA method (chicken ELISA Kits; MyBioSource.com, USA). The sensitivity of corticosterone kit was 1.0 ng/ml. The intra-assay precision (precision within an assay) and the inter-assay precision (precision between assays) were CV=4.2% and CV=6.5%, respectively.

Values were expressed as mean±SEM (standard error of mean). Statistical evaluation of significant difference between means, for multiple comparisons of all groups, was performed with one-way analysis of variance (ANOVA) followed by the Tukey's multiple range test, using the SPSS16 program. The significance level was considered P<0.05.

Results

Rectal temperature and corticosterone level

The data showed that heat stress significantly caused to increase in rectal temperature at 4th h of the experiment compared with the control group (P<0.05). Tryptophan treatment (25 and 50 mg/kg) decreased heat stress-induced hyperthermia (P<0.05) (Table 2).

Table 2: Effect of tryptophan and heat stress on rectal temperature

Groups	Rectal temperature (ºC) (Means±SEM)	Minimum-Maximum Values (ºC)
Control Temperature	$39.66^{a} \pm 0.088$	39.3-40.2
Heat stress	$41.2^{b} \pm 0.057$	40.9-41.4
Heat stress +Tryptophan 25 (mg/kg)	$40.35^{c} \pm 0.076$	39.8-40.6
Heat stress +Tryptophan 50 (mg/kg)	$40.2^{c} \pm 0.055$	40-40.5

"a, b, c At each column, different superscript letters show significant difference between the groups (P<0.05)."

Analysis of serum corticosterone data showed that corticosterone levels were significantly increased in heat stress exposed chicks in comparison to the control group (P<0.05); While in heat stress tryptophan treated groups corticosterone was decreased (P<0.05) (Table 3).

Table 3: Effect of tryptophan and heat stress on serum corticosterone level

Groups	Corticosterone (ng/ml) (Means±SEM)	Minimum-Maximum Values (ng/ml)
Control Temperature	$49.98^{a} \pm 0.73$	49.5-51.1
Heat stress	$59.77^{b} \pm 1.33$	58.2-61.4
Heat stress +Tryptophan 25 (mg/kg)	$49.83^{a} \pm 1.88$	48.5-52.4
Heat stress +Tryptophan 50 (mg/kg)	$50.07^{a} \pm 1.85$	48.7-52.2

"a, b, c At each column, different superscript letters show significant difference between the groups (P<0.05)."

Heat stress significantly reduced the brain mRNA level of tryptophan hydroxylase 1, 2 and bdnf (Figure 1, 2 and 3) (P<0.05). While treatment with tryptophan (50 mg/Kg BW) in this condition significantly increased gene expression of TH 1 and TH 2. Gene expression of BDNF mostly incremented by tryptophan administration especially in chickens that received 50 mg/Kg body weight tryptophan.

Agarose gel electrophoresis of cDNA amplification products was presented in Figure 4.

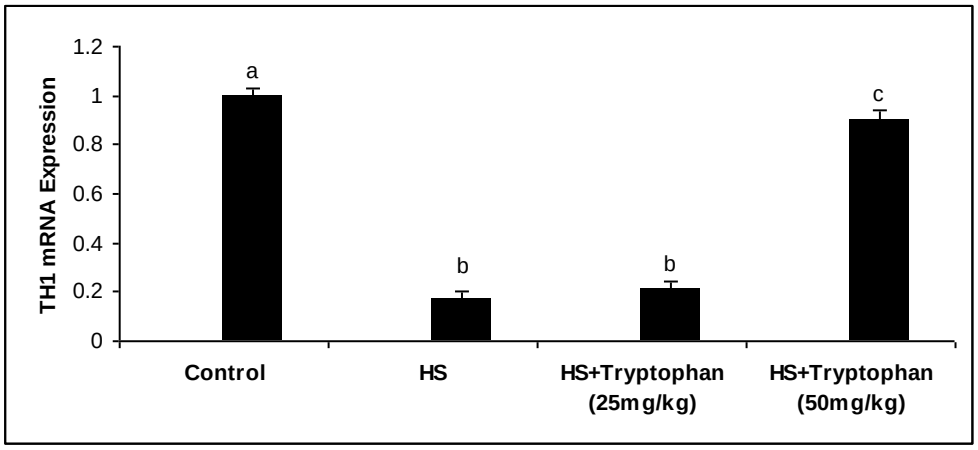

Figure 1: Effect of tryptophan and heat stress on diencephalic TH1 expression relative to GAPDH (Means±SEM).
"Different superscript letters show significant difference between the groups (P<0.05)."

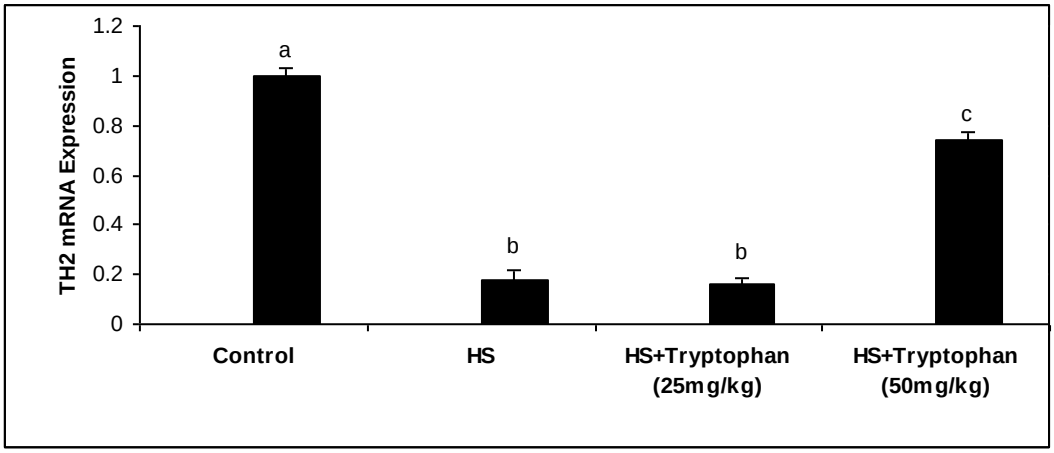

Figure 2: Effect of tryptophan and heat stress on diencephalic TH2 expression relative to GAPDH (Means±SEM).
"Different superscript letters show significant difference between the groups (P<0.05)."

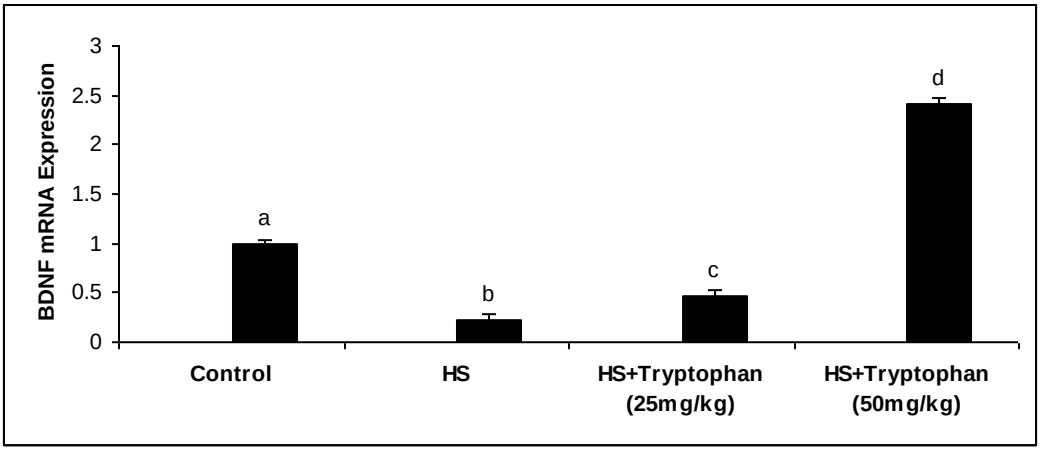

Figure 3: Effect of tryptophan and heat stress on diencephalic BDNF expression relative to GAPDH (Means±SEM).
"Different superscript letters show significant difference between the groups (P<0.05)."

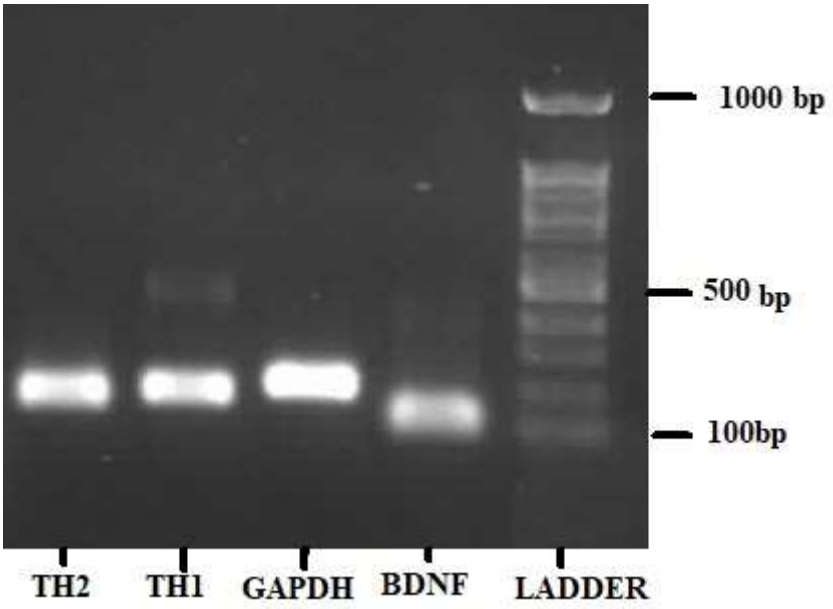

Figure 4: Agarose gel electrophoresis of TH1 (180 bp), TH2 (200 bp), GAPDH (162 bp) and BDNF (109 bp) cDNA amplification products under UV light.

Discussion

In the present study, the influence of L-tryptophan administration before heat stress exposure was investigated on body temperature, serum corticosterone and diencephalic mRNA level of tryptophan hydroxylase 1, 2 and BDNF. The data indicated that heat stress could elevate body temperature, serum corticosterone concentration and reduce the gene expression of tryptophan hydroxylase 1, 2 and BDNF. Tryptophan treatment during heat stress significantly compensated the changes associated to the heat stress exposure (Nakagawa et al, 2016).

In the present study, administration of tryptophan reduced the increased rectal temperature after thermal stress. Similar changes were seen in the case of corticosterone. Of course, changes in the amount of corticosterone with tryptophan intake are more effective and its amount is closer to the control group.

It has been reported that the disturbance in body temperature regulation is related to the hypothalamic serotonin reduction during rodent-heat stress exposure. Tryptophan hydroxylase is first rate-limiting enzyme of serotonin production. In depression rat models, serotonin decline is due to the reduction of brain tryptophan hydroxylase 1 and 2 and their dysfunction (Chen et al, 2017). In line with our findings, heat stress exposure reduced diencephalic tryptophan hydroxylase 1 and 2 genes expression. Over-activation of HPA axis and monoaminergic system and elevation of blood corticosterone and catecholamines levels are the most common feature of physical, mental and thermal stress during heat stress condition (K Ito et al, 2014).

It has been shown that the central injection of tryptophan (400 and 800 nmol) in 5-6 day-old chicks significantly counteracts with CRH-augmented social isolation (Yoshida et al, 2015). In addition, Zhang et al (2004) reported that the central injection of serotonin acts as a CRH-induced behaviors modulator. Accordingly, in the current study, tryptophan, as a precursor of serotonin, has influenced the modulation of the CRH axis and prevented the increase of corticosterone.

In the recent experiment, tryptophan administration before heat stress exposure leds to reduce rectal temperature and serum corticosterone level. Glucocorticoids are

known as catabolic hormones and body heat increment is a result of increased catabolism. In agreement with our results, oral supplementation of tryptophan (20, 40, and 60 mg/kg, for 3 days) causes a significant decrease in blood cortisol and heart rate horses (Davis et al, 2017).

Also it has been reported that increase in the amount of plasma corticosterone, decrease in plasma serotonin and enhancement in sensitivity to the stress in tryptophan-deficient diet in rats have occurred (Tanke et al, 2008).

It has been demonstrated that there is a reciprocal relationship between BDNF and serotonergic system so that serotonin depletion promotes BDNF down-regulation and synthesis (Zhou et al, 2008) and central injection of BDNF increases tryptophan hydroxylase mRNA expression and serotonin production (Siuciak et al, 1998). Therefore, it seems logical that in our study, heat stress exposure reduced chicken brain BDNF mRNA level and tryptophan supplementation had a compensatory effect and increased brain BDNF gene expression.

Negative effects of elevated glucocorticoids during stress on brain BDNF production have also been reported (Schaaf et al, 1998). Heat stress exposed chicken in our research had high serum corticosterone and low BDNF gene expression. In contrast, Tanizawa et al (2014) reported a reduced corticosterone level and no changes in brain BDNF gene expression following 3 hours heat stress conditioning repeated 15 minutes every day for 4 consecutive days. Based on the previous reports, at least 5 hours exposure to high temperature is required to induce heat stress in chicken (K Ito et al, 2014). It has been shown that the diencephalic and plasma tryptophan levels are decreased during acute heat stress (K Ito et al, 2014; Kentaro Ito et al, 2015). This might be a conservative and protective mechanism to overcome heat stress. During heat stress gluconeogenesis occurs by amino acids breakdown and decreases in their reserve pool. This might lead to the reduction of blood and brain tryptophan concentration. Therefore, a tryptophan supplementation will help to prevent heat stress side-effects. It has been reported that peripheral serotonin can control thermogenesis by inhibiting the beta-adrenergic effects on adipose tissue (Crane et al, 2015). In agreement to our results, dietary tryptophan increases blood serotonin and decreases corticosterone levels and improves gut micro-flora (U. Bello et al, 2018). In contrast, using a selective serotonin agonist for TH1A receptors reduces body temperature but increases corticosterone in rats (Newman-Tancredi et al, 2018).

Although serotonin is a product of tryptophan metabolism, kynurenine is the main product of tryptophan and about 95 percent of tryptophan is converted to kynurenine (Mellor et al, 1999). A key product of kynurenine pathway is kynurenic acid that acts as a glutamate receptor antagonist and thereby antagonizes dopamine release (Amori et al, 2009; Fujigaki et al, 2017). It has been reported that a significant elevation in the blood glutamate, norepinephrine and dopamine is found in heat stress exposed rats (Chauhan et al, 2017). Chronic heat exposure for 4 days activates blood kynurenine metabolites pathway in chickens (Tomonaga et al, 2018).

The above implications show a significant relationship between heat stress and kynurenine metabolite and emphasize the role of tryptophan metabolite for buffering the heat stress consequence.

Based on our results, it can be concluded that heat stress disturbs brain sertonergic system by reducing tryptophan hydroxylase enzymes gene expression. Furthermore, high blood corticosterone levels and body temperature elevation as well as decrease in brain BNDF gene expression are other consequences of acute heat stress in chicks.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Original Article

The Effect of hydroalcoholic extract of *Astragalus membranaceus's* root on the hematological parameters and bone marrow cells after the administration of Mitomycin C in male rats

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Abstract

Mitomycin C (MMC) is an anti-cancer drug; but it has a side-effect which is bone marrow depression. *Astragalus membranaceus* is used in traditional Chinese medicine. This plant has various pharmacological effects, including hematopoietic, angiogenesis and immunostimulant effects. This study evaluated the effects of *Astragalus* root hydroalcoholic extract on the hematological and bone marrow cells after administering MMC. Twenty-eight male Sprague-Dawley rats (250±30 g) were divided into four groups: control (without treatment), Mitomycin group (MMC, 2 mg/kg, i.p.), Astragalus extract (*Astragalus* root extract, 500 mg/kg for 14 days) and treated group (MMC, 2 mg/kg, i.p. and *Astragalus* root extract, 500 mg/kg for 14 days). The hematological parameters (hematocrit, hemoglobin, red blood cell (RBC) count, red cells indices, total white blood cells (WBC), platelets, and differential leukocyte count) and bone marrow cells (erythroid and myeloid series) were measured. After receiving MMC, the hematological parameters and bone marrow cells were reduced. The results showed that hydroalcoholic extract of *Astragalus membranaceus* could increase significantly the hematological parameters including hematocrit, hemoglobin, RBC, WBC, and platelets as well as bone marrow cells. This study showed that *Astragalus* root hydroalcoholic extract positively affected anemia signs.

Key words: *Astragalus membranaceus*, Anemia, Mitomycin C, Bone marrow

Introduction

Anemia is a common complication that leads to a decrease of hemoglobin and red blood cells (Patel et al., 2016). Various causes and many factors, such as mutations in the gene for hemoglobin, acute and chronic bleeding, inadequate nutritional intake, inflammation, low levels of erythropoietin, iron deficiency, and failure to produce red blood cells can induce anemia. Anemia is interference with the oxygen delivery to tissues, weakness, increased risk of preterm delivery, low birth weight, headache, pallor, shortness of breath, poor concentration, psychosis, seizures, strokes, and even death (Kassebaum, 2016). Anemia may also be induced by drugs like isoniazid, sulfonamides, cyclophosphamide, and

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Mitomycin (Barragán-Ibanez, 2016). Mitomycin C is an anticancer drug that could be converted into an alkylating agent and used for treating a range of cancers, including esophagus, breast, bladder, stomach and cervix cancer. Mitomycin C also causes decreased hemoglobin levels, hemolytic anemia, inhibition in the activity of bone marrow, leukopenia, thrombocytopenia, kidney disorders, and hypertension, as side effects (Shah et al, 2013).

The dried root of *Astragalus membranaceus* is one of the oldest and most commonly used treatments in traditional Chinese medicines (Wong, 2006). A wide range of medicinal properties also have been reported for Astragalus root, including its blood-forming activity, anti-hyperglycemia, anti-inflammatory, anti-bacterial, and anti-viral effects (Bian and Li, 2009). Saponins, isoflavonoids, polysaccharides, and astragalosides are all biologically active constituents of *Astragalus membranaceus* root (Bian and Li, 2009; Liu et al, 2017). This study aimed to evaluate the effects of Astragalus root hydroalcoholic extract on the hematological parameters and bone marrow cells after administering Mitomycin C in rats.

Materials and Methods

Root extraction and drug preparations

Astragalus root powder (255 g) was mixed with ethanol (550 ml of 25%) and stirred for several hours. After 24 h, the mixture was filtered and made smooth. Then, the vacuum distillation to a quarter of the initial volume was condensed; after lyophilization, the powder was kept in the freezer (-20 ° C). In this study, Mitomycin C (2 mg vials) (Kowya, Japan) was used (Kumari et al, 2016).

Animals

Twenty-eight male Sprague Dawley rats (250±30) were divided randomly into four groups (n=7) and kept in separate cages as in the following groups:

1. The control group received no treatment.
2. Mitomycin group: 2 mg/kg Mitomycin C (1 mL) was injected intraperitoneal (i.p.) and after four weeks, for 14 days, distilled water gavage was done.
3. Astragalus extract group: distilled water (1 mL) was injected (i.p.), and after four weeks, 500 mg/kg of Astragalus root extract was given for 14 days.
4. Treated group: 2 mg/kg Mitomycin C was injected (i.p.), and after four weeks, 500 mg/kg Astragalus root extract was given for 14 days (Shah et al, 2013).

Animal ethics

The experiment was performed under the approval of the state Committee on Animal Ethics, Shiraz University, Shiraz, Iran (IACUC no: 4687/63). In addition, the recommendations of the European Council Directive (86/609/EC) of Nov 24, 1986, regarding protecting animals used for experimental purposes were considered.

Blood and bone marrow analysis

At the end of the test, the rats were anesthetized by CO_2 and blood samples were taken from the heart and collected in EDTA tubes. Blood parameters including hematocrit, hemoglobin concentration, white blood cells, red blood cells, differential leukocyte count, and platelet count were measured. The femur was selected for bone marrow, and the smears were prepared. Bone marrow slides were used to evaluate the cellularity, particle distribution and average megakaryocyte numbers per 20 fields and to classify the erythroid and myeloid precursors (Voigt and Swist, 2011; Latimer, 2011). Bone marrow cell counts, erythroid and myeloid cell counts, and the proportion of the myeloid cells to erythroid cells (M/E ratio) were also measured.

Statistical analysis

The one-way analysis of variance (ANOVA) and Duncan test were

conducted. P values ≤ 0.05 were considered significant.

Results

Blood factors

The amount of hemoglobin and red blood cells in the Mitomycin group showed a significant decrease compared to the amounts observed in the control group. Hemoglobin and the number of red blood cells in the Astragalus extract group increased compared to the control group, but this difference was insignificant. Hemoglobin in the treated group increased compared to the Mitomycin group (Table 1).

Table 1: Comparison of erythrocytes in different groups (Mean ± SEM)

Factors / Groups	Hct (%)	Hb (g/dl)	WBC (/µl)	RBC $\times 10^6$ (/µl)	PLT $\times 10^5$ (/µl)	MCV (fl)	MCH (pg)	MCHC (g/dl)
Group 1(control)	47.57 ± 0.48^b	15.83 ± 0.15^b	8917.29 ± 354.90^b	8.64×10^6 ± 2^b	5.33×10^5 ± 5758.75^b	55.16 ± 0.96^b	18.36 ± 0.33^b	33.27 ± 0.04^b
Group 2(C1)	40.83 ± 3.59^a	13.51 ± 1.19^a	7414.50 ± 179.76^a	5.76×10^6 ± 5.59^a	4.30×10^5 ± 7846.09^a	71.39 ± 1.27^a	23.62 ± 0.44^c	32.92 ± 0.18^b
Group 3(C2)	49 ± 0.44^b	17.47 ± 0.57^b	1.12×10^4 ± 552.6^c	8.80×10^6 ± 1.07^b	5.43×10^5 ± 25539.89^b	55.71 ± 0.39^b	19.86 ± 0.63^b	35.64 ± 1.04^b
Group 4(T)	45.43 ± 2.10^{ab}	13.59 ± .28^a	8988.14 ± 504.37^b	8.32×10^6 ± 3.40^b	5.47×10^5 ± 9413.77^b	58.71 ± 1.41^b	16.45 ± .57^a	29.50 ± 1.64^a

Control: control group; C1: Mitomycin group; C2: Astragalus extract group; T: treated group treated with Mitomycin C and Astragalus root extract. Lowercase letters in each column show a significant difference in various groups' $p < 0.05$.

Hct: Hematocrit
Hb: Hemoglobin
WBC: White Blood Cell
RBC: Red Blood Cell
PLT: Platelets
MCV: Mean Corpuscular Volume
MCH: Mean Corpuscular Hemoglobin
MCHC: Mean Corpuscular Hemoglobin Concentration

The MCV decreased significantly in the treated group compared to the Mitomycin group, with no significant differences with the control group. MCH and MCHC in the treated group showed a significant decrease compared with the Mitomycin group (Table 1).

White blood cell counts in the treated group showed a significant increase compared to the Mitomycin group. However, it showed no significant difference compared to the control group (Table 1). Lymphocytes in the treated group showed a significant decrease compared to that of the Mitomycin group, but it showed no significant difference compared to the control group. The number of neutrophils in the treated group showed a significant increase compared to that observed for the Mitomycin group. However, it showed no significant difference compared to the control group (Table 2).

The platelet counts in the treated group significantly increased compared to those of the Mitomycin group, but it showed no significant difference compared to the control group (Table 1).

Table 2: Comparison of blood leukocytes in different groups (Mean ± SEM)

Factors / Groups	BAS (%)	BAS (/µl)	EOS (%)	EOS (/µl)	MON (%)	MON (/µl)	LYM (%)	LYM (/µl)	NUT (%)	NUT (/µl)
Group 1(control)	0	0	0.14 ± 0.14[a]	12.48 ± 12.48[a]	5.35 ± 0.73[a]	477.07 ± 65.09[a]	63.18 ± 1.99[a]	5633.94 ± 177.45[a]	28.42 ± 2.12[b]	2534.29 ± 189.04[b]
Group 2(C1)	0	0	0.17 ± 0.17[a]	12.60 ± 12.60[a]	4.59 ± 0.80[a]	340.32 ± 59.31[a]	78.07 ± 2.37[b]	5788.50 ± 175.72[b]	17.17 ± 2.63[a]	1273.06 ± 195[a]
Group 3(C2)	0	0	0[a]	0[a]	5.85 ± 0.78[a]	655.2 ± 87.36[a]	61.36 ± 3.04[a]	6872.32 ± 340.48[a]	31.36 ± 2.85[b]	3512.32 ± 319.2[b]
Group 4(T)	0	0	0.14 ± 0.14[a]	12.58 ± 12.58[a]	3.74 ± 0.48[a]	336.15 ± 43.14[a]	61.61 ± 1.75[a]	5597.59 ± 157.29[a]	36.31 ± 1.74[b]	3263.59 ± 156.39[b]

Control: control group; C1: Mitomycin group; C2: Astragalus extract group, T: treated group treated with Mitomycin C and Astragalus root extract. Lowercase letters in each column show a significant difference in various groups' $p < 0.05$.

A: absolute
BAS: Basophils
EOS: Eosinophils
MON: Monocytes
LYM: Lymphocytes
NUT: Neutrophils

Bone marrow factors

After administering Mitomycin C in all groups compared to the negative control group, all cells of the myeloid line were significantly reduced, except the band cell. Myeloid cell lines including myeloblast (MBS), promyelocyte (PMS), myelocyte (MS), meta myelocyte (MTMS) and erythroid cell lines including rubriblast (RRS), prorubricyte (PRS), basophilic rubricyte (RS), and polychromatic rubricyte (MRS) demonstrated in Figure 1. In the test group, all the cells of the myeloid line (except the band cell) and all the erythroid cell lines increased compared to the positive control group. The proportion of the myeloid cell line to the erythroid cell line in the positive control, comparative control, and test groups increased compared to the proportion observed for the negative control group (Table 3).

Table 3: Comparison of erythroid and myeloid cells in the bone marrow in different groups (Mean ± SEM)

Factors / Groups	MBS (%)	PMS (%)	MS (%)	MTMS (%)	BAND (%)	RRS (%)	PRS (%)	RS (%)	MRS (%)	M/E
Group 1(control)	0.372 ± 0.26[a]	2.2 ± 0.38[a]	16.82 ± 0.99[b]	18.37 ± 0.86[b]	17.31 ± 3.88[a]	0.82 ± 0.34[ab]	5.62 ± 0.77[b]	27 ± 1.49[b]	11.05 ± 5.33[b]	1.23 ± 0.05[a]
Group 2(C1)	0.26 ± 0.21[a]	2.3 ± 0.56[a]	13.8 ± 1.69[a]	16.66 ± 0.99[a]	0.34 ± 3.16[c]	0.63 ± 0.31[a]	4.83 ± 0.48[a]	0.20 ± 1.06[a]	6.56 ± 2.90[a]	2.08 ± 0.04[c]
Group 3(C2)	0.34 ± 0.42[a]	2.25 ± 0.48[a]	17.2 ± 0.53[b]	18.62 ± 0.80[b]	17.68 ± 5.12[a]	0.97 ± 0.34[b]	5.74 ± 0.86[b]	27.4 ± 1.17[b]	9.86 ± 5.15[b]	1.28 ± 0.05[a]
Group 4(T)	0.28 ± 0.29[a]	2.22 ± 0.40[a]	16.94 ± 0.97[b]	17.14 ± 0.92[a]	26.8 ± 7.60[b]	0.86 ± 0.53[a]	5.31 ± 0.72[b]	21.4 ± 6.61[a]	8.91 ± 1.46[a.b]	1.74 ± 0.10[b]

Control: control group; C1: Mitomycin group; C2: Astragalus extract group; T: treated group treated with Mitomycin C and Astragalus root extract. Lowercase letters in each column show a significant difference in various groups' $p < 0.05$

RRS: Rubriblast; PRS: Prorubricyte; RS: Basophilic Rubricyte; MRS: Polychromatic rubricte
MBS: Myeloblast; PMS: Promyelocytes; MS: Myelocytes; MTMS: Meta myelocytes

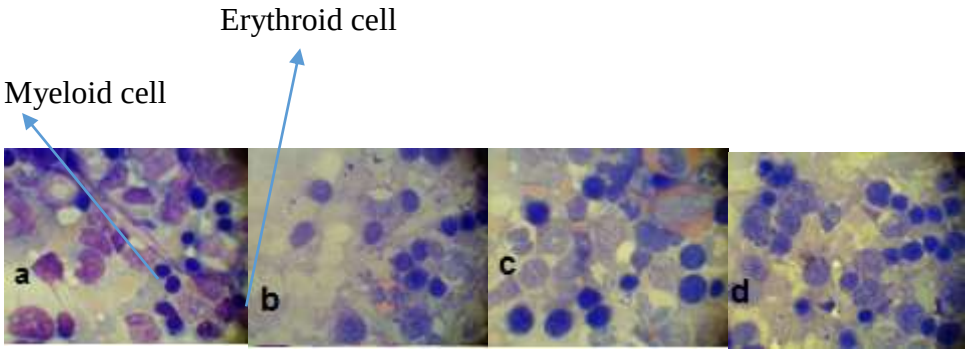

Figure 1: Pathology image of bone marrow cells
a): bone marrow cells in the control group (100 ×).
b): bone marrow cells in the Mitomycin group (100 ×).
c): bone marrow cells in the Astragalus extract group (100 ×).
d): bone marrow cells in the treated group (100 ×).

Discussion

This study showed that *Astragalus* root hydroalcoholic extract positively affected the treatment of anemia. Plants have always been a suitable source for the production of pharmaceuticals (Ashraf et al, 2016). Astragalus contains active biological compounds in its root (Ionkova et al., 2010). In the present study, bone marrow erythroid and myeloid categories decreased after the administration of Mitomycin C. These results show that Mitomycin C is a bone marrow suppressor.

Cytotoxicity and the side - effects of Mitomycin C are inhibiting the metabolism and forming free radicals in the cells (Siegel et al, 1990). The drug acts by two mechanisms, namely bioreductive alkylation and generation of free radicals such as superoxide and hydroxyl radicals through its metabolic activation (Bass et al, 2013; Dusre et al, 1989). One of the side - effects of Mitomycin C is bone marrow depression (Crooke and Bradner, 1976). Mitomycin C induces genotoxicity and cytotoxicity in healthy cells (Siegel et al, 1990). The extract of *Dorema aucheri* on hematologic factors in rats showed no significant difference in the total number of red blood cells, white blood cells, lymphocytes percentage, basophils, eosinophils and monocytes (Khoshvaghti et al, 2013). *Dorema aucheri* extract is rich in flavonoids and flavonoid compounds and has strong antioxidant properties (Khoshvaghti et al, 2013; Tavana et al, 2015). The intraperitoneal injection of aqueous and organic extracts of *Eukalyptoseucalyptose globulus* on blood biochemical factors was studied, and the results showed that ethanol extract could have a significant effect on the level of white blood cells, red blood cells, hemoglobin, and hematocrit but no significant effect on the platelet count (Sheikhzadeh et al, 2011).

Zataria multiflora is a grassy and annual plant that contains different constituents, including carvacrol, and can increase the number of white blood cells, and the percentage of neutrophils, monocytes, eosinophils, and lymphocytes (Boskabady and Jalali, 2016). In the present study with Astragalus root extract, the increase in the number and percentage of leukocyte parameters are less.

In a study on oxidative stress, Trifolium extract showed an antioxidant effect on platelet count. Trifolium has polyphenolic compounds, phenolic acids, flavonoids, isoflavones, clovamides, and had powerful antioxidant properties. The extract increased in platelet levels (Kolodziejczyk-Czepas et al, 2013).

The roots of *Astragalus membranaceus*, known pharmaceutically as radix Astragali, are rich in saponins, polysaccharides, isoflavonoids, γ-aminobutyric acid, and various trace elements (Wagner et al.,

1997). *Dorema aucheri* extract also has flavonoid compounds; however, future studies should identify effective compounds (Tavana et al, 2015).

Hydro-alcoholic propolis extract showed a Hindi protective effect on gene toxicity and cytotoxicity of Mitomycin C (Kumari et al, 2016). Mitomycin C can cause a significant increase in cellular apoptosis in bone marrow (the polar and non-polar compounds). The propolis extract is an antioxidant and inhibits the product of radicals induced by the Mitomycin C (Kumari et al., 2016). The reduction of erythroid lineage cells and decreased bone marrow myeloid cells, myelocytes and metamyelocytes class by Mitomycin C also corresponded with the findings of the present study and corroborated our findings (Kumari et al, 2016).

Polysaccharides of Angelica root are the most important compound with anti-tumor properties (Hui et al, 2006). They also correspond with the growing diversity of plant species because the most important part is the root of many of the compounds. After suppressing of the bone marrow cells using cyclophosphamide, Angelica extract can improve deficiently (Hui et al., 2006). Cyclophosphamide reduced the number of white blood cells and the number of blood vessels. The Astragalus extract polysaccharides stimulate and increase the production of stem cells in bone marrow (Li et al, 2014). It also enhances the hematopoietic cells, but this difference is not significant. This is probably due to the same effect in both plant polysaccharides, a finding consistent with the present study (Li et al, 2014). A study found that Astragalus root could treat bone diseases such as osteoporosis (Bian and Li, 2009). Astragalosides can initiate osteoblast proliferation and differentiation and may also be from early to late-stage osteoblast differentiation (Bian and Li, 2009).

Astragalus root as a medicinal plant has all of the above-described compounds, in turn, today, as a new drug used to treat a variety of diseases (Kim et al., 2009). Because of the superior efficacy and completeness of *Astragalus membranaceus* to other plants, the composition is effective as a whole compound (Liu et al, 2017). In the present study, Astragalus root extract could increase the erythroid and myeloid lineage cells in the bone marrow and blood. Different compounds in the Astragalus root extract (Liu et al, 2017) can improve the toxic effects of Mitomycin C on blood factors and bone marrow cells, and therefore suggested that this extract has hematopoietic activity.

Astragalus root extract positively affected the blood and bone marrow factors. In other words, this extract could increase the erythroid and myeloid lineage cells in the bone marrow and blood. Further research is needed in the immune, cardiovascular, and reproductive systems so that the types of compounds in the Astragalus root could be identified.

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Conflict of interest

There is no conflict of interest to disclose.

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Original Article

Comparison of four postoperative pain evaluation scales in dogs undergoing ovariohysterectomy

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Abstract

This study aimed to compare four pain scales, including simple descriptive scales (SDS), visual analog scale (VAS), the University of Melbourne Pain Scale (UMPS), and the short form of Glasgow Composite Pain Scale (GCPS-SF) to assess postoperative pain in dogs that underwent ovariohysterectomy (OHE). Twenty-two female mixed-breed dogs were allocated into three treatments to receive incisional (n=7), transverse abdominis plane (TAP, n=7), and rectus sheet (RS, n=8) blocks. After premedication with acepromazine (0.05 mg/kg) and morphine (0.5 mg/kg), anesthesia was induced (4 mg/kg) and maintained (0.4 mg/kg/min) with propofol. Each dog randomly received one analgesic method, and then OHE was performed. Postoperative analgesia was evaluated up to 6 hours after the operation with the above-mentioned pain scales. The results showed that with the GCPS-SF, the scores at 4, 5, and 6 hours after surgery in the Incisional and RSB were higher than the baseline. In the UMPS, in the RSB, at 2, 3, 4, 5, and 6 hours after surgery, the pain score was significantly higher than the baseline. With the VAS, the pain score in the RSB was higher than the baseline at 3, 4, 5, and 6 hours after surgery. In the SDS, the Incisional and RS pain scores were higher at 3, 4, 5, and 6 hours after surgery than the baseline values. In conclusion, the UMPS might detect pain earlier and is more sensitive than the other three methods. Further studies should be done to confirm the results.

Key words: Glasgow Composite Measuring Pain Scale, University of Melbourne Pain Scale, Visual Analog Scale, Simple Descriptive Scale, Ovariohysterectomy

Introduction

As a part of standard medical practice, veterinarians must relieve pain and suffering of their patients. The definition, recognition, and management of pain may be challenging in dogs and cats. Veterinarians must recognize pain and administer adequate analgesics for ethical reasons related to animal welfare and to diminish postoperative complications such as immunosuppression or self-trauma. Therefore, pain assessment should be a standard physical examination component for every patient undergoing surgical procedures.

Various methods have been developed to assess pain in small animals, which rely on

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physiological and behavioral changes. However, these methods have limitations, as relying only on physiological variables is insufficient, and depending solely on behavioral changes can be subjective among veterinarians. As a solution, numerical pain scoring systems were developed as a more objective tool for pain quantification by interpreting both behavioral and physiological changes (Fowler et al, 2003; Hernandez-Avalos et al, 2019). These scoring tools allow for more reliable pain assessment and reduce observer subjectivity and bias (Epstein et al, 2015).

The Simple Descriptive Scale (SDS) is a pain scoring system that uses numbers from 0 to 3 to indicate the pain level, ranging from no to severe. However, this scale may not be sensitive enough to detect slight changes in pain intensity (Grimm et al, 2015). The Visual Analog Scale (VAS) is a 10 cm straight line labeled on both ends, with one end indicating no pain and the other showing severe pain. In veterinary medicine, since animals cannot assess their own pain, the observer determines the degree of pain (Hernandez-Avalos et al, 2019). The Glasgow Composite Measuring Pain Scale (GCPS) is a scoring system based on evaluations of spontaneous behavior, interactions with animals, and clinical observations. A shorter version of this scale (GCPS-SF) was developed for routine clinical use, emphasizing speed, ease of use, and guidance for analgesia provision (Holton et al, 2001; Reid et al, 2007). The University of Melbourne Pain Scale (UMPS) is a multidimensional scale used to evaluate dogs' pain by focusing on their behavior and physiological constants. This scale considers six variables: physiological constants, auscultation response, activity, emotional state, posture, and vocalizations (Firth et al, 1999; Hernandez-Avalos et al, 2019; Saritas et al, 2015).

To the knowledge of the authors, there is no report of comparison of these four pain scales in dogs to recognize if there is any difference between scales or if any scale is more accurate, practical, and valuable to assess postoperative pain in dogs. Therefore, the objective of the current study was to compare SDS, VAS, GCPS-SF, and UMPS in dogs anesthetized with propofol and underwent ovariohysterectomy (OHE).

Material and Methods

In this study, 22 dogs were selected based on their physical status (ASA I-II) and body condition score (3-6 on a scale of 1-9). These dogs weighed an average of 19.3 ± 2.7 kg and were between 1.5-2.5 years old. The dogs underwent a thorough physical examination, CBC, and TP measurements to ensure their health status before being transferred to the Veterinary Hospital. They were kept in individual cages for at least two weeks before the study. The dogs were fed twice a day and provided water ad libitum, with a fasting period of 12 hours and water limitation for 2 hours before each experiment.

On the day of the experiment, the dogs were given premedications of acepromazine (0.05 mg/kg; Neurotranq, Acepromazine maleate, Alfasan, Holland) and morphine (0.5 mg/kg; Morphin Sulfate, 10 mg/mL, Darou Pakhsh, Iran) through intramuscular injection. After 20 minutes, a 20 gauge intravenous catheter was aseptically inserted into the left saphenous vein, and a lactated ringer's solution infusion was started at a rate of 5 mL/kg/hr. Propofol (6 mg/kg; Pofol, Propofol 1%, Dong Kook Pharm, Chng Cheong, Korea) was given intravenously (IV) to induce anesthesia, which was adjusted according to the effect. Following tracheal intubation, all dogs were connected to a re-breathing system and given 100% oxygen, initially at 30 mL/kg/min for 10 minutes and then 10 mL/kg/min. Anesthesia was maintained with a constant rate infusion of propofol (0.4 mg/kg/min) using a syringe pump

(Daiwha, Medifusion, Gangnam-gu, Seoul, Korea). Throughout anesthesia, various variables such as heart rate (HR), non-invasive systolic, diastolic, and mean blood pressure (SBP, DBP, and MBP), respiratory rate (f_R), rectal temperature (RT), end-tidal carbon dioxide (ETCO2, Capnograph, Respironics, USA), and peripheral arterial oxygen saturation (SPO2) were continuously monitored using a multiparameter monitor (Trismed, Vitapia 7000k, Daejeon, Korea). The baseline values for these parameters were recorded 5 minutes before surgery commenced.

During the experiment, the dogs were breathing on their own, but if the levels of EtCO2 fell outside the normal range (35-45 mmHg), manual ventilation was used to restore them. Rectal temperature was maintained at 37-38ºC, achieved via a heating pad and warm water. After 15 minutes, the surgical area was meticulously cleaned using an aseptic technique.

Following standard procedures, an ovariohysterectomy was carried out routinely. If any limb movement was observed during the operation, propofol (1 mg/kg) would be administered. If HR or BP increased by 20% or more compared to baseline values, one mcg/kg fentanyl (Fentanyl citrate, 0.5 mg/10 mL, Caspian Tamin Pharmaceutical, Rasht, Iran) would be given intravenously (IV), up to a maximum of two doses. If these parameters did not improve, a continuous infusion of fentanyl at a rate of 0.5 mcg/kg/hr would be initiated.

The dogs were randomly assigned one of the three treatments: incisional (Incisional, n=7), Transverse Abdominis Plane (TAP; n=7), or Rectus Sheath (RS; n=8) blocks. All dogs were placed in dorsal recumbency, and the skin from the pubis to the xiphoid and laterally to 10 cm on either side of the ventral midline was shaved and prepared aseptically before block administration.

Incisional, TAP, and RS blocks were done according to methods described elsewhere. Briefly, for incisional line block, bupivacaine 0.25% (1 mg/kg; Bupivacaine HCL 0.5%, Mungmoon Pharm, Seoul, Korea) diluted to 0.8 mL/kg, injected in an 8cm line using a 20-gauge, 2-inch hypodermic needle (Fitzpatrick et al. 2010). For the TAP block using an ultrasound (Landwind, Wellkang tech. Shenzhen, China) to identify the layers of the abdominal wall, a 22-gauge 90-mm spinal needle (Disposable spinal needle, Dr. Japan), attached to an extension set, was inserted into the facial plane between the internal abdominal oblique and transversus abdominis muscles and then Bupivacaine 0.25% (1 mg/kg, diluted to 20 mL with normal saline) was injected in both sides of the abdomen (Schroeder et al., 2011). For the RS block, with the aid of ultrasound, a spinal needle (22 gauge, 90 mm) was inserted into the plane between the internal rectus sheath and the rectus abdominis muscle, and a bupivacaine 0.25% (0.5 mL/kg) was injected into both sides of the abdominal wall (James et al., 2010).

A single individual (H.I.R) administered all injections, while an experienced veterinarian performed all surgeries. The same investigator (M.K) recorded and measured the data. Intraoperative analgesia assessment involved monitoring parameters such as HR, blood pressure, propofol consumption, and frequency of injected fentanyl. Postoperative analgesia was evaluated using the GCPS-SF, UMPS, VAS, and SDS assessed independently by two individuals blinded to the treatment (MK and MEG). If the pain score exceeded 5/20 or 6/24 in the GCPS-SF or 8 in the UMPS or was greater than 3 in the VAS, fentanyl would be administered intravenously (IV) at a dose of 1mcg/kg, with each animal allowed to be injected up to two times at most. Ketoprofen (1.1 mg/kg; Ketoprofen 1%, Rooyan Daroo, Tehran, Iran) was administered to all animals at the end of the study.

The data were analyzed using GraphPad Prism software version 9.0.0. Agreement between researchers for given pain scores

was checked using the Weighted kappa coefficient. The correlation was evaluated based on Altman's model (1990) as very good (K=0.81-1.00), good (K=0.61-0.80), moderate (K = 0.41-0.60), relatively weak (K=0.21-0.40), and weak (K<0.20). The normal distribution of the data was evaluated by the Shapiro-Wilk test. The Friedman test was employed for statistical analyses. Data were expressed as median (minimum-maximum). The significant level was considered at P<0.05.

Results

All animals tolerated the analgesia, anesthesia, and surgery procedures well and completed the study. Table 1 provides the results of the pain scores after the operation. The inter-rater agreement among the observers was very good (K=0.92).

Table 1: Median (minimum and maximum) of the pain score in the postoperative period in dogs undergoing OHE under constant rate infusion of propofol receiving incisional (Incisional, n=7), transverse abdominal plane (TAP, n=7) and rectus sheath (RS, n=8) blocks

Groups		baseline	30 minutes	1 hour	2 hr	3 hr	4 hr	5 hr	6 hr	*p* value
Composite Measuring Pain Scale-Short Form (GCPS - SF)	Incisional	0 (0-5)	1 (0-7)	1 (0-7)	3 (2-10)	4 (3-11) £	6 (3-11) *, §	6 (5-11) *, £	6 (5-11) *, †, £, §	0.0001>
	TAP	0 (0-3)	1 (0-4)	1 (0-7)	1 (0-8)	3 (0-4) £	3 (0-4) §	4 (1-6) *, £	4 (1-6) *, £, §	0.0066
	RSB	0 (0-2)	1.5 (0-4)	1.5 (0-6)	2 (1-6)	3.5 (1-8) £	4 (0-11) *, §	5 (1-11) *, £	5.5 (3-11) *, £, §	0.0001>
	p value	0.6993	0.9331	0.9261	0.3368	0.5803	0.1223	0.0919	0.0284	
The University of Melbourne Pain Scale (UMPS)	Incisional	0 (0-7)	1 (0-8)	2 (3-9)	5 (3-9) ‡	5.5 (2-9) ‡	8 (4-9) *, ‡	8.5 (5-9) *, †, ‡	9 (5-9) *, †, £, §	0.0001>
	TAP	0 (0-1)	1 (0-7)	1 (0-11)	1 (0-11) ‡	4 (0-7) ‡	4 (1-7) ‡	5 (1-6) *, ‡	5 (1-7) *, £, §	0.0039
	RSB	0 (0-4)	2 (0-4)	2 (0-5)	5 (1-7) *, ‡	5 (1-9) *, ‡	5 (0-9) *, ‡	6 (2-10) *, ‡	7 (4-10) *, £, §	0.0001>
	p value	0.5649	0.8725	0.9845	0.2210	0.3213	0.0504	0.0195	0.0175	
Visual Analogue Scale (VAS)	Incisional	1 (0-3)	1(0-4)	1 (0-4)	2 (1-4) £	2.5 (1-4)	3 (2-5) *	4 (3-5) *, †	4 (3-5) *, †	0.0001>
	TAP	0 (0-3)	1 (0-3)	1 (1-4)	1 (1-2) £	2 (1-3)	2 (0-3)	2 (1-3)	2 (1-3)	0.0657
	RSB	0 (0-1)	1 (0-3)	1.5 (1-3)	2 (1-4) £	2.5 (1-3) *	3 (0-4) *	3 (0-4) *	3.5 (2-4) *	0.0001>
	p value	0.3045	0.9834	0.9163	0.2564	0.4657	0.1825	0.0129	0.0031	
Simple Descriptive Scale (SDS)	Incisional	0 (0-2)	1 (0-3)	1 (1-3)	1.5 (1-3)	2 (1-3) *	3 (2-3) *	2.5 (2-3) *	3 (2-3) *	0.0001>
	TAP	0 (0-1)	0 (0-3)	2 (0-3)	1 (0-3)	2 (0-3)	2 (0-3) *	2 (0-3) *	2 (1-3) *	0.0001>
	RSB	0 (0-1)	0 (0-2)	1 (0-3)	2 (0-3)	2 (1-3) *	2 (1-3) *	2 (1-3) *	3 (2-3) *	0.0012
	p value	0.5802	0.6111	0.9376	0.6578	0.6941	0.6255	0.3148	0.2908	

* Significantly different from baseline (P<0.05), † Significantly different from the TAP treatment (P<0.0.5), ‡ Significantly different from other pain scales (P<0.05), £ Significantly different from the SDS (P<0.05), § Significantly different from the VAS (P<0.05).

Comparison of the pain scores within each treatment showed that with the GCPS-SF, the scores at 4, 5, and 6 hours after surgery in the Incisional were higher than the baseline (P=0.0257, 0.0016, and 0.0010, respectively). The TAP treatment's pain score at 5 and 6 hours after surgery was significantly higher than the baseline (P=0.0090). The pain scores at 4, 5, and 6 hours after surgery in the RSB were also significantly higher than the baseline (P=0.0109, 0.0001, and 0.0001, respectively). In the UMPS, in the Incisional, at 4, 5, and 6 hours after surgery,

the pain score was significantly higher than the baseline (P=0.0102, 0.0011, 0.0007, respectively). In the TAP, at 5 and 6 hours after surgery, the pain score was significantly higher than the baseline (P=0.0109 and 0.0018, respectively). In the RSB, at 2, 3, 4, 5, and 6 hours after surgery, the pain score was significantly higher than the baseline (P=0.0348, 0.0208, 0091, 0.0013, and 0.0001, respectively). With the VAS, the pain score was significantly higher in the Incisional at 4, 5, and 6 hours compared to the baseline (P=0.0393, 0.0055, and 0036, respectively). Also, the pain score in the RSB was higher than the baseline at the 3, 4, 5, and 6 hours after surgery (P=0.0410, 0.0215, 0.0044, and 0.0001, respectively). In the SDS, the pain scores were higher than the baseline values at 3, 4, 5, and 6 hours after surgery (P=0.0025, 0.0036, 0.0017, and 0.0003, respectively). The pain scores in the TAP were significantly higher than baseline at 4, 5, and 6 hours after surgery (P=0.0091, 0.0076, and 0.0007, respectively). In the RSB, the pain scores were higher at 3, 4, 5, and 6 hours after surgery compared to the baseline values (P=0.0254, 0.0025, 0.0009, and 0.0005, respectively).

Comparison of the pain scores among treatments showed that with the GCPS-SF, the pain score at 6 hours after surgery was significantly higher in the Incisional than in the TAP (P=0.0057). In the UMPS, the pain score at 5 and 6 hours after surgery in the Incisional was significantly higher than the TAP (P=0.0209 and 0.0249, respectively). With the VAS, the pain scores at 5 and 6 hours after surgery in the Incisional were significantly higher than in the TAP (P=0.0143 and 0.0065, respectively).

In comparison among the pain scores at 2, 3, 4, and 5 hours after surgery, the UMPS had the highest score than the GCPS-SF, VAS, and SDS (P<0.01). VAS and SDS are different from each other (P<0.01). At 3 hours, there was a significant difference between the GCPS-SF and SDS (P<0.001). At 4 hours after surgery, the GCPS-SF differed significantly from the VAS (P<0.01). At 5 hours, Comparing GCPS-SF and SDS showed a significant difference (P<0.001). At 6 hours after surgery, the UMPS and GCPS-SF had significant differences with the VAS and SDS (P<0.001).

Discussion

The present study used four pain scales and scoring methods to evaluate dog analgesia after ovariohysterectomy. The results showed that all four procedures can be easily performed in dogs. The UMPS detected pain faster than other scales. It appeared that VAS might detect pain faster than GCPS-SF; however, VAS did not show pain in the TAP treatment.

The pain changes the animal's behavioral and physiological responses to reduce or eliminate the possibility of injury and improve recovery. Although there are inter-individual and inter-species differences, some behaviors that change in animals in pain include: behavior patterns, appearance, posture, gait, appetite, and touch response. The difficulty in diagnosing pain has been mentioned as one of the main reasons for not administering enough analgesics (Anil et al, 2002). Testing the validity of pain scales is inherently difficult because there must be a gold standard to which comparisons can be made. VAS is considered the gold standard in humans because it is a self-reporting scale. Since it is impossible for an animal patient to report their pain, a trained observer must do a pain assessment (Williams et al, 2000). Currently, there is no universally accepted gold standard technique for pain assessment in veterinary medicine.

Pain scaling systems can generally be divided into unidimensional and multidimensional methods. In the unidimensional approach like SDS and VAS, the observer should give a pain score to an animal according to their impression. As a result, they are utterly dependent on the observer evaluation which might differ

widely among various individuals. In contrast, multidimensional approaches like GCPS-SF and UMPS use behavior and physiologic parameters to score the pain and are less dependent on the observer. The current study used SDS, VAS, GCPS-SF, and UMPS to evaluate pain in dogs who underwent OHE.

The SDS and VAS methods are designed based on observing dogs' behaviors and physical states. Although heart rate and respiratory rate changes are discussed in the VAS, they have no particular point. The GCPS-SF and UMPS methods are multidimensional scales; however, physiological factors play a more definitive role in the UMPS than GCPS-SF. The GCPS-SF is a modified and simplified form of the Glasgow Composite Measuring Pain Scale (GCPS), introduced by Reid et al. (2007). It has also been stated that the GCPS can differentiate between mild, moderate, and severe pain in both soft and hard tissues (Murrell et al. 2008). For instance, an increase in heart and respiratory rates lead to an increase in the total score in UMPS but have no point in the GCPS-SF (Hernandez-Avalos, 2019).

In the previous investigations, one (Portela et al, 2014; Campoy et al, 2022), two (Carpenter et al, 2004; Fitzpatrick et al, 2010; Campagnol et al, 2012; Cavaco et al, 2022) or three (Lambertini et al, 2018) pain scoring scales were used. Holton et al, (1998) compared VAS, SDS, and NRS to evaluate acute pain in dogs and concluded that the NRS method was the most appropriate clinical method when there was more than one observer; but in total more than one observer can produce a high amount of variability in scores (Carpenter et al, 2004). Carpenter et al, (2004) used VAS and composite pain scale (CPS; based on UMPS) approach and stated that VAS was more reliable than CPS. Campagnol et al, (2012) scaled the pain using VAS and numeric rating scales (NRS) and found that, except for one hour, pain in VAS and NRS was similar. Lambertini et al. (2018) utilized three pain scales, including GCPS-SF, dynamic interactive visual analog scale (DIVAS), and mechanical nociceptive threshold (MNT). They found that GSPS-SF had higher ranges than others, but DIVAS and MNT had no difference. GCPS-SF was the only scale able to see a difference at higher time points. Cavaco et al, (2022) used NRS and GCPS-SF, and they did not find significant differences in the results.

In the present study, UMPS showed a significant difference at 2, 3, 4, and 5 hours after the operation compared to other employed pain scales. The UMPS detected significant differences concerning pain scores at 2 hours after surgery; the other three methods were not so which may indicate that the UMPS can show pain faster than other pain evaluation methods. The SDS showed a significant difference in the Incisional treatments 3 hours after surgery which UMPS and VAS did not confirm. At 3 hours after the operation, the UMPS, VAS, and SDS delivered a significant difference from the baseline, but GCPS-SF did not. It might be because GCPS-SF is more accurate at higher time points, not the first few hours. The SDS showed a significant difference in the Incisional treatments 3 hours after surgery which UMPS and VAS did not confirm. The GCPS-SF, UMPS, and SDS showed significant differences at 4, 5, and 6 hours after the surgery in the three treatments compared to the baseline. The VAS showed no significant difference in the TAP treatments at all time points compared to the baseline which may be due to the ineffectiveness of the VAS in detecting pain in some dogs due to high dependency on the observer evaluation. For pain comparison among treatments, the UMPS and VAS showed significant differences between the Incisional and TAP treatments at 5 and 6 hours, while the GCPS-SF indicated a significant difference only at 6 hours post-operation.

In conclusion, it appeared that all four employed pain scale methods could be used relatively easily in dogs that underwent OHE. Although all four methods can be used reliably, it seems that the UMPS might detect the pain earlier and is a more sensitive method during 6 hours after the surgery compared to the other three methods.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Prevalence of subclinical streptococcal mastitis in dairy cows in Chaharmahal and Bakhtiari province

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Abstract

Mastitis is an inflammatory disease of the mammary gland, caused by many infectious agents such as bacteria, fungi, and viruses. Streptococci are reported to be among the major pathogens causing bovine mastitis around the world, which may cause clinical and subclinical forms of mastitis. Mastitis is one of the primary diseases of dairy cows and is responsible for remarkable economic losses due to reduction in quantity and quality of milk, cost of treatment, and the early culling of the cows. Considering the existence of substantial industrial and traditional dairy cattle farms in Chaharmahal and Bakhtiari province and the fact that mastitis is the most common disease in dairy industries, this study aimed to identify the role of streptococci in subclinical mastitis in dairy cows in Chaharmahal and Bakhtiari province. For this purpose,134 subclinical mastitis milk samples were collected from 8 dairy farms in Chaharmahal and Bakhtiari province, based on the california mastitis test (CMT) results and screened for the streptococcal cause of mastitis. DNAs were extracted from collected specimens and PCR was performed with specific primers for *Streptococcus agalactiae*, *Streptococcus uberis*, and *Streptococcus dysgalactiae*. Out of 134 mastitis milk samples 10 (7.5%), 14 (10.4%), and 5 (3.7%) samples were positive for *S. agalactiae*, *S. uberis*, and *S. dysgalactiae* respectively. The result of this research shows that 21.6 (29:134) percent of mastitis in dairy cattle farms in the studied region may be due to streptococci. The obtained data can be used in management and prevention strategies for cattle mastitis control in Chaharmahal and Bakhtiari province.

Key words: Mastitis, *Streptococcus*, Cattle, PCR, Chaharmahal and Bakhtiari

Introduction

Mastitis, characterized by the inflammation of the mammary gland, is a pervasive and economically significant disease affecting dairy cows worldwide (Libera et al, 2021). As the most common infectious ailment in the farm industry, it poses substantial challenges, necessitating a comprehensive understanding of its etiology, classification, symptoms, economic implications, and diagnostic methodologies. The mammary gland's vulnerability to infection is heightened

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during the post-milking period when the teat canal remains open for 1-2 hours. This path of susceptibility is crucial, as it correlates with a reduction in local antimicrobial protection, creating an opportune environment for pathogens to invade (Reshetka, 2013; Zverzhanovskiy et al, 2017; Serdyuchenko et al, 2018; Sobol et al, 2017; Zykova et al, 2018; Donnik et al, 2017; Ruegg, 2017; Phuektes et al, 2001).

Although viruses, fungi, and algae are acknowledged as potential mastitis causes, the predominant cause of bovine mastitis is the invasion of the udder by pathogenic bacteria. Among the lot of bacterial strains associated with mastitis, *Staphylococcus aureus, S. agalactiae, S. dysgalactiae,* and *S. uberis* stand out as the most common causes (Phuektes et al, 2001). Streptococci causing mastitis are classified into contagious and environmental groups (Cheng and Hen, 2020). Contagious pathogens are adept at surviving within the host and spreading among cows during the milking process, facilitating easy dissemination within the herd. On the other hand, environmental pathogens can survive outside the host and are part of the cow's normal microflora (Hogan and Smith, 2003). *S. agalactiae* is categorized as a contagious pathogen, *S. uberis* is considered environmental, and instances of contagion are observed. The classification of *S. dysgalactiae* as either environmental or contagious remains a subject of ongoing investigation, underscoring the complexity of mastitis pathogenesis (Oliver et al, 2003).

The clinical presentation of mastitis in cows encompasses a spectrum of symptoms, including udder swelling, heat, pain, abnormal milk appearance, raised body temperature, lethargy, and anorexia (Heringstad et al, 2000; Kibebew, 2017). Mastitis is stratified into three classes - clinical, subclinical, and chronic (Cheng and Hen, 2020). Clinical mastitis is characterized by visible abnormalities in both the cow and the milk. Changes are limited to alterations in milk yield and somatic cell count, distinguishing it from subclinical mastitis. Subclinical mastitis is estimated to occur at rates 15–40 times higher than clinical mastitis. Lack of visible clinical symptoms is one of the challenges of timely diagnosis of this type of mastitis (Martin et al, 2018).

The economic ramifications of mastitis encompass the costs of treatment and veterinary interventions, reduced milk production, and the culling of livestock. In addition to economic costs, it affects the overall productivity and profitability of dairy products (Sordelli et al., 2000). Consequently, there is an urgent need for correct and timely treatment, necessitating fast and reliable diagnostic tools for mastitis (Godkin et al, 1993).

Traditional diagnostic methods such as microbial culture have historically been employed for mastitis diagnosis. However, their time-consuming nature prompted the adoption of molecular methods, including polymerase chain reaction (PCR), as valid alternatives with high sensitivity and specificity (Hassan et al, 2001). The PCR method offers a swift and reliable option for identifying bacterial pathogens. It enables the recognition of pathogens within hours, compared to the days required by traditional cultural methods. The heightened sensitivity of PCR allows for the detection of pathogens in the early stages of infection and in the carrier animals, even when bacterial concentrations in milk are minimal. However, the excessive sensitivity of PCR lies in its susceptibility to misdiagnosis due to minor contaminants in samples (Phuektes et al, 2001).

This study aims to investigate the prevalences of bovine streptococcal (*S. dysgalactiae, S. uberis,* and *S. agalactiae*) subclinical mastitis, in Chaharmahal and Bakhtiari province, by PCR method. By considering the prevalence of these streptococci, the research endeavors to provide valuable insights into the epidemiology and management of this prevalent dairy cattle ailment.

Materials and Methods

From June to September 2023, 134 subclinical mastitis milk samples were collected from eight distinct dairy farms located in Chaharmahal and Bakhtiari province. Mastitis suspected samples were collected based on CMT. Following the exclusion of the initial three milkings from each quarter, starting from the fourth milking, approximately 2-3 ml of milk was deposited into the corresponding compartment of the CMT container and the CMT procedure was conducted according to the company (Bovivet, Denmark) instructions.

The CMT positive samples were collected in sterilized test tubes and subsequently transported to the laboratory for further analysis. The samples underwent a centrifugation process at 6000 rpm for 30 minutes. The resulting sediments were then earmarked for molecular testing.

DNA was extracted from all 134 subclinical milk samples, and also positive controls including *S. agalactiae* (PTCC:1768), *S. uberis* (IBRC-M 10804), and *S. dysgalactiae* (PTCC:1236) using DNP Kit EX6071 (Sinaclon, Iran) following the specified instructions meticulously. Briefly, 1 ml of each milk sample was centrifuged at 3000 rpm for 10 minutes and then 100 µl protease buffer and 5 µl of protease were added to the precipitate and vortexed and incubated at 55° C for 30 minutes. After that, 400 µl of lysis solution was added to it and homogenized by vortexing for 15-20 seconds. In the next step, 300 µl of precipitation solution was added and mixed by vortexing for 5 seconds. The lysed sample was centrifuged at 12000 rpm for 10 minutes. The supernatant was decanted by gently inverting the tube and placing the tube on tissue paper for 2-3 seconds. Washing of precipitate was done with the addition of 1 ml of wash buffer and mix by 3-5 seconds vortexing. In the next step, the sample was centrifuged at 12000 rpm for 5 min and the supernatant was poured off and the pellet was dried at 65° C for 5 minutes. The pellet was suspended in 50µl of solvent by gentle shaking and placing at 65° C for 5 min. The purified DNA was harvested by centrifugation at 12000 rpm for 30 seconds and the supernatant was stored at -70 ℃ and used as a template in the PCR reaction.

The concentration of extracted DNA was calculated at a wavelength of 260/280 nm using a nanodrop spectrophotometer (Eppendorf, Germany). Pure DNA was defined as samples having 260/280 absorbance ratios of less than 1.8.

A pivotal aspect of this study accomplished the Polymerase Chain Reaction (PCR) method. In this process, a total volume of 20 µL was employed for DNA amplification. This comprised 10 µL of PCR 2x Master Mix (GeneDireX, Inc., Taiwan), 1µL (10 picomole) of each of forward and reverse primers supplied by Metabion in Germany, 3µL of template DNA, and 5µL of nuclease-free water. The sequence of selected primers, as outlined in Table 1, played a crucial role in targeting specific genetic sequences associated with *S. agalactiae*, *S. uberis* and *S. dysgalactiae*. In each PCR reaction along with samples, DNA from the above-mentioned strains was used as positive control and distilled water as a negative control.

The thermal cycling conditions for the PCR process were meticulously optimized for each streptococcal species. For *S. agalactiae* and *S. uberis*, the cycling involved an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. In the case of *S. dysgalactiae*, the cycling conditions were adapted, incorporating an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes.

The PCR products were analyzed through 1.5% agarose gel electrophoresis. This meticulous step, post-staining with a safe stain (Yekta Tajhiz Azma, Iran) and visualization under UV light (Uvitec, England), served as the cornerstone for confirming the success of DNA amplification and providing a visual representation of the molecular composition of the mastitis-causing streptococcal species in the examined samples.

Table 1: The sequence of primers used in the PCR

Species	Sequence (5′–3′)	PCR product size (bp)	Reference
S. agalactiae	F: AAGGAAACCTGCCATTTG R: TAACCTAGTTTCTTTAAAACTAGAA	270	(Phuektes et al., 2001)
S. dysgalactiae	F: GAACACGTTAGGGTCGTC R: AGTATATCTTAACTAGAAAAACTATTG	264	(Phuektes et al., 2019)
S. uberis	F: CGCATGACAATAGGGTACA R: GCCTTTAACTTCAGACTTATCA	445	(Momtaz et al., 2012)

The statistical analysis of the obtained data was conducted through a descriptive analysis utilizing Microsoft Excel (Microsoft Corporation, 2018).

Result

Based on the CMT performed on milk samples, all collected samples were positive. According to the PCR results, 10 (7.5%) samples were positive for *S. agalactiae*, 14 (10.4%) samples were positive for *S. uberis*, and 5 (3.7%) samples were positive for *S. dysgalactiae* (figure 1).

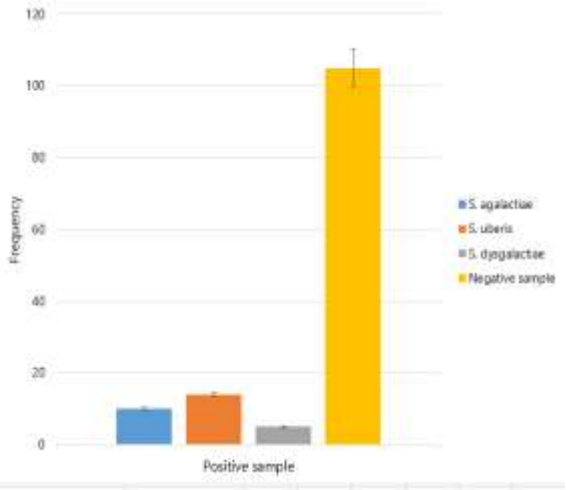

Figure 1: Frequency chart of bovine streptococcal mastitis in 134 individual mastitic millk samples from Chaharmahal and Bakhtiari province.

Figure 2: Agarose gel electrophoresis of the *S. agalactiae* PCR products. Lane 1: 100 bp DNA marker; lane 2: positive control (270 bp); lane 3: negative control and lane 4 is a positive sample.

Figure 3: Agarose gel electrophoresis of the *S. uberis* PCR products. Lane 1: 100 bp DNA marker; lane 2: positive control (445 bp); lane 3: negative control and lane 4 a positive sample.

Figure 4: Figure 2. Agarose gel electrophoresis of the *S. dysgalactiae* PCR products. Lane 1: 100 bp DNA marker; lane 2: positive control (264 bp); lane 3 and 4 two positive sample and lane 4: negative control.

Discussion

Mastitis is the most prevalent disease affecting dairy cows worldwide (Ziv, 1992). Subclinical mastitis is one of the most important diseases causing substantial economic loss to the farmers and the dairy industries due to the long-term effects of chronic infections (Abdella, 1996) and also through decrease in milk quality and quantity. Subclinical mastitis in dairy cattle is a chief and silent problem whose early detection is critical to prevent its associated economic losses and also to make decisions for its rapid and effective treatment (Chagunda et al, 2006). Laboratory methods should be used to identify it because mostly remains unnoticed by the farmer (Singh et al, 2008). Streptococci are among important cow subclinical as well as clinical mastitis bacterial agents (Kibebew, 2017); so, the present research was conducted to detect the importance of streptococcal subclinical dairy cows mastitis in Chaharmahal and Bakhtiari province. In line with the findings of various researchers, our study underscores the role of streptococci as causative agents in subclinical cases of cattle mastitis.

The role of specific streptococcal species in subclinical mastitis has been explored in several studies, providing valuable insights into the diverse microbial landscape associated with this bovine ailment. In agreement with our results, Phuektes et al, (2001), in their research for developing a multiplex PCR assay for the simultaneous detection of the four major bacterial causes of bovine mastitis including *S. agalactiae*, *S. aureus S. dysgalactiae* and *S. uberis* reported that these agents play respective roles in 13.7%, 3.4%, 1.7% and 0.85% of bovine subclinical mastitis. Moatemedi et al, (2007) revealed the role of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* respectively in 20%, 12.5% and 0.83% of subclinical mastitis in dairy cattle in Ahvaz. Ehsani et al, (2024) reported that the contamination rates of bulk tank milk with *S. uberis* in Isfahan were 16% and 20% as determined by culture and RT-PCR methods, respectively. Comparing their results with the present study, the role of *S. uberis* was more prominent than those of the two streptococci. This discrepancy may be due to the difference in time, region, sampling season and the type of milk (bulk tank and individual sample) in the two studies (Song et al, 2020). In accordance with our results, Emadi et al, (2013) also reported that *S. uberis* is involved in 6.6% of subclinical mastitic cow milk samples in Tehran province.

Molecular methods have created a fundamental revolution in the detection and identification of microorganisms; these methods are simple, low-cost and highly sensitive (Rezazadeh Zarandi et al, 2017). Nithin Prabhu et al, (2013) reported that the PCR method can be successfully used for the identification of the major mastitis caused streptococci, especially *S. agalactiae*, *S. dysgalactiae*, and *S. uberis*. Vojgani et al, (2006) investigated the contamination of the bulk milk tanks of livestock farms with mastitis ascociated pathogens by using universal primers and reported that the use of this primer was able to identify the main cause of mastitis. Soltao et al, (2017) highlighted the utility of PCR assays for identifying mastitis pathogens in bulk tank milk. While our study focused on

individual cow samples, the insight from bulk tank milk examinations emphasizes the potential of repeated testing as a valuable monitoring tool, particularly when considering *S. dysgalactiae, S. agalactiae*, and *S. uberis*. Wang et al, (2016) investigated the frequency of mastitis-causing pathogens in bulk tank milk and reported that *S. aureus*, *S. agalactiae, S. dysgalactiae* and *Trueperella pyogenes* are the most frequently detected pathogens in bulk tank milk samples.

Economically, bovine mastitis incurs significant disadvantages in livestock production. The compromised milk quality and quantity directly impact the profitability of dairy operations. Timely diagnosis and intervention are essential to curtail economic losses associated with decreased milk yield, veterinary expenses, and potential culling of infected animals. Implementing preventive measures based on accurate diagnostic tools can contribute to substantial economic benefits by minimizing treatment costs, preserving milk production, and maintaining the overall health of the dairy herd (Wang et al, 2016; Kibebew, 2017).

In conclusion, our research reinforces the significant contribution of streptococci, particularly *S. uberis.* and *S. agalactiae* to subclinical cases of bovine mastitis in Chaharmahal-Bakhtiari. Regular testing, coupled with preventive measures, is crucial for controlling the incidence of mastitis in this region. The PCR-based test demonstrated specificity in identifying and determining the prevalence of these bacterial pathogens. The economic advantages of timely diagnosis and intervention cannot be overstated, as they play a pivotal role in sustaining the economic viability of dairy operations.

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Conflict of interest

The authors declare no conflict of interest.

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Radiographic evaluation of bone disorders in referred dogs to Veterinary Hospital of Shahid Chamran University of Ahvaz

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Abstract

Skeletal disorders are included in companion animals relatively significant percentage between referred cases to the Hospital or Clinic in every region. The major skeletal problems have been reported among growing young dogs and large breeds; while small breed dogs are prone to some bone diseases. Lack of balanced nutrition (especially for calcium and phosphorus) is one of the effective factors in the arising of bone disorders. The aim of the present study was to determine the incidence and types of bone defects such as fractures, infections, neoplasia and other skeletal acquired complications in the limb organs, head and vertebral column. The present survey was done during eleven years (2004 to 2014), based on the prepared radiographs in Veterinary Hospital of Shahid Chamran University of Ahvaz; in the following, factors such as age, gender, breed and location were detected for their relationship with bone complications. In this study, bone disorders were detected such as fractures, osteomyelitis and osteoarthritis, neoplasias, dislocations in dogs and other complications like panosteitis, osteochondrosis and osteodystrophy in young animals. The results are presented as descriptive statistics. A total of 4355 referred cases to Radiology Department, 1054 cases (24.20%) were related to dogs. Out of these, 425 cases (40.32%) had skeletal disorders, out of which 46.59% and 53.41% were related to large and small breeds respectively. Skeletal disorders included fractures, luxations and other complications. The most important of these cases were radial fracture (26.71%), femur (28.34%), tibia (22.46%) and ulna (27.95%). The age of the studied animal, were in the range of two months to nine years-old. In term of gender, 62.35% of the dogs were male and 37.65% female. No significant difference was seen for age between mature (51.29%) and immature (48.71%), gender, location and breeds (large and small) statistically. In conclusion, the highest incidence of skeletal disorders was femural (28.34%) and ulna (27.95%) fractures, respectively. The obtained results showed that radiography is a valuable method to recognize skeletal disorders and the detection of the frequency in dogs.

Key words: Radiography, Bone disorders, Fracture, Dog, Ahvaz

Introduction

Bone disorders are included a relatively significant percentage of referred dogs to hospitals or Veterinary clinics in each region. Orthopedic disorders consiste of hip dysplasia and osteochondrosis, panosteitis, hypertrophic osteodystrophy, dislocation of patellar joint, non-infectious necrosis of the femural head, intervertebral disc diseases and rickets in small animals. Most bone problems have been reported in growing young dogs of large breeds, while small breed dogs are also prone to some other

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bone diseases. Imbalance in the diet (especially in terms of calcium and phosphorus) is considered as one of the effective factors in causing bone diseases. Bone diseases can be controlled by modifying the diet to some extent. Food control for prevention of weight gain and the balance of minerals, vitamins, protein and other indices, play a role in control of animal weight, is a very important issue in pets (Hazewinkel, 2005).

Incidence of bone fracture in dogs has been reported differently. In our country, Irans, eferred cases of bone problems always constitute a relatively significant percentage of dogs to hospitals and other therapeutic centers. The clinical signs can be varied according to the bone which is affected and may be characterized as lameness, swollen and bleeding (Smith et al, 2001; Vandenberg et al, 2013).

It is important that studies of the skeleton be made in standard positions. The standard views for limb bones are craniocaudal (dorsopalmar, dorsoplantar) and mediolateral. At least two views, taken at right angles to one another, are required for proper evaluation of the status of a bone. It is necessary to know the positions of the various centers of ossification in the young animals and the times at which the physes close. Young animals appear to have very wide joint spaces, because the cartilaginous models on which the epiphyses and the small bones of the carpus and tarsus are developing. Growth is completed in dogs by aproximately 10 to 14 months of age. However, considerable variations may occur in the times of physeal closure, even in animals of the same breed. In the long bones, the proximal humeral epiphysis is the last to mineralize. The pelvic symphysis may not fuse for several years. Variations occur in the appearance of bones in some breeds, such as chondrodystrophic animals (Kealy, 2010).

There are several principle causes of bone fracture in dogs and cats, such as road traffic accident, falling from heights, human abuse, animal biting, indoor trauma and unknown trauma. Several studies had reported that the most common cause of bone fracture was road traffic accident accidents (Libardoni et al, 2016; Uwagie-Ero et al, 2018). Incidence in epidemiology is a measure of the probability of occurrence of a given medical condition in a population within a specified period of time. Considering the practical aspects of the current investigation and the emphasis on conducting applied research, the researchers' prediction is that by conducting a comprehensive survey in this field, unknown aspects of orthopedic problems in dogs are identified; therefore, the aim of the present study was to determine the incidence and types of bone defects such as fractures, infections, neoplasia and other skeletal acquired complications in the limb organs, head and vertebral column. The present study was done during eleven years from early 2004 to late 2014, based on the prepared radiographs in Veterinary Hospital of Shahid Chamran University of Ahvaz; for this purpose the factors such as age, gender, breed and location were detected for their relationship with bone complications also.

Materials and methods

Ethical approval

This survey was approved by the Animal Care and Research Committee of Shahid Chamran University of Ahvaz. It was conducted based on the Guidelines for Animal Care and Use (Ethical code: 95581148).

Data collection

Data were collected from the owner of medical records at referral Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz. Searches were made on all skeletal disorders as a retrospective study during eleven years (from 2004 to 2014). Dogs with skeletal complication were confirmed by the history, clinical, orthopedic, and

radiographic examinations, then were submitted to surgery department which was not included in this survey.

During this time, a total of 4355 referred cases to Radiology Department, 1054 cases (24.20%) were related to dogs. Of these, 425 cases (40.32%) had skeletal disorders. In the meantime, 46.59% and 53.41% were related to large and small breeds respectively. The age of the studied animals were in the range of two months to nine years-old and were divided as into mature and immature. In this study, 62.35% of the dogs were male and 37.65% female. The percentage of skeletal lesions was calculated and then the disorders such as types of fractures and their diversity in bone, osteomyelitis, malignant, osteoarthritis, and dislocation of joints, and in young dogs complication such as panosteitis, osteochondrosis and osteodystrophy (hypertrophic and fibrosis) were in vestigated. Fractures were classified according to sources (Shales, 2008). Classification of bone fracture had been done based on the body parts, and specific bone fractures which included: A-Incidence of specific limb (forelimbs / hind limbs) fractures, B- Incidence of specific appendicular bone fractures. Forelimb (Humerus, radius and ulna), Hindlimb (Femur, tibia and fibula), and the number of the other bone fractures were also recorded.

It should be noted that in the first stage, while taking accurate statistics of the number of referred cases to the Radiology department, radiographs related to bone complications were separated from the rest of the files in dogs. In addition, the files without complete owner information or radiographs with inappropriate and uninterpretable quality were not used in this research. To review the radiology images, regardless of the radiologist's previous diagnosis, all the images were re-examined and interpreted, and their diagnosis was recorded. Then the profile registration forms were reviewed and finally matched with the new diagnosis.

Statistical test

Data about age, sex, and breeds were collected, and then were added to the Microsoft Excel, stored separately and exported to analytical software using the Chi-square test. Values of $P \leq 0.05$ were considered as statistically significant.

Results

In the present study, there were 4355 referral files from different departments of the hospital to the radiology department within a range of 11 years. Of these, 425 cases (40.32%) had skeletal disorders. In the meantime, 46.59% and 53.41% were related to large and small breeds respectively. Skeletal disorders included fractures, luxations and other complications. The most important of these cases were radial fracture (27.95%), femur (22.46%), tibia (17.11%) and ulna (1.07%). The age of the studied animals, was in the range of two months to nine years-old. In this study, 62.35% of the dogs were male and 37.65% female. Statistically no significant difference was seen for age between mature (51.29%) and immature (48.71%), gender, location and breed (large and small) ($P>0.05$).

By reviewing the documents, the used foods included: homemade, commercial and mixed foods. Since in most cases, there was a combination of the above food types in the diet of dogs, it was not possible to analyze them accurately; therefore, it was not possible to investigate the effects of diet on the skeletal disorders.

In table 1, 374 cases (88%) were related to fractures, 6 cases (1.41%) with tumors or osteomyelitis, 6 cases (1.41%) with orthopedic diseases of young and growing dogs and 39 other cases (9.18%) with articular complications. Out of 183 cases, 161 (87.98%) were related to fractures in fore limb, 187 cases (90.78%) in hind limb, and 26 cases (72.22%) in head and vertebral column. The highest fracture disorder was in hind limb and the lowest one in head and

vertebral column. More details are given in the section below (Table 1).

Types of disorders and skeletal complications are shown in fore limb in Table 2. Out of 183 cases of bone disorders related to the fore limb, 15 cases (9.32%) were observed in scapula, 39 cases (24.22%) in Humerus, 43 (26.71%) in radius, 45 cases (27.95 %) in ulna, three cases (1.86 %) in carp, six cases (3.73%) in metacarpus and 10 (6.21%) in digits. The highest complication was in ulna (27.95%) and the lowest one in carp (1.86%) in the fore limb. More details are given in the section below (Table 2).

Table 1: Types of disorders and bone complications in different organs during 11 years (2004-2014) in referred dogs to Veterinary Hospital of Shahid Chamran University of Ahvaz

Bone disorders / organ	Fractures	Tumors and osteomyelitis	Orthopedic diseases in young and growing dogs	joints	Total
Fore limb	161 (87.98%) Aa	3 (1.64 %) Ac	6 (3.28%) Abc	13 (7.10%) Ab	183
Hind limb	187 (90.78%) Aa	2 (0.97%) Ac	-	17 (8.25%) Ab	206
Head and vertebral column	26 (72.22%) Ba	1 (2.78%) Ac	-	9 (25%) Ab	36
Total	374	6	6	39	425

The difference in uppercase letters indicates the existence of a significant difference between the indicates of each column. The difference in lowercase letters indicates a significant difference in each row.

Table 2: Types of disorders and bone complications in forelimb during 11 years (2004-2014) in dogs referred to Veterinary Hospital of Shahid Chamran University of Ahvaz

Bone complications / organ	Scapula	Humerus	Radius	Ulna	Carp	Metacarpus	Digits	Total
fractures	15 (9.32%) Ab	39 (24.22%) Aa	43 (26.71%) Aa	45 (27.95%) Aa	3 (1.86%) ABd	6 (3.73%) Acd	10 (6.21%) Abc	161
Tumors and osteomyelitis	-	2 (66.67%) Ba	-	-	1 (33.33%) Ba	-	-	3
Orthopedic diseases of young and growing dogs	-	2 (33.33%) Ba	3 (50%) Ba	1 (16.67%) Ba	-	-	-	6
joints	1 (7.69%) Bb	4 (30.77%) Bab	-	-	6 (46.15%) Aa	-	2 (15.38%) Bab	13
Total	16	47	46	46	10	6	12	183

The difference in uppercase letters indicates the existence of a significant difference between the indicates of each column. The difference in lowercase letters indicates a significant difference in each row.

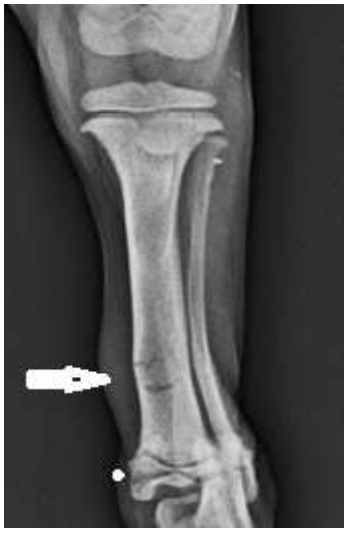

Figure 1: Incomplete fracture in tibia

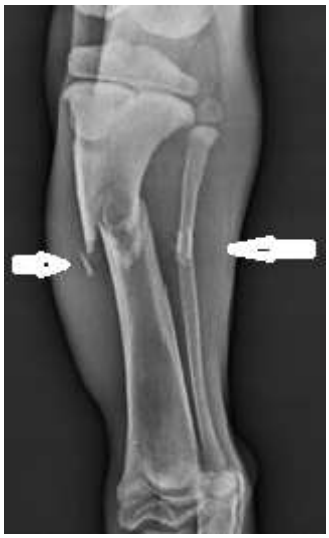

Figure 2: complete fracture in tibia and fibula

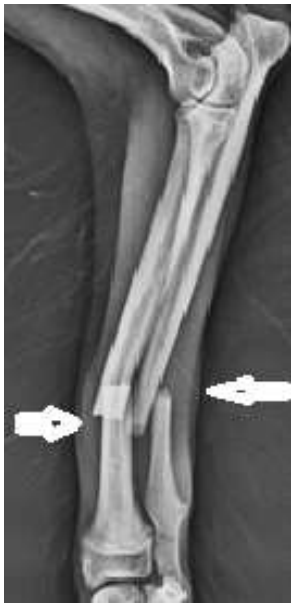

Figure 3: Overriding fracture in radius and ulna

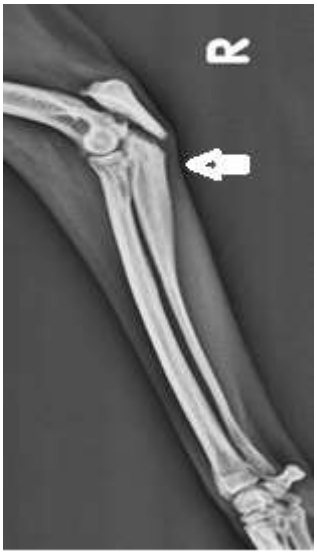

Figure 4: fracture in olecranon

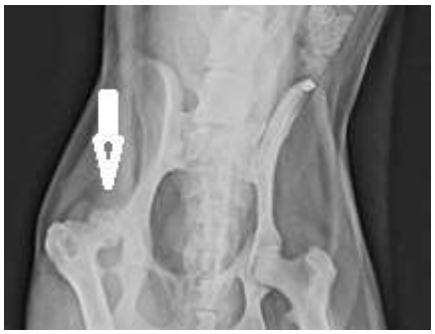

Figure 5: Luxation in right hip joint

Types of disorders and bone complications are observed in hindlimb in the studied dogs. Out of 206 cases of bone complications related to hind limb, 12 cases (6.42 %) were observed in ilium, 15 cases (8.02%) in ischium, 14 cases (7.49%) in pubis , 5 cases (2.67 %) in pelvic symphysis, 53 cases (28.34%) in femur, 42 cases (22.46%) in tibia, 32 cases (17.11 %) in fibula, two cases (1.07 %) in tarsus, four cases (2.14 %) in metatarsus and eight cases (4.29 %) in digits. The highest complication was observed in femur (28.34%) and the lowest one in tarsus (1.07%). Regarding the joint complications17 cases, of which 9 cases (52.94%) were related to the hip joint, where about 50% (4 case) of the complications of the mentioned joint are related to complete and incomplete dislocation of this joint. More details are given in the section below (Table 3).

Table 3: Types of disorders and bone complications in hindlimb during 11 years (2004-2014) in dogs referred to Veterinary Hospital of Shahid Chamran University of Ahvaz

Bone complications / Organ	Ileum	Ischium	Pubis	Symphysis	Femur	Tibia	Fibula	Tarsus	Metatarsus	Digits	Total
Fractures	12 (6.42%) Acd	15 (8.02%) Ac	14 (7.49%) Ac	5 (2.67%) Adc	53 (28.34%) Aa	42 (22.46%) Aab	32 (17.11%) Ab	2 (1.07%) Ac	4 (2.14%) Ac	8 (4.29%) Acde	187
Tumors and osteomyelitis	-	-	-	-	-	1 (50%) Ba	-	-	1 (50%) Aa	-	2
Joints	Hip 9 (52.94%) Aa	Stifle 5 (29.41%) Bab	-	-	-	-	-	3 (17.65%) Ab	-	-	17
Total	21	20	14	5	53	43	32	5	5	8	206

The difference in uppercase letters indicates the existence of a significant difference between the indicates of each column. The difference in lowercase letters indicates a significant difference in each row.

Table 4: Types of disorders and bone complications in skull and vertebral column during 11 years (2004-2014) in dogs referred to Veterinary Hospital of Shahid Chamran University of Ahvaz

Bone complications / organ	Skull	Maxilla	Mandible	Face	Vertebrae				Total
					Neck	Thorex	Lumbar	Tail	
Fractures	-	6 (23.08%) Aa	4 (15.38%) Aa	2 (7.69%) Aa	-	5 (19.23%) Aa	6 (23.08%) Aa	3 (11.54%) Aa	26
Tumors and osteomyelitis	-	-	1 (100%) Aa -	-	-	-	-	-	1
Joints	-	-	-	-	-	3 (33.33%) Aa	6 (66.67%) Aa	-	9
Total	-	6	5	2	-	8	12	3	36

The difference in uppercase letters indicates the existence of a significant difference between the indicates of each column. The difference in lowercase letters indicates a significant difference in each row.

Out of the thirty six cases of bone complications for the skull and vertebral column, 26 cases were fractures, one case tumors and osteomyelitis (in mandible), and 9 cases joint complications. Out of 26 fractures, 6 cases (23.08%) were related to the maxilla, 4 cases (15.38%) the mandible, 2 cases (7.69%) face, 5 cases (19.23%) thorax vertebrae, 6 cases (16.67%) lumbar vertebrae and 3 cases (8.33%) tail vertebrae. Tumors and osteomyelitis were 1 case (100 %) in the mandibl bone. Out of the nine of the bone complications for the joints, 3 cases (8.33%) were in the thorax vertebrae and 6 cases (16.67%) in the lumbar vertebrae.

Discussion

The obtained results showed that the highest incidence of skeletal disorders in the population of referral dogs in Ahvaz region was femural and ulna fractures at 28.34% and 27.95%, respectively. Considering that Veterinary Hospital of Ahvaz is the only specialized center for companion animal diseases in Khuzestan province, most cases of accidents and bone diseases refer to this center (even those cases that go to outside clinics) due to the lack of diagnostic equipment; so the results are considered with a large extent to indicate that the prevalence of skeletal complications is relatively high in dog's population of Ahvaz region.

Most of the articles are as case reports in the field of bone complications in pets, including dogs, both in Iran and other countries. Harris and Langley-Hobbs (2013) reported a case of idiopathic ischemic necrosis in the carpal bone of a dog. The affected dog was six years-old, female, ovariohysterectomized and was of Mixed breed. The animal was referred with a 6-week history of lameness. Radiographic findings confirmed bone complications (bone lysis) in the involved bone. Kishimoto et al, (2009) examined the femural-pelvic joint in 22 healthy Border collie dogs. In their study, mean values of dorsolateral subluxation score was 45.7% (±10.2%) using CT-scan technique. In the present study, about 50% of joint complications are related to complete or incomplete dislocation of the hip joint.

Ghadiri et al, (2011) reported a case of Lumbosacral Transitional Vertebra (LTV) Type-3 in a German shepherd dog. In their research, the affected dog was seven years-old and was referred to Veterinary Hospital of Ahvaz with a 2-week history of intermittent lameness and pain in the sacro-lumbar area. In another research, Ghadiri et al, (2007) reported the radiographic findings of HOD in a Mixed breed puppy. They reported HOD-related lesions consisting of new periosteal bone formation around the distal metaphysis of the radius, ulna, and tibia. Soroori et al, (2012), reported that among 1896 cases referred to the radiology department 49 dogs were suffering from osteoarthritis complications. In their research, it was shown that in 15 cases of the studied dogs, there was complications in the vertebral column and in 34 other cases, the disorders were in forelimb and hindlimb organs. In addition, bone complications were reported in large breed more than small breed dogs. Old age and overweight were reported as two important and predisposing factors in the development of degenerative changes in joints.

Chalmers et al, (2006) used the method of bone density determination in the femur head to diagnose osteoarthritis in the early stages. In another study, Smith et al, (2001), investigated the effect of various factors such as age, gender, breed and weight on the occurrence of radiographic signs of osteoarthrosis in the Pennsylvania Veterinary College. It was determined that weight was the most important risk factor in the development of osteoarthrosis in all breeds. But the gender was not a determining factor in the occurrence of complications. Also, the level of infection in the German shepherd breed was 4 times higher than other breeds. Barder et al, (1983), in a study conducted on 130 cases of humerus fractures in dogs and cats, reported that the percentage of fractures in different parts of the humerus was as follows: four percent in the proximal part, 47% in the body, 12% in the condyles and 37% in the distal part of the bone.

Pelvic fractures are relatively common in Veterinary Medicine, and in some reports, it accounts for 20-30% of fractures. In addition, several cases of pelvic fractures are related to multiple fractures. Also, 59 to 83 percent of hip dislocations have been reported in connection with trauma (Unger et al., 1990). In Veterinary reports, 20-25% of fractures have been reported in femur bone. In stifle joint dislocations, 75 to 80% of dislocations were occurred in the medial region (Unger et al., 1990). Tibia fractures are relatively common in dogs and cats also, and up to 21% of fractures are related to long bone (Unger et al, 1990). In the present study, the percentage of fractures in different parts of the pelvis was 24.60 percent which is consistent with the above results. In the present study, the percent of femoral fractures was obtained 28.34% which is relatively similar with other researche results.

Eatezadi et al, (2006), conducted a retrospective study of dog hindlimb fractures by radiology. In the above study, in total, 187 cases of different fractures

were investigated, seventiy four cases were in the femur, 50 cases in the tibia, 25 cases in the fibula, 21 cases in the pubic bone and 17 cases in the ilium. Also, in their study, it was found that a high percentage of fractures in immature animals and the highest incidence of fractures were in the femur. In the present study, the highest incidence of skeletal disorders was in femur bone at 28.34% that was similar with results of above research.

In another research by the Keosengthong et al, (2019), the incidence of bone fractures was 1.7% and 1.1% in dogs and cats, respectively. Regarding the breeds in both the dogs and cats, mongrel breed were the most affected at 40.6% and 66.3%, respectively. Abo-Soliman et al, (2020) showed that male dogs and cats had a higher incidence than females, as well as, the highest records of fractures were in mongrel breed dogs and cats. The bone fracture mostly occurred in dogs younger than one-year-old, and in cats with age range of one to three years.

Laflamme (2001) and Linda et al, (2019) showed that excessive dietary calcium can cause bone deformities and even cause deficiency in other nutrients, including zinc. Tryfonidou et al, (2002) showed that excessive caloric intake and disproportionate amounts of calcium affect skeletal diseases, especially in large breed puppies. These researchers showed that if the amount of calcium is higher than 3% of the dry matter of the diet, it can lead to bone deformities in dogs. Hazewinkel, (2005) reported that the ratio of calcium to phosphorus should be in the range of 1 to 1.5, but at the same time, the absolute amounts of each mineral are more important than the above ratio. For example, if the absorption of energy is not controlled in the diet, or if the amount of minerals in the diet is not within the normal range, it causes development of bone diseases. It should be noted that other factors such as genetics, exercise, and trauma play an important role in causing bone injuries, especially in puppies (Stockman et al, 2013).

In a study by Kitshoff et al, (2013), 109 dogs were reviewed with 135 mandibular fractures. Small breed dogs and dogs less than eight months of age predominated (102 cases). Dog fights were the most common aetiology in their study (68). The molar region was the most commonly affected region (56). Evaluation of the radiographs revealed that transverse, relatively unstable, and displaced fractures were the most common regiods. The majority of fractures involved teeth in the fracture line, with the first molar frequently involved. The majority of fractures were open. Eliasi et al. (2014) conducted a survey on the radiographic files of the population of dogs referred to the radiology department in the field of breed, age and gender factors on the complication of pelvic-femural joint dysplasia (2011-2013). The incidence of hip dysplasia in large breed dogs (German shepherd and Doberman pinscher) was more common than in small breed dogs (Perkins and Dachshund). In regard gender, they were seen more in females, and for age, it was seen more in range of 5 months to 2 years. There were no differences between different groups for age, gender and breed. In another survey, Samani et al, (2014) worked on cats (40 cases) referred to Veterinary Hospital of Ahvaz University. They observed that most fracture problems were related to lumbo-sacral fractures. The occurrence rate of the recent fracture was 50% and it was reported in young ages between 1.5 and 8 months, which was significantly different from older age groups, but it was not significant for gender. The current work was carried out in continuation of the previous studies with a greater extent.

Metatarsal fractures are considered for 1.8 to 11% of all fractures in dogs (Ness et al, 1996). Benjamin et al, (2010) announced that most metatarsal fractures are caused by trauma. Greyhound dogs were more prone to metatarsal fractures. The ages of dogs

were different for metatarsal and metacarpal fractures reported from 2 months to 10 years. Okumura et al. (2000) reported a metatarsal fracture in a 12-year-old male pit bull terrier, and Seibert et al. (2011) announced this bone complication in a 4-year-old female pit bull terrier. The most important clinical findings are pain, swelling, presence of wound and cryptan sound during examination in metatarsal fractures (Fitzpack et al, 2011). The present results were nearly similar to the previously recorded findings by other researchers.

In the study conducted on the effects of ozone therapy on experimental fracture healing in the rabbit model, they reported that ozone was effective in improving the fracture repair process (Mohammad Hoseni et al, 2022).

In another survey on radiographic assessment of hip joint after femoral head and neck ostectomy and its relationship with clinical findings in dogs, it was concluded that revision surgery to resection of ossicles, especially in the neck region and lesser trochanter, can improve the patient's condition (Tayebi et al, 2023).

In conclusion, the most important of these cases were radial fracture (27.95%), femur (22.46%), tibia (17.11%) and ulna (1.07%). The highest incidence of skeletal disorders in dogs was femural and ulna fractures at 28.34% and 27.95%, respectively, from all the obtained results, regarding the causes of fractures in different parts, 45.32 percent are related to accidents, 24.43 percent are due to falling from a height, 17.13 percent of fractures are due to conflicts between animals, and 13.12% were due to unknown causes. The results showed that radiography is a valuable method to recognize skeletal disorders together with their frequency in dogs.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Original Article

Evaluation of the regenerative effect of chitosan scaffold and hyaluronic acid with and without mesenchymal stem cells on wound healing in rats

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Abstract

This study aimed to evaluate the restorative effect of chitosan hyaluronic acid scaffold (CHAS) with and without mesenchymal stem cells (MSCs) on the wound healing process in rats. The different wound treatment groups were as follows: no treatment or control (C), wound treatment with CHAS, wound covering with CHAS with MSCs. The wound healing effect was measured by measuring the wound area in each mouse on days 3, 5, 9, and 14. Then, for histopathological evaluation in the above days, each wound and 5 mm of normal skin tissue around each wound were separated and fixed. The results demonstrated that on the third and fifth days after the wound formation, the area of the remaining wound in the CHAS group was significantly smaller than the CHAS with MSCs but no significant difference was observed in the group C. Also, the area of the remaining wound on the ninth and fourteenth days in the studied groups did not show a significant difference. However, on day 14, the mean wound area in the CHAS group with MSCs was smaller than the other two groups. Histological examinations of the wound site were studied in terms of collagen arrangement, inflammation, vascular formation, granulation tissue, and epithelial regeneration. Studies in terms of collagen arrangement, granulation tissue formation, and vascular formation showed that on the third day. There was a significant difference between the groups, while no statistically significant difference was found between the groups in terms of inflammation and epithelial regeneration on the studied days. All these results demonstrate that there is no significant difference between the CHAS group and the CHAS group with MSCs as well as in group C.

Key words: Chitosan, Hyaluronic acid, Mesenchymal stem cell, Wound healing

Introduction

Wounds are one of the most common health problems and their complete healing represents a major challenge worldwide (Boateng and Catanzano, 2015). Wound healing is traditionally divided into four sequential phases: hemostasis, which lasts from a few minutes to a few hours after a skin injury, acute inflammation, which lasts 1- 3 days, Proliferation, generally lasting several days to a month, and eventual skin

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remodeling or scarring (Raziyeva et al, 2021). The inflammatory phase is characterized by the presence of pro-inflammatory neutrophils and macrophages at the lesion site. The proliferative phase of wound healing involves keratinocytes, fibroblasts, macrophages and endothelial cells whose active cooperation promotes re-epithelialization, angiogenesis and fibroplasia (Oliveira et al, 2022). Tissue remodeling mainly involves fibroblasts which are responsible for replacing the fibrin clot with scar tissue, slowing down angiogenesis and changing collagen composition (Yang et al, 2021). Several strategies have been proposed to improve wound healing. They can be broadly divided into biological agents, biomaterials, and cellular strategies (Son et al, 2019). Many studies have shown that cell therapies improve wound healing by improving angiogenesis and re-epithelialization to utilize cellular technologies, bone marrow-derived mesenchymal stem cells, adipose-derived cells, epidermal cells, and others (Shojaei et al, 2019).

Mesenchymal stem cells (MSCs) are involved to varying degrees in all three phases of wound healing. An important element of the mechanism of action of MSCs is that they directly reduce the inflammatory response and modulate immune system activity (Jiang and Xu, 2020). Studies have shown that the addition of MSCs to an active immune response reduces the secretion of the pro-inflammatory cytokines TNF-α and interferon while increasing the production of the anti-inflammatory cytokines, interleukin-10 and IL-4. This demonstrates the importance of the anti-inflammatory and immunomodulatory effects of MSCs in wound healing the detailed mechanisms of which have been described in several reviews (Harrell et al, 2019). In addition, MSCs have an antimicrobial effect which is very important in cleaning the wound from infections. The antimicrobial activity of MSCs occurs through two mechanisms: directly through the secretion of antimicrobials such as LL-37 and indirectly through the secretion of immune factors that regulate bacterial killing and phagocytosis by immune cells (Johnson et al, 2022). Current data suggest that the persistence and survival of MSCs at the lesion site are limited and need to be overcome by tissue engineering (TE) approaches (Shafiq et al, 2021).

One of the goals of skin tissue engineering is to use engineering techniques to facilitate the natural wound healing cascade by providing appropriate physicochemical and biochemical factors via natural or synthetic polymers (Nour et al, 2021). Chitosan (CS) is a de-acetylated derivative of chitin found primarily in the exoskeletons of arthropods including shrimps, crabs, and insects. In fact, CS is a polysaccharide composed of two di-acetyl units (D-glucosamine linked to β-(1-4)) and acetylated units (N-Acetyl-D-glucosamine) (Bakshi et al, 2020). The glucosamine moieties of CS have been reported to be a potent accelerator of wound healing. CS promotes wound healing through two main pathways (Eivazzadeh-Keihan et al, 2022). First, the N-Acetyl-D-glucosamine moiety of CS contributes to fibroblast proliferation and collagen production. Its positive charge interacts electrostatically with glycosaminoglycans leading to the uptake of growth factors. In the second phase, macrophages are activated by N-Acetyl-D-glucosamine to phagocytose and release mediators such as TGF-β1 and platelet-derived growth factor (Shariatinia, 2019). Furthermore, CS controls the production of IL-1 which controls fibroblast proliferation and collagen synthesis (Ribeiro, 2021). CS has also been reported to induce IL-8 secretion by fibroblasts leading to angiogenesis and neutrophil migration. CS can act as a hemostatic agent, promoting blood clotting by absorbing fibrinogen and plasma proteins. In addition, it can block nerve endings, thereby relieving pain (Guo et al, 2023). Therefore, it contributes to rapid wound healing and prevents scarring.

Numerous studies have shown that components and mimetics of the extracellular matrix play an important role in the repair of various tissues (Amorim et al, 2021). Scientists believe that hyaluronic acid (HA) can improve the mechanical properties and cell affinity of scaffolds through chemical combinations and surface modifications. HA is an important component of the synovial fluid and extracellular matrix with a disaccharide structure consisting of di-glucuronic acid and N-acetyl glucosamine (Chen et al, 2021). HA influences cell proliferation, differentiation and tissue repair. Long-chain HA activates fibroblast proliferation and migration and increases collagen deposition (Prajapati and Maheriya, 2019). During the wound healing process, HA can bind to fibrinogen during clot formation. At high concentrations, hyaluronic acid forms a porous network structure that allows the diffusion and migration of cells and proteins. During angiogenesis, HA binds to CD44 which promotes the formation of new blood vessels (Graça et al, 2020). It can also lead to wound healing through proliferation, migration, and increased collagen deposition in keratinocytes (Kawano et al, 2021). HA relieves pain by reducing nerve sensitivity (Li et al, 2020).

In fact, the speed and quality of repair of damaged tissue are very important (Kolimi et al, 2022). Therefore, this study was designed and implemented with the aim of evaluating the regenerative effect of chitosan and hyaluronic acid scaffold with and without MSCs on wound healing in rats.

Material and Methods

Preparation steps of 2% chitosan (CS) scaffold:

Medium molecular weight Sigma CS powder (Sigma, USA) was prepared, and the powder was dissolved in 0.5 molar acetic acid to produce and fabricate porous tissue. To produce 0.5 molar acetic acid according to the formula N1 V1 = N2 V2, 2.89 cm3 of this acid was poured into a 100 cm3 volumetric flask and the solution was then filled up with water. Then 2 grams of chitosan powder were added to this solution. To completely dissolve the CS-powder, this solution was stirred for 5 hours at 50 degrees Celsius on a Steer device using a magnet. After this time, a clear solution was obtained. The resulting solution was placed in the refrigerator at -4 °C for one day, then placed in the freezer at -18 °C for one day, and after leaving the freezer, it was placed in a freeze dryer for one day. The mechanism of this device is that when freezing at a temperature of about -50°C, porosity is created in the desired material using a vacuum pump. After completing these phases, a white, spongy and porous substance was finally obtained. In the final step, the obtained material was placed in a freezer at -18 °C for 24 hours and then placed in a non-sterile device for sterilization (Garg, Chanana & Joshi, 2012).

Scaffold impregnation with hyaluronic acid (HA)

To impregnate a series of scaffolds with HA, the first HA solution was prepared at a concentration of 3 mg/ml (0.3%). To avoid any contamination and to ensure the sterility of the solution, the preparation and impregnation phases of the solution were carried out under a laminar hood. In addition to using sterile PBS to prepare the HA solution, the resulting solution was also filtered using a filter on the syringe head. At this stage, the scaffolds are grown in containers. Six houses were set up, HA solution was added, and kept in an incubator at 37 °C for 24 hours (Figure 1).

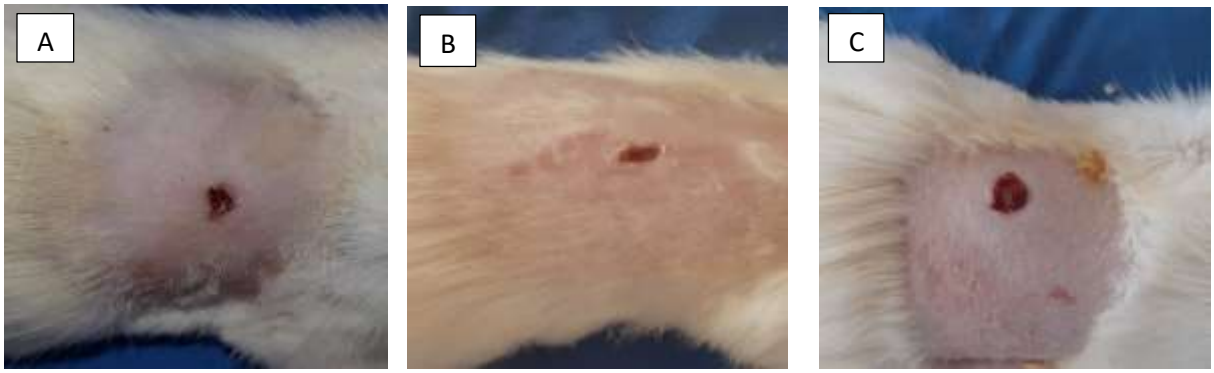

Figure 1: Wound site on the third day after creating a lesion: a) control group b) chitosan hyaluronic acid group c) chitosan hyaluronic acid + mesenchymal stem cells group

Mesenchymal Stem Cell (MSC) Culture

In this study, the cultured cells were human MSCs donated by Imam Khomeini Hospital in Tehran and transferred to the Animal Embryo Technology Research Institute of Shahrekord University in the frozen state during the first passage in order to produce there. Under the same conditions during the third passage, they were grown on research scaffolds. In the cell growth and proliferation study on the CH scaffold, the scaffolds were sterilized under UV- light for at least 2 hours and then placed in 24-well plates. In the third step, the cells were cultured on CH pieces in DMEM medium at 10% FBS for three days. Finally, they were transferred to the surgical department for use in the reconstruction process (Figures 2, 3).

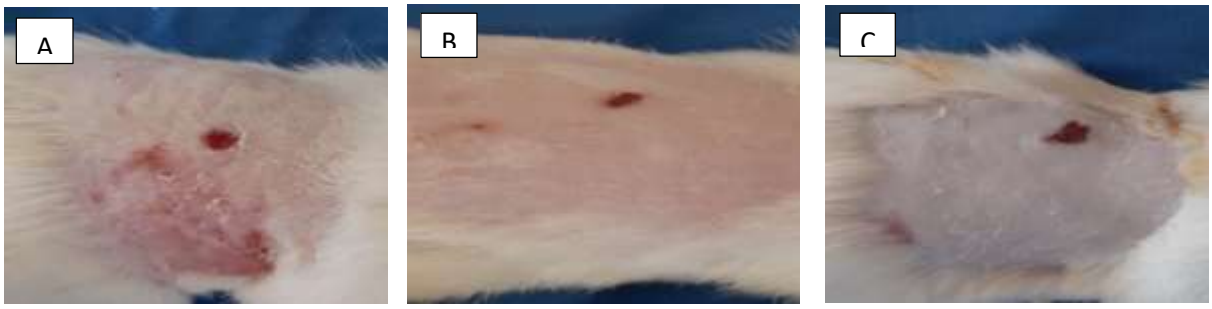

Figure 2: wound site on the fifth day a) control group b) chitosan hyaluronic acid group c) chitosan hyaluronic acid + mesenchymal stem cells group

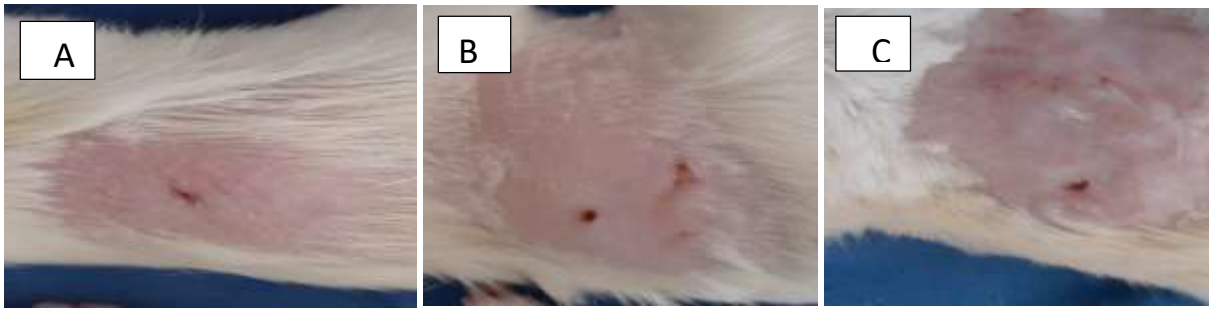

Figure 3: wound site on the ninth day a) control group b) chitosan hyaluronic acid group c) chitosan hyaluronic acid + mesenchymal stem cells group

Animal study

To carry out this research, the first 60 male rat pieces weighing about 200-250 gr were collected from the animal shelter of Shahrekord University. To adapt to the new environment, they were kept in cages for two weeks and given sufficient water and standard food. The animals were randomly divided into three groups of twenty animals each. In the negative control group (C), no compound was applied to the wound. In the second group, chitosan and hyaluronic acid (CH) scaffolds were placed on the wound, while in the third group, CH scaffolds with MSCs (CHM) were placed on the wound. It should be noted that the scaffold was sterilized under ultraviolet light for one hour before use.

To create a wound on the skin, the hair on both sides of the spine on the animal's back was first shaved. Subsequently, a combination of ketamine (at a dose of 40 to 87 mg/kg) and xylazine (at a dose of 5 to 13 mg/kg) was used to induce anesthesia. After setting the appropriate depth of anesthesia, the required wound area was disinfected with betadine scrub solution and the necessary measures were taken to establish sterile conditions. Before performing the wounds, the exact location of the wounds was marked. A 5 mm diameter skin punch device was used to create wounds of equal size (Figure 4). After dressing the wounds to prevent infection, the wound areas were covered with a sterile dressing and the rats were examined until complete healing. Each of them was then kept and cared for in a separate cage. After recovering and regaining consciousness, the animals were reintroduced into the herd and received enrofloxacin10%, by intramuscular injection at a dose of 10 mg/kg every three days. The day the wound formed was considered day zero.

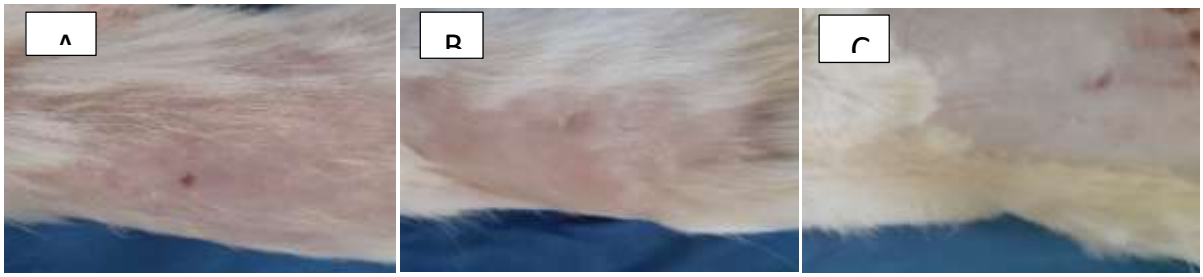

Figure 4: wound site on the fourteenth day a) control group b) chitosan hyaluronic acid group c) chitosan hyaluronic acid + mesenchymal stem cells group

Gross examination of wounds

On the 3rd, 5th, 9th and 14th days after the operation, wound location and wound appearance, such as inflammation, heat, redness, swelling and compatibility of materials with the wound were investigated. Photographs were then taken with a digital camera at a distance of 20 cm from the wound. The Image J software was used to evaluate and evaluate the prepared images.

Histopathological examination

Five samples were collected from each group on days 3,5,9 and 14 to prepare histopathological sections. Therefore, the rats were painlessly euthanized using the maximum anesthetic dose and appropriate tissue samples were collected from the wound. In this way, by cutting with a scalpel, a portion of the skin tissue at the edges of the wound, both healthy and damaged, was collected in its entire thickness and, after washing with

physiological serum, was fixed in container with buffered formalin to 10%. Five-micrometer sections were then prepared from these samples using a microtome and stained with hematoxylin-eosin as routine method. The wound healing process was evaluated and scored from the histopathological point of view of the prepared samples using evidence such as inflammation, fleshy tissue formation, collagen fiber alignment, angiogenesis and epithelial tissue regeneration (Gupta and Kumar, 2015).

Statistical analysis

The SPSS program was used to perform statistical tests. The macroscopic results obtained were analyzed using one-way anova and the microscopic results were analyzed using the nonparametric Kruskal-Wallis statistical test.

Results

The results of macroscopic examination of wound healing

On the third day after wound formation, there was no significant difference between the CH group and the control group, but there was a significant difference between this group and the CH group along with MSC (p=0.018). Also, there was a significant difference (p=0.018) between the control and CHM group. On day 5, there was no significant difference between the control group and CH group (p=0.065). The data show that there is a significant difference between the CH and the CHM group (P=0.045) and the wound area was smaller in the CH group. On the 9th day, no significant difference was observed between the groups, but the area of the wound in the CHM group was smaller than the other groups. On the 14th day, no significant difference was observed between the groups, but the wound area in the CH group was less that in the CHM group (Table 1, Figure 1).

Table 1: Area of wounds in mm2 scale in different groups

Groups	Days of study			
	3	5	9	14
Control	12.67±1.67[a]	8.14±1.89[a]	3.77±1.08[a]	1.40±0.44[a]
Chitosan/hyaluronic acid	12.63±3.36[a]	6.79±4.34[a]	3.70±1.01[a]	0.67±0.22[a]
Chitosan/hyaluronic acid /MSC	12.63±1.44[b]	8.42±1.49[b]	2.63±0.44[a]	0.95±0.65[a]
P value	P<0.05	P<0.05	p>0.05	p>0.05

The results of histopathology studies of wound healing

In the microscopic study of wound samples on days 3, 5, 9, and 14, the degree of wound healing was evaluated using evidence such as inflammation, formation of fleshy bud tissue, orientation of collagen fibers, vascularization, and epithelial tissue regeneration. A score of 0 to 4 was used to evaluate the healing of wounds based on the mentioned evidence.

The results of examining the arrangement of collagens

The results of examining the arrangement of collagens show that on the third day of the study, a significant difference can be seen among the studied groups (p<0.05). On the fifth, ninth, and fourteenth days of the study, the study of the collagen composition in the groups showed that there was no statistically significant difference between them (p<0.05) (Table 2).

Table 2: The results of the arrangement of collagen in the studied groups.

P≤0.05 indicates a significant difference. middle (minimum - maximum)

Groups	Days of study			
	3	5	9	14
Control	0(0-0)	1(1-2)	2(2-2)	3(3-3)
Chitosan/hyaluronic acid	1(1-1)	1(1-1)	2(2-3)	3(3-3)
Chitosan/hyaluronic acid /MSC	1(1-1)	2(2-2)	3(2-3)	3(3-4)
P value	0.030	0.061	0.067	0.368

The results of investigating the formation of granulation tissue

The results of examining the formation of granulation tissue show that there is a significant difference between the groups on the third day of the study ($p<0.05$). No significant difference was observed between the groups on days 5, 9 and 14 ($p<0.05$) (Table 3).

Table 3: The results of investigating the formation of granulation tissue in the studied groups. P≤0.05 indicates a significant difference. middle (minimum - maximum)

Groups	Days of study			
	3	5	9	14
Control	0(0-0)	3(3-2)	3(3-4)	3(3-4)
Chitosan/hyaluronic acid	1(1-1)	3(3-3)	4(3-4)	4(3-4)
Chitosan/hyaluronic acid /MSC	1(1-1)	3(3-4)	4(4-4)	4(4-4)
P value	0.018	0.102	0.264	0.565

Inflammation examination results

The results of the investigation of inflammation show that there is no significant difference between the investigated groups at all investigated times ($p<0.05$). On the ninth day, despite the absence of inflammation in the control and chitosan hyaluronic acid groups, no significant difference was observed between all groups ($p=0.67$) (Table 4)

Table 4: The results of investigating inflammation in the studied groups. P≤0.05 indicates a significant difference. middle (minimum - maximum)

Groups	Days of study			
	3	5	9	14
Control	2(2-2)	1(0-1)	0(0-0)	0(0-0)
Chitosan/hyaluronic acid	2(1-2)	1(2-1)	1(0-1)	0(0-0)
Chitosan/hyaluronic acid /MSC	1(1-1)	1(0-1)	0(0-0)	0(0-0)
P value	0.061	0.306	0.670	1

Results of vascularization

Examining the results of angiogenesis in the present study, it was observed that there was no significant difference between the investigated groups on the fifth, ninth and fourteenth day ($p<0.05$). But on the third day, angiogenesis in the hyaluronic acid chitosan group and the hyaluronic acid chitosan group with stem cells was better than the control group ($p<0.05$), and also the hyaluronic acid chitosan group was better than the hyaluronic acid chitosan group with stem cells ($p<0.05$) (Table 5).

Table 5: The results of investigating the formation of blood vessels in the studied groups.

P≤0.05 indicates a significant difference. middle (minimum - maximum)

Groups	Days of study			
	3	5	9	14
Control	0(0-0)	2(2-2)	2(2-1)	1(1-0)
Chitosan/hyaluronic acid	3(3-2)	2(2-3)	2(3-1)	2(1-2)
Chitosan/hyaluronic acid /MSC	2(3-2)	3(3-3)	2(3-2)	2(3-1)
P value	0.046	0.061	0.061	0.152

The results of examining the regeneration of the epithelium

The results of the examination of the regeneration of the epithelium are given in Table 6-4. As can be seen, there is no significant difference between the investigated groups in all investigated times ($p<0.05$) (Table 6).

Table 6: The results of investigating the regeneration of the epithelium in the studied groups. P≤0.05 indicates a significant difference. middle (minimum - maximum)

Groups	Days of study			
	3	5	9	14
Control	0(0-0)	1(2-1)	2(2-2)	3(4-3)
Chitosan/hyaluronic acid	0(0-0)	2(2-1)	3(3-3)	4(4-3)
Chitosan/hyaluronic acid /MSC	0(0-0)	2(2-2)	3(4-3)	4(4-4)
P value	1	0.102	0.110	0.061

Results of examining microscopic images of wounds in different groups

On the third day (Figure 5 A), there was a surface clot on the surface of the wound (star) and hyperemia was observed in the wound space in the control group (arrow). Also, the presence of a surface clot on the wound surface with a small accumulation of fibrin (star) and a small number of inflammatory cells in the wound space in the CH group (arrow) was observed (Figure 5 B). The presence of surface clot on the surface of the wound along with the accumulation of fibrin (star) and a small number of inflammatory cells and edema in the wound space in the CHM group (arrow) were recorded (Figure 5 C). On the fifth day, the presence of surface clot and the formation of edematous granulation tissue (star) and the formation of blood vessels in the wound space (arrow) were seen (Figure 6 A). The absence of surface clot (star) and the formation of granulation tissue with strings Irregular spots were recorded in the wound space in the CH group (arrow) (Figure 6 B). The Surface clot and filling of the wound space by irregular granulation tissue (star) and formation of blood vessels in the wound space were present in the CHM group (arrow) (Figure 6 C). On the 9th day, the filling of the wound space by immature granulation tissue (arrow) and the presence of newly formed vessels in the control group (star) were recorded (Figure 7 A). The formation of the epidermis and the filling of the wound space by fibrous granular tissue (arrow) were seen in CH group (Figure 7 B). Also, the filling of the wound space by relatively thick and regular granulation tissue was observed in the CHM group (arrow) (Figure 7 C). On the 14th day, in the control group, the formation of the epidermis was evident along with the filling of the wound space by relatively thick and irregular collagen fibers (arrow) (Figure 8 A). The formation of keratinized epidermis along with the filling of the wound space by relatively thick and regular collagen fibers (arrow) and the reduction of blood vessels were also seen in the CH group (star) (Figure 8 B). The formation of keratinized epidermis along with the filling of the wound space by thick and regular collagen fibers (arrow) and the reduction of blood vessels were also observed in the CHM group (star) (Figure 8 C).

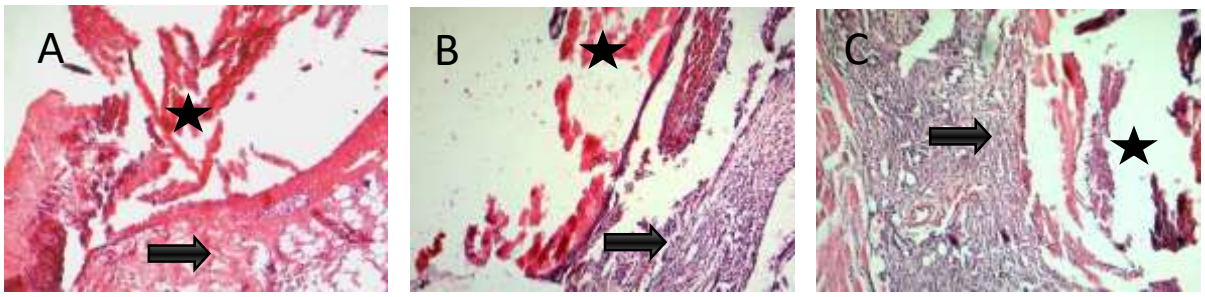

Figure 1: Microscopic sections of the wound site on day 3 after wound formation. (H&E, X10)

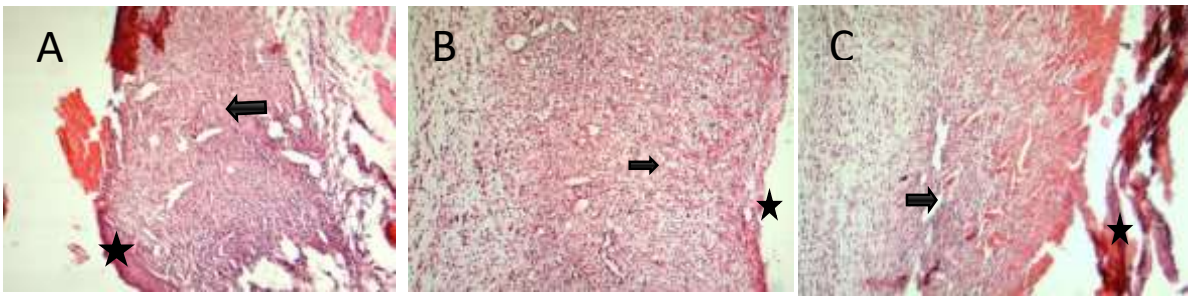

Figure 2: Microscopic sections of the wound site on the 5th day after wound formation. (H&E, X10)

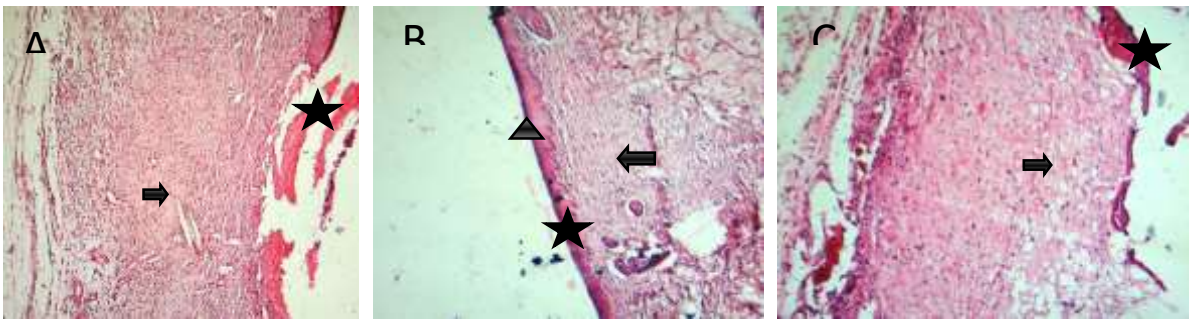

Figure 3: Microscopic sections of the wound site on the 9th day after wound formation. (H&E, X10)

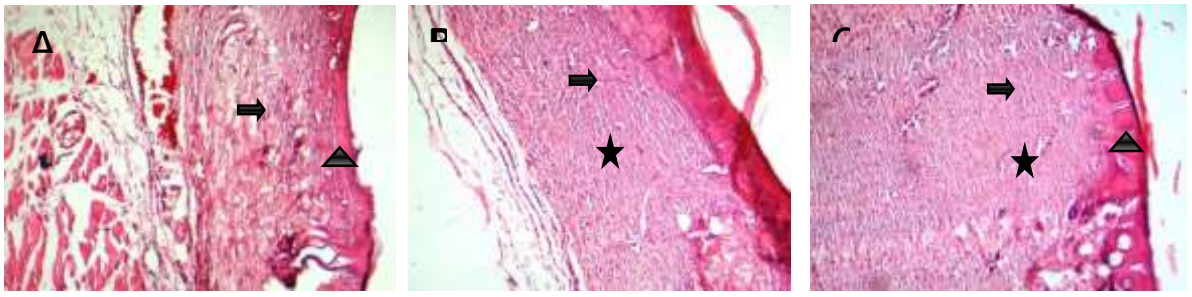

Figure 4: Microscopic sections of the wound site on the 14th day after wound formation

Discussion

Skin may be a delicate tissue that secures the entire body and inside tissues against warm, electrical adjust and physical harm (Kirwann and Pignataro, 2015). Skin dressings anticipate pathogens and organisms from entering the harmed zone and body water loss and lead to acceleration of wound closure and reduction of scar formation (Okur et al, 2020). The wound healing process may be a complex prepare, and in case of extreme tissue harm, the tissue reclamation may not lead to the return of the ordinary tissue structure, and the connective tissue may be damaged and a

scar or wound may be shaped (Tabassum et al, 2021). Nowadays, the utilization of dressings of biological origin plays a viable part in wound healing and scar decrease (Farahani and Shafiee, 2021). Moreover, broad considers on the utilization of topical treatments or modern dressings are continuously being conducted (Borda et al, 2016). Until presently, different dressings have been made for the treatment and healing of wounds, but most of them have issues such as incompatibility within the natural environment, need of antibacterial and biodegradable properties (Bianchera et al, 2020). In the new tissue designing strategies, engineered polymers such as chitin, chitosan, and collagen are utilized to create scaffolds due to their suitable biological properties such as adhesion, compatibility, and antimicrobial properties (Ahmed et al, 2018). MSCs have moreover attracted the consideration of numerous analysts due to their anti-bacterial properties and healing situations (Krasnodembskaya et al, 2010).

In this study, the effect of chitosan-hyaluronic acid scaffold with and without bone-derived mesenchymal stem cells (BMSC) on wound healing in rats was examined. On the third and fifth day, the region of the wound was significantly less compared to the CHM group. But there was no significant difference with the control group. On the 9th and 14th days of the study, there was no significant difference between the groups in terms of wound area. Sadik et al, (2015) examined chitosan gel and MSCs and found that chitosan improved wound healing compared to the control, but none of the wounds closed within 15 days. According to this study, the recovery and speed of wound healing was higher within the groups treated with MSCs, and it has also been shown that intradermal injection of MSCs has a faster healing rate than systemic injection (sadik et al, 2015). In addition to the positive effects of chitosan and hyaluronic acid on wound healing, bone-derived mesenchymal stem cells cause the growth and chemotaxis of fibroblasts, thereby increasing the speed of wound healing (Ha et al, 2020). In contrast to our results, in other studies, the effect of using hyaluronic acid, chitosan and MSCs in reducing the wound area is clear.

The results of examining the arrangement of collagen bundles in the present study showed that on the third day, CHM group and CH group had significantly better collagen arrangement than the control group. The local use of hyaluronic acid on the wound by increasing the movement of fibroblasts towards collagen sponges and the formation of collagenous tissue in the early stages accelerates the healing handle, and bone-derived stem cells increment type one and three collagens in the wound healing process (Thönes et al, 2019). In the study of Berce et al, (2018), the role of the coating containing chitosan and hyaluronic acid has been emphasized in reducing scar formation by improving the rearrangement of collagen bundles (Berce et al, 2018). Also, in the study of Sadik et al, (2018), it was shown that the systemic injection of MSCs in complete repair Collagen plays a significant role in wound healing and skin repair (sadik et al, 2015).

The results of the present study, in terms of examining the granulation tissue, showed that on the third day of the study, there was a significant difference between the CH group and the CHM group and the control group. In spite of the fact that the granulation tissue formation was better in the CH group on days five, nine and fourteen of the study than the other two groups, no significant difference was observed between the groups. HA increases vascularization, regeneration of epithelium and formation of granulation tissue, as well as migration of endothelial cells and improvement of regeneration of epithelium at the wound site (Hussain et al, 2017). BMSCs discharging different particular cytokines and chemokines, have higher amounts of VEGF-α, IGF-1, EGF, keratinocyte growth factor, angiopoietin-1,

derived factor 1 compared to skin fibroblasts. From the stroma, they secrete macrophage inflammatory protein-1 alpha and beta and erythropoietin. Also, the factors released by BM-MSC attract macrophages and endothelial cells into the wound and thus increase wound healing (Chakravorty and Shukla, 2023).

The results of the investigation of inflammation in the present study show that there is no significant difference between the studied groups. HA does not play a significant role in promoting inflammation and speeding up healing (Frenkel, 2014). Chitin, chitosan, its monomers and oligomers accelerated the wound healing process by increasing the activities of inflammatory cells such as polymorphonuclear leukocytes, macrophages and fibroblasts (Sharifi et al, 2022). It has been found that dressings impregnated with chitosan reduced inflammation and created significant antimicrobial effects compared to the control group. In fact, chitosan plays an effective role in faster wound healing by increasing the presence of multinucleated white blood cells, increasing the migration of fibroblasts to the wound surface and improving their growth and multiplication, and increasing the migration of macrophages (Yazarlu et al, 2021). The role of mesenchymal stem cells in reducing inflammation is related to the presence of cytokines and is very short-term (Van Buul et al, 2012).

In the present study, the results of angiogenesis show that there is a significant difference between the groups only on the third day of the study, and no critical difference was seen on the other days. It has been demonstrated that bone-derived stem cells increase epithelization and vascularization in the healing process of diabetic and non-diabetic wounds in mice (Pountos et al, 2014). It has also been found that chitosan induces inflammatory cells and increases blood vessels and collagen in the new tissue (Deng et al, 2010). In the study of Yang et al., 2021, the effect of nanoparticles containing hyaluronic acid on the inflammatory process and angiogenesis was evaluated and proved by measuring α-SMA and CD31 during the healing process of diabetic wounds (Yang et al, 2021).

The results of this study showed the appropriate performance of chitosan/hyaluronic acid scaffold impregnated with mesenchymal stem cells in wound healing, especially in the early days of wound healing. In fact, the nearness of progenitor cells, cytokines and growth factors in mesenchymal stem cells increases the performance of other components within the wound recuperating process. The manner and duration of use and the dose of the material used compared to other studies cause differences in the final results, and additional studies are needed in this field.

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Conflict of interest

The authors declare that there is no conflicts of interest regarding the authorship and publication of this research article.

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Effects of Alpha-pinene on oxidative stress and inflammatory response in acute gastric ulcers in rats

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Abstract

Despite the many therapeutic advances, gastric ulcers continue to be prevalent. Natural compounds have been found to play a crucial role in preventing gastric ulcers in various phytochemical studies. The study aimed to investigate the protective effect of alpha-pinene against ethanol-induced gastric ulcers in rats by evaluating its impact on pro-inflammatory cytokines and oxidative stress markers. Male Wistar rats were orally administered alpha-pinene (50 and 100 mg/kg) prior to being induced with gastric ulceration using ethanol, and the gross morphological lesions, pro-inflammatory cytokine levels, and oxidative stress markers in gastric tissues were evaluated. Alpha-pinene treatment reduced gross morphological lesions in comparison to untreated animals. In ethanol-treated rats, alpha-pinene at 50 and 100 mg/kg also reduced oxidative stress, as verified by a decrease in tissue myeloperoxidase activity and malondialdehyde levels. In addition, alpha-pinene at both doses increased GSH and CAT levels compared to the untreated group. Alpha-pinene at both doses also lowered IL-1β and TNF-α production compared to the untreated group. Alpha-pinene may have a beneficial therapeutic role in gastric damage induced by ethanol as it reduces oxidative stress and pro-inflammatory factors.

Key words: Alpha-pinene, Inflammatory cytokines, Oxidative responses, Acute gastric injury

Introduction

Many diseases, including gastric ulcers and gastric carcinoma, are caused by oxidative stress (Tandon et al, 2004; Taheri Otaghsara et al, 2023). This results from high levels of reactive oxygen species production, leading to depletion of tissue antioxidant defense factors (Suzuki et al, 2012). Ethanol exposure disrupts the balance between protective and invasive factors of the gastric mucosa, stimulating pro-inflammatory cytokines and reactive oxygen species (ROS) (Pan et al, 2008), as well as myeloperoxidase (MPO) activity in neutrophils, causing gastric mucosal

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damage (Chatterjee et al, 2007). The previous studies have found that cytokines and oxidant/antioxidant mediators such as malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH), and superoxide dismutase (SOD) play an important role in acute gastrointestinal injury caused by ethanol (Almasaudi et al, 2017; J I Choi et al, 2010; Rozza et al, 2014).

Chemical anti-ulcer drugs have side effects (Koyyada, 2021), like omeprazole, a proton pump inhibitor (PPI) that inhibits gastric acid secretion (Toh et al, 2015). However, it may increase Clostridium difficile infection incidence (Trifan et al, 2017), induce hypomagnesemia, or attenuate anticoagulant drug efficiencies (Kenngott et al, 2010). Anti-inflammatory and antioxidant compounds can scavenge free radicals (Kinjo et al, 2008). Medicinal plant compounds have received attention for their anti-inflammatory and antioxidant effects in treating gastric ulcers (Danisman et al, 2023; Sumbul et al, 2011).

Several natural products have proved to have both protective and curative effects against gastric ulcer (Solmaz et al, 2009; Wang et al, 2011). Compounds such as *Apium graveolens* L, ginger, resveratrol, Quercus, Cirsium vulgare, and Falcaria vulgaris have beneficial effects in gastric ulcers due to their antioxidant effects (Basatinya et al, 2021; Solmaz et al, 2009; Wang et al, 2011). Alpha-pinene is a bicyclic monoterpene found in plants and acts as a repellent agent. It has many medicinal properties, including anti-inflammatory, antioxidant, and antibiotic activities (X Huang et al, 2013; Mercier et al, 2009). Alpha-pinene has been shown to improve the activity of SOD, CAT, and GPx, and reduce the concentration of MDA, NO, and IL-6 in the hippocampus and cortical tissue following stroke (Khoshnazar et al, 2019). Moreover, it can inhibit intracellular ROS production and significantly induce the expression of antioxidant enzymes such as SOD, CAT, glutathione peroxidase (GPx), glutathione reductase (GR), and heme-oxygenase 1 (HO-1) (Porres-Martínez et al, 2016). Additionally, it has anti-ulcer activity against ethanol-induced gastric ulcers and protects against gastric mucosal damage (Al-Juhaishi, 2014). However, detailed information about the mechanism of alpha-pinene effect on gastric ulcers is unavailable.

The animal model of ethanol-induced gastric injury is often used to evaluate the anti-ulcer activity of natural products and drugs (Abdelwahab et al, 2013; Brzozowski et al, 1998). Our study analyzed the impact of alpha-pinene on gastric tissue in rats with ethanol-induced gastric ulcers by scrutinizing the macroscopic changes and measuring levels of inflammatory and antioxidant factors.

Materials and methods

Animals

Thirty male adult Wistar rats weighing 250±10 grams were obtained from the Animal Care Center of Shahid Chamran University of Ahvaz. They had free access to food and water and were kept under a 12-hour light/dark cycle at a temperature of 23±2°C. The animal use followed the Guidelines for the Care and Work of Laboratory Animals (NIH Publication No. 23-86). The animal work ethics committee of Shahid Chamran University of Ahvaz approved the study protocol (EE/1401.2.241.402.25/scu.ac.ir).

Experimental Design

Thirty Wistar rats were randomly divided into five groups, with each group comprising six rats. The study involved five groups of rats. The first group (control) received oral liquid paraffin (Chemicenter, Iran) for seven days followed by an intraperitoneal injection of normal saline on the day of the experiment. The second group (Eth) received oral liquid paraffin for seven days, followed by the induction of stomach ulcers through intragastric

administration of ethanol (Merck, Germany) at a dose of 1ml/200gr bw. The third group received alpha-pinene (Sigma Alderich, St. Louis, MO, United States) (Zhang et al, 2020) at a dose of 50mg/kg for seven days, followed by the creation of gastric ulcers. The fourth group received alpha-pinene at a dose of 100mg/kg for seven days, followed by the creation of gastric ulcers. The fifth group received omeprazole (Irannajo Pharmaceutical Co, Iran) at a dose of 20mg/kg (Taheri Mirghaed et al, 9900) for seven days, followed by the induction of gastric ulcers. The rats were not allowed to eat but had access to water for 24 hours before receiving the ethanol. Rats were anesthetized with thiopental sodium (Loghman, Iran) (50 mg/kg, i.p.) and euthanized by decapitation 1.5 hours after Eth administration. The stomachs from each group were then isolated (Figure 1).

Evaluation of mean gastric ulcer index

The stomach tissue was cut from the dorsal surface, and then the area of ulcers (mm) was counted. When counting the ulcers, the names of the groups were blinded. The mean gastric ulcer index (MUI) was determined using the following formula:

MUI = total ulcer area/millimeter of rats ulcerated

% Inhibition Ulcer = (MUI of ethanol treated – MUI of rat pretreated)/MUI of ethanol treated * 100

Determination of Inflammatory Markers

The tissues were homogenized by pounding in a mortar. Pro-inflammatory cytokines such as IL-1β (IL-1β, Kiazist, Hamedan, Iran cat# E0119Ra), TNF-α (TNF-α, Kiazist, Hamedan, Iran cat# E0764Ra) were measured by enzyme-linked immunosorbent assay (ELISA) methods. The total protein content was measured by the Bradford method (Kiazist, Hamedan, Iran cat#KBRD96). To measure protein concentration, tissue samples were mixed with Bradford's reagent and distilled water. Absorbance was measured at 495 nm using a plate reader. A standard curve of bovine serum albumin protein was used to calculate the protein concentration of the samples.

Determination of oxidant/antioxidant factors

Oxidant/antioxidant factors such as MPO (MPO, Navand LAB Kit, Iran cat#KSOD96), MDA (MDA, Kiazist, Hamedan, Iran cat#KMDA96), GSH (GSH, Kiazist, Hamedan, Iran cat#KTHI96), and CAT (CAT, Kiazist, Hamedan, Iran) were analyzed by enzyme-linked immunosorbent assay (ELISA) methods.

Statistical Analysis

SPSS version 16 was utilized to analyze data. One-way ANOVA was conducted to compare the groups, followed by Tukey's statistical test for post-hoc comparison. *P<0.05, **p<0.01 and ***p<0.001 were considered statistically significant.

Results

Gastric Tissue Macroscopic changes

Our results revealed that the average ulcer size (measured in mm) was significantly greater in the ethanol, alpha-pinene 50 mg/kg, alpha-pinene 100 mg/kg, and omeprazole 20 mg/kg groups compared to the control group (P<0.001, P<0.01, P<0.05, and P<0.05 respectively). However, the average ulcer size was lower in alpha-pinene 50 mg/kg, alpha-pinene 100 mg/kg, and omeprazole 20 mg/kg groups when compared to the ethanol group (P<0.05, p<0.01, and P<0.01 respectively) (Figures 2 and Figure 3 A and C).

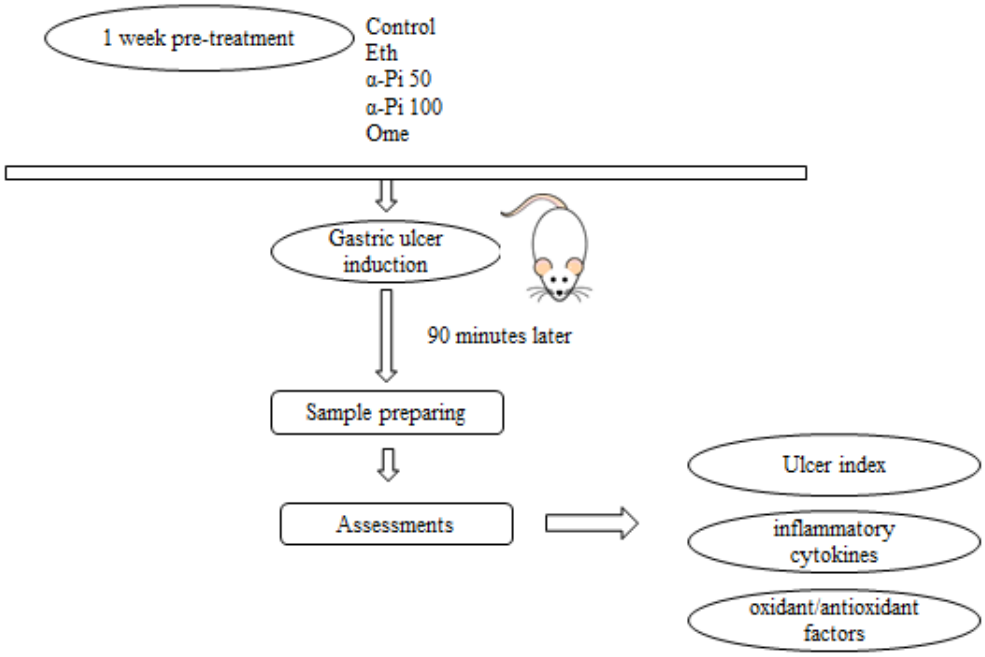


Figure 1: The schematic diagram

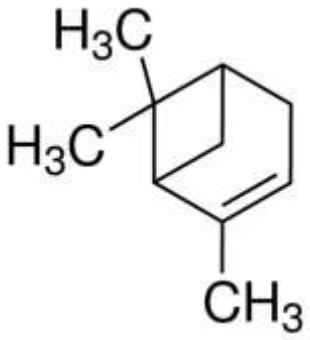
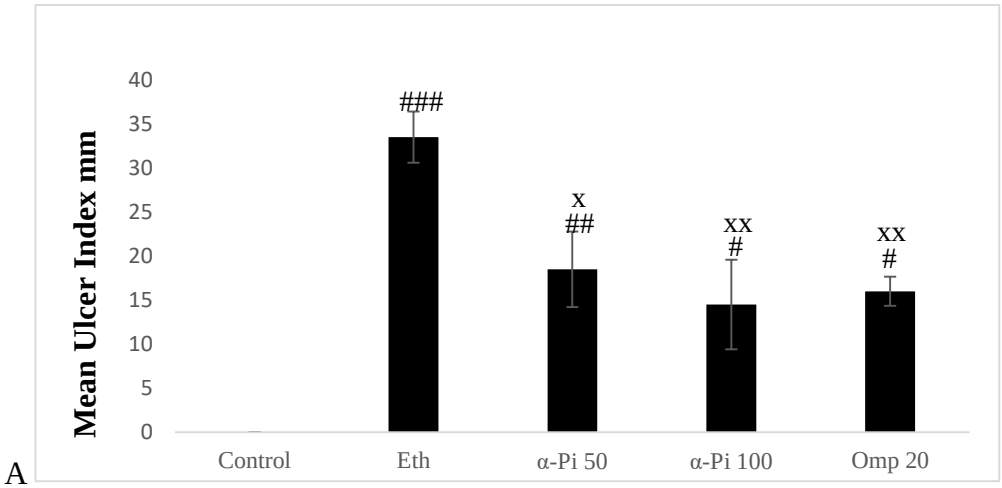
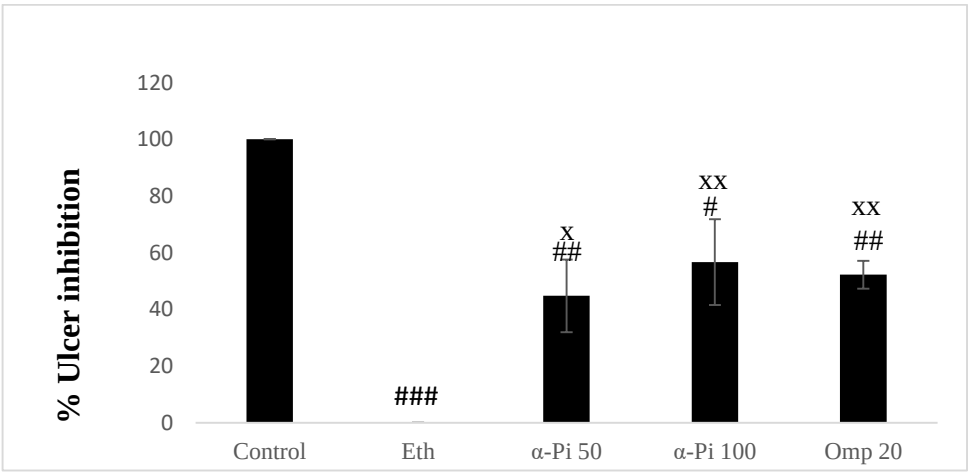


Figure 2: Chemical structures of α- pinene (C10H16)



A



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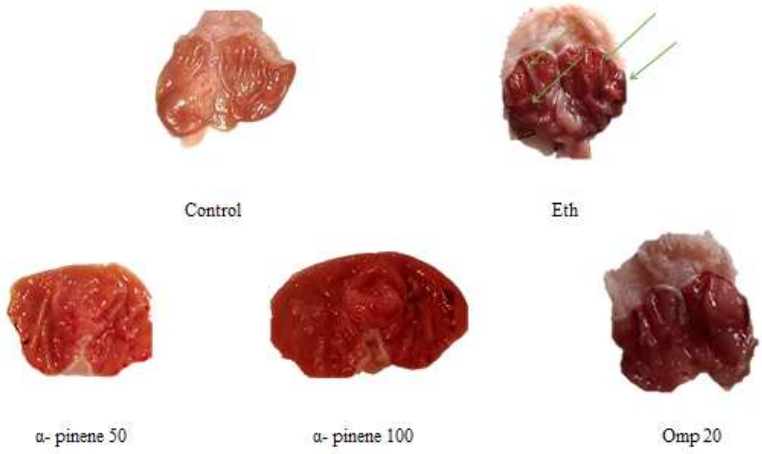

C

Figure 3: Ulcer index in study groups. (Mean±SEM) in control, ethanol (Eth), alpha-pinene 50 and 100 mg/kg groups + ethanol, and Omeprazole group with a dose of 20 mg/kg + ethanol (Omp 20). (A) Mean ulcer index. (B). %Ulcer inhibition. (C). Macroscopic image. # p< 0.05, ## p< 0.01, and ### p< 0.001 show a significant level difference compared to the control group. x p< 0.05, xx p< 0.01, and xxx p< 0.001 show a significant level difference compared to the ethanol group (n=6).

In the study, it was shown that the groups given ethanol, alpha-pinene 50 mg/kg, alpha-pinene 100 mg/kg, and omeprazole 20 mg/kg had a lower percentage of ulcer inhibition compared to the control group (P<0.001, P<0.01, P<0.05, P<0.01 respectively). Also, the groups given 50 mg/kg alpha-pinene, 100 mg/kg alpha-pinene, and 20 mg/kg omeprazole had a higher percentage of ulcer inhibition compared to the ethanol group (P<0.05, P<0.01, and P<0.01 respectively) (Figures 2 and 3B).

The concentrations of inflammatory cytokines in gastric tissue

TNF-α levels are significantly increased in ethanol, alpha-pinene 50 mg/kg, alpha-pinene 100 mg/kg, and omeprazole 20 mg/kg compared to the control group (P<0.001). However, TNF-α levels are significantly decreased in alpha-pinene 50 mg/kg, alpha-pinene 100 mg/kg, and omeprazole 20 mg/kg groups compared to the ethanol group (P<0.001) (Figure 4 A).

In addition, IL-1β levels are significantly increased in ethanol, alpha-pinene 50 mg/kg, alpha-pinene 100 mg/kg, and omeprazole 20 mg/kg compared to the control group (P<0.01). However IL-1β levels are significantly decreased in alpha-pinene 50 mg/kg, alpha-pinene 100 mg/kg, and omeprazole 20 mg/kg groups compared to the ethanol group (P<0.01) (Figure 4 B).

The levels of oxidant/antioxidant factors in gastric tissue

The groups treated with ethanol and alpha-pinene 50 mg/kg showed a significant increase in the MDA levels compared to the control group (P<0.001 and P<0.05, respectively). The levels of MDA in the groups treated with alpha-pinen 100 mg/kg and omeprazole 20 mg/kg did not differ from the control group. However, the levels of MDA in the groups treated with alpha-pinene 50 mg/kg, alpha-pinene 100 mg/kg, and omeprazole 20 mg/kg were significantly lower than the ethanol group (P<0.01, P<0.001, and P<0.001, respectively) (Figure 5 A).

The group treated with ethanol showed a significant increase in the MPO levels compared to the control group (P<0.01). The levels of MPO in the groups treated with alpha-pinene 50 mg/kg, alpha-pinen 100 mg/kg, and omeprazole 20 mg/kg did not differ from the control group. The levels of MPO in the groups treated with alpha-pinene 50 mg/kg, alpha-pinene 100 mg/kg, and omeprazole 20 mg/kg were significantly lower than the ethanol group (P<0.05) (Figure 5 B).

The groups treated with ethanol and alpha-pinene 50 mg/kg showed a significant decrease in the GSH levels compared to the control group (P<0.001 and P<0.01, respectively). The levels of GSH in the groups treated with alpha-pinen 100 mg/kg and omeprazole 20 mg/kg did not differ from the control group. However, the levels of GSH in the groups treated with alpha-pinene 50 mg/kg, alpha-pinene 100 mg/kg, and omeprazole 20 mg/kg were significantly higher than the ethanol group (P<0.05, P<0.0501, and P<0.0501, respectively). In addition, the levels of GSH in the groups treated with alpha-pinene 100 mg/kg, and omeprazole 20 mg/kg were significantly higher than the alpha-pinene 50 mg/kg (P<0.01) (Figure 5 C).

The group treated with ethanol showed a significant decrease in the CAT levels compared to the control group (P<0.001). The levels of CAT in the groups treated with alpha-pinene 50 mg/kg, alpha-pinen 100 mg/kg, and omeprazole 20 mg/kg did not differ from the control group. The levels of CAT in the groups treated with alpha-pinene 50 mg/kg, alpha-pinene 100 mg/kg, and omeprazole 20 mg/kg were significantly higher than the ethanol group (P<0.05) (Figure 5 D).

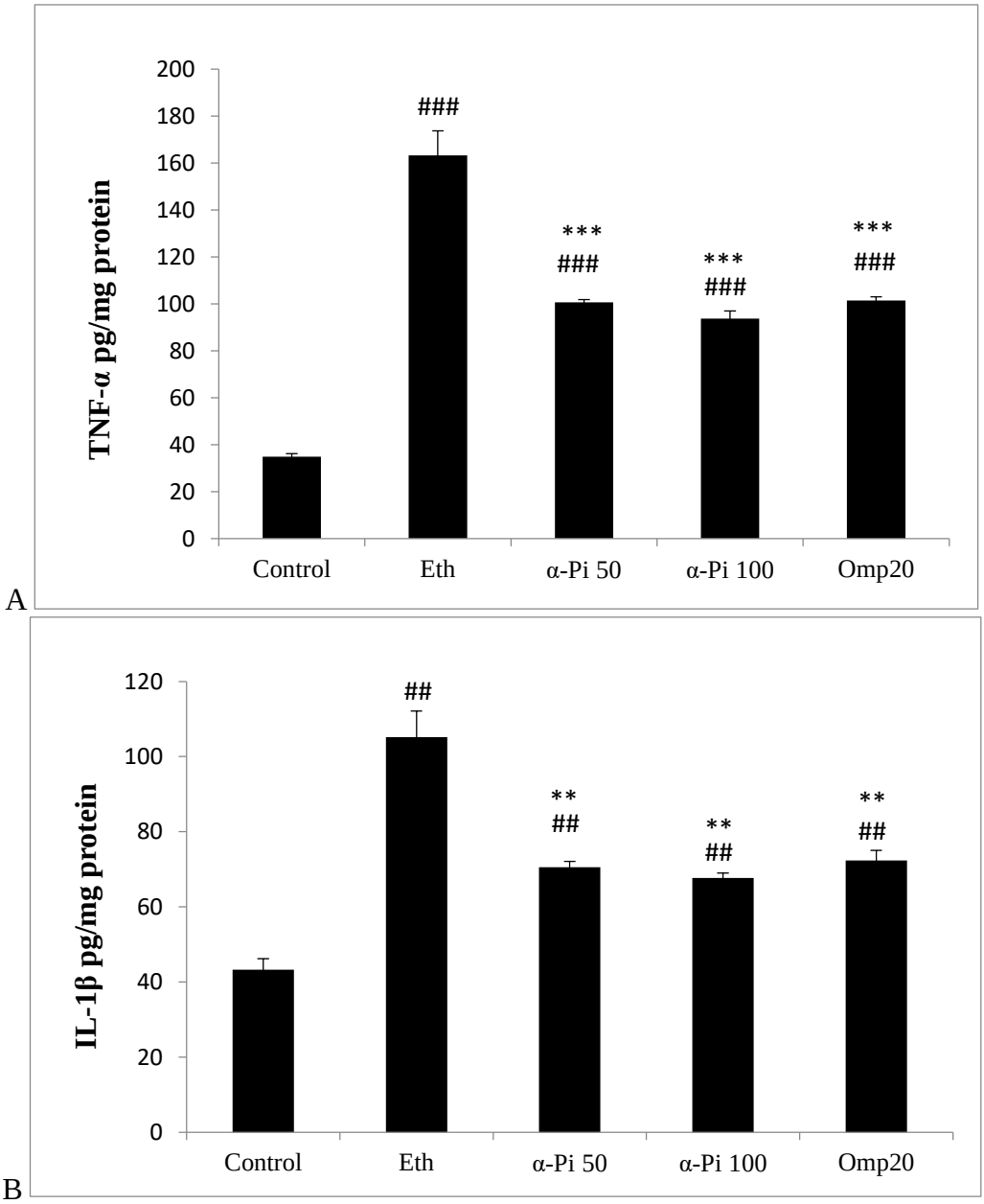

Figure 4: The effects of α- pinene on the levels of (A) TNF-α and (B) IL-1β. (Mean±SEM) in control, ethanol (Eth), alpha-pinene 50 and 100 mg/kg groups + ethanol, and Omeprazole group with a dose of 20 mg/kg + ethanol (Omp 20). # p< 0.05, ## p< 0.01, and ### p< 0.001 show a significant level difference compared to the control group. *p< 0.05, **p< 0.01, and ***p< 0.001 show a significant level difference compared to the ethanol group (n=6).

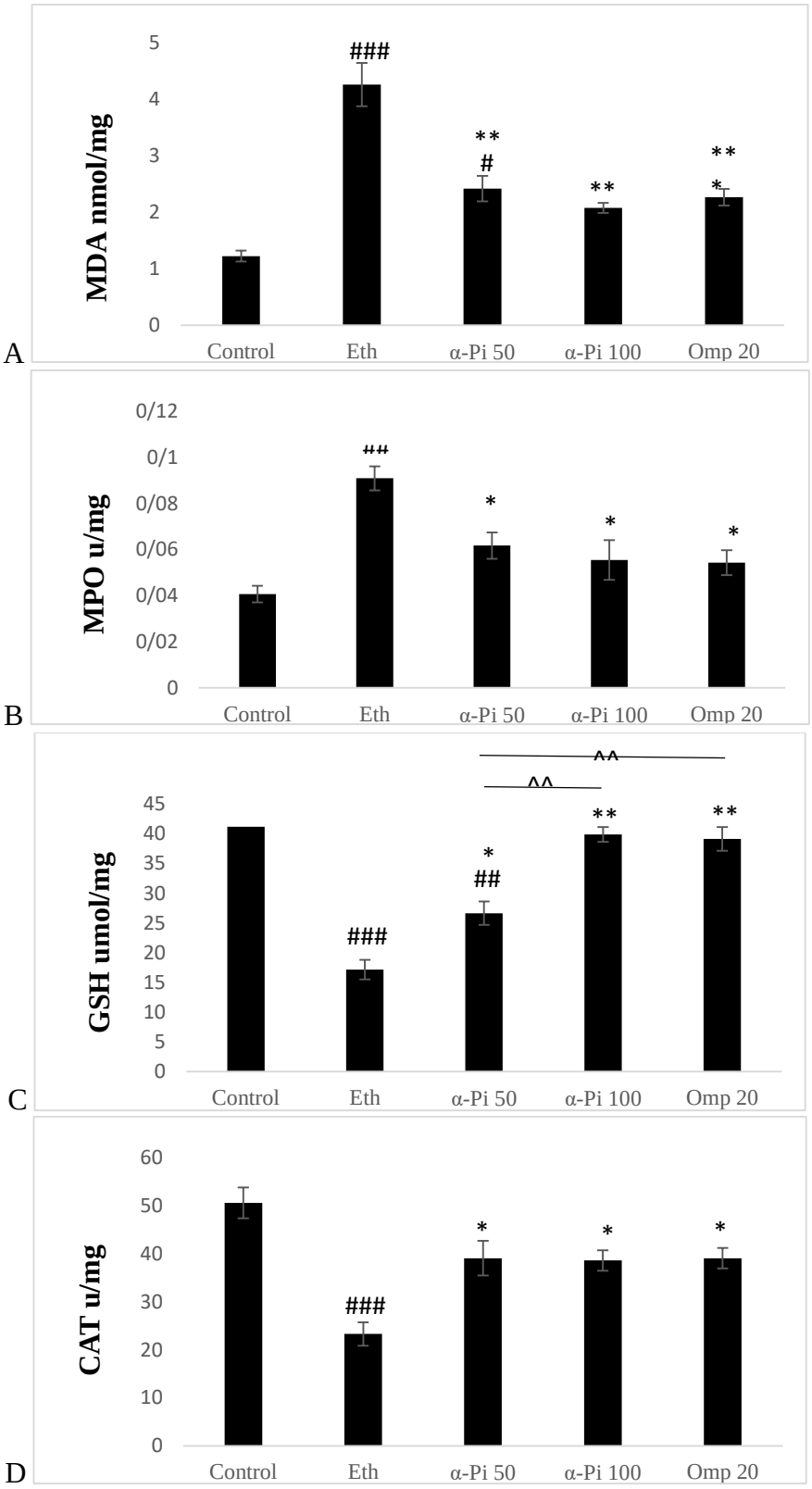

Figure 5: The effects of α- pinene on the levels of (A) MDA, (B) MPO, (C) GSH, and (D) CAT. (Mean±SEM) in control, ethanol (Eth), alpha-pinene 50 and 100 mg/kg groups + ethanol, and Omeprazole group with a dose of 20 mg/kg + ethanol (Omp 20). # p< 0.05, ## p< 0.01, and ### p< 0.001 show a significant level difference compared to the control group. *p< 0.05, **p< 0.01, and ***p< 0.001 show a significant level difference compared to the ethanol group. ^^p< 0.01 show a significant level difference alpha-pinene 50 + ethanol compared to the alpha-pinene 100 mg/kg + ethanol and Omeprazole group with a dose of 20 mg/kg + ethanol groups (n=6).

Discussion

The oral administration of ethanol induces a gastric ulcer model that is similar to the human gastric ulcer disorder. This model is useful for investigating the anti-ulcer effects of different drugs (Huang et al, 2013; Kim et al, 2005). Many studies have investigated the effect of medicinal plants and their substances on stomach ulcers, highlighting its significance (Bi et al, 2014). Our study evaluated the potential therapeutic effects of α- pinene in an ethanol-induced gastric ulceration model in rats. In our study, pretreatment with alpha-pinene (50 and 100 mg/kg) exhibited a gastroprotective effect against ethanol-induced gastric ulceration, reducing the gastric ulcer index.

The previous studies have shown that alpha-pinene has protective effects against inflammation and oxidative stress in vitro. For instance, in lipopolysaccharide (LPS)-stimulated macrophages, alpha-pinene reduces TNF-α, IL-6, and nitric oxide (NO) production by suppressing the nuclear factor kappa B (NF-κB) pathway (Kim et al, 2015; Kwak et al, 2019). Moreover, alpha-pinene has exhibited antioxidant and anti-inflammatory properties in ischemia and stroke experimental models (Choi et al, 2010). We studied the impact of alpha-pinene on the levels of pro-inflammatory cytokines in rat stomach tissue damaged by ethanol consumption (Abdelwahab, 2013; Badr et al, 2019; Li et al, 2018; Raish et al, 2021; Ren et al, 2020; Su et al, 2019). Our study showed that alpha-pinene can reduce inflammation in gastric tissue by suppressing TNFα and IL1β production at doses of 50 and 100 mg/kg.

Excessive production of reactive oxygen species (ROS) is a harmful factor in the progression of tissue damage (Corsini et al, 2018; Juránek et al, 2013; Nikitovic et al, 2013). Antioxidant systems in the body consist of various enzymes and biomolecules and defend against the adverse effects of ROS (Kıran et al, 2023). The first line of defense against oxidative stress involves enzymes such as CAT, GPX, and SOD. SOD neutralizes superoxide, producing hydrogen peroxide (H2O2), which is then eliminated by CAT (Ighodaro et al, 2018). Also, GPX is responsible for the reduction of H2O2 (Gebicka et al, 2019; Sharifi-Rad et al, 2020). Our study found that pretreatments with 50 and 100 mg/kg of alpha-pinene increased CAT levels compared to an ethanol-treated group. GSH reacts with ROS and non-enzymatic antioxidants, protecting cells against oxidative damage caused by H2O2, superoxide anion, hydroxyl radicals, and alkoxyl radicals. It also prevents inactivation of enzymes and proteins (Asantewaa et al, 2021). Our findings indicated that pretreatment with alpha-pinene and omeprazole led to higher levels of GSH when compared to a group treated with ethanol. Acute ethanol administration increases MDA and MPO activity (Li et al, 2011; Li et al, 2021). MPO promotes ROS production (Chen et al, 2020), which reacts with cell membranes causing, lipid peroxidation. This leads to oxidative damage in gastric tissue (Yu et al, 2017). Our study showed that pretreatment with alpha-pinene and omeprazole reduces levels of MDA and MPO compared to an ethanol-treated group. These results suggest that alpha-pinene could improve oxidative stress and inflammation in gastric tissue caused by ethanol exposure. Additionally, the previous research has found that alpha-pinene has antioxidant effects which could prevent UVA-induced aging by increasing antioxidant enzymes (CAT, GPX, and SOD) and reducing lipid peroxidation in an aging model caused by UVA (Karthikeyan et al, 2019).

Previously, the effect of pretreatment with alpha-pinene on gastric ulceration induced by ethanol in male Swiss rats was investigated. Alpha-pinene reduced gastric juice volume and acidity while increasing gastric wall mucus (Pinheiro Mde et al, 2015). GC/MS analysis revealed alpha-

pinene as the main component of Pistacia atlantica Deaf essential oil. The oil's protective impact against ethanol-induced gastric ulcer was evaluated in an animal model. Administering oral doses of 25, 50, and 100 mg/kg of Pistacia atlantica Deaf essential oil one hour before wound induction by ethanol led to a reduction in the destruction and necrosis of stomach tissue (Memariani et al, 2017). Studies suggest that Pistacia atlantica, Cymbopogon citratus, and Origanum vulgare L can protect against ethanol-induced gastric ulcer (Périco et al, 2020). Gastroprotective effects of alpha-pinene in ethanol-induced gastric ulcers have been demonstrated (Al-Juhaishi, 2014). However, the mechanism of pinene's effect on the digestive system is not well understood. According to its chemical structure, pinene has antioxidant and anti-inflammatory effects (Salehi et al, 2019). Thus, alpha-pinene may offer protection against gastric ulcers by reducing inflammation and oxidative stress.

Alpha-pinene pretreatment suppressed abnormal gastric changes caused by ethanol-induced gastric ulcers. Alpha-pinene had protective effects against gastric ulcers by reducing oxidative stress and inflammatory cytokine production. However, there are limitations to the experimental research methods. One limitation is that experiments may not reflect the real-world situations. Further research is needed to generalize the results from animal models to humans.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Removal of lodged esophageal foreign bodies by gastrotomy in two dogs

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Abstract

One of the most serious emergency situations in dogs is esophageal obstruction. Bones are mostly reported as foreign bodies which cause obstruction. This study reports two similar situations where dogs had a bone stuck in their esophagus. The dogs showed symptoms such as regurgitation, respiratory distress, salivation, and retching. The endoscopy had been attempted to migrate the bone orally in the previous veterinary clinics, but it failed and both cased were referred to the Veterinary Teaching Hospital of Shiraz University. Plain radiography was repeated to confirm the presence of the foreign body. Initially, our treatment plan involved moving foreign objects toward the stomach before proceeding with a gastrostomy procedure. Due to the fact that both foreign bodies were lodged in the esophageal mucosa and the esophagus should not be incised as much as possible, a long fine-tips alligator forceps was used to pull out both foreign bodies from the gastrotomy incision. In both patients, the foreign bodies were removed from the esophagus without causing any mucosal damage. After wo weeks, the cases showed no complications.

Key words: Esophagus, Foreign body, Surgery

Introduction

One of the most common emergency situations in dogs is the presence of a foreign body in the esophagus. The cases with esophageal foreign body (EFB) often show the clinical signs including regurgitation, vomiting, salivation, retching/gagging, coughing and dysphagia, and halitosis (Luthi and Neiger, 1998). The most commonly reported EFBs in veterinary medicine include bones, wood, sewing needles, toys, fishing hooks, and other food items (Brisson et al, 2018; Dunlap and Risselada, 2019). Reports indicate that EFBs are frequently observed in small breed dogs, such as poodles, West Highland White Terriers, Yorkshire Terriers (Deroy et al, 2015; Luthi and Neiger, 1998). Although some studies stated that younger dogs are more susceptible, others stated that very young and old dogs can equally be affected (Deroy et al, 2015; Dunlap and Risselada, 2019). In many cases, a plain radiograph or endoscopy can be used to diagnose the presence of foreign body and determining its type and location. The present study describes two similar cases that the foreign bodies pulled out through the gastrotomy.

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Case descriptions

Case I

A 4-year-old, intact, male Pomeranian dog (4 kg), exhibiting respiratory distress, salivation, and retching from previous day, was referred to the Veterinary Teaching Hospital of Shiraz University on 9 July 2022. The history indicates that the dog had been visited by another clinic earlier in order to remove a foreign body from esophagus, but due to unsuccessful endoscopic attempts, it was subsequently referred. Immediately, diagnostic imaging was performed to determine the presence, location, and size of the foreign body. Radiography revealed a foreign body with an osseous tissue nature and dimensions of 3.1 cm × 3.7 cm at the base of heart (Figure 1a). Laboratory findings showed only a mild dehydration without any other abnormality.

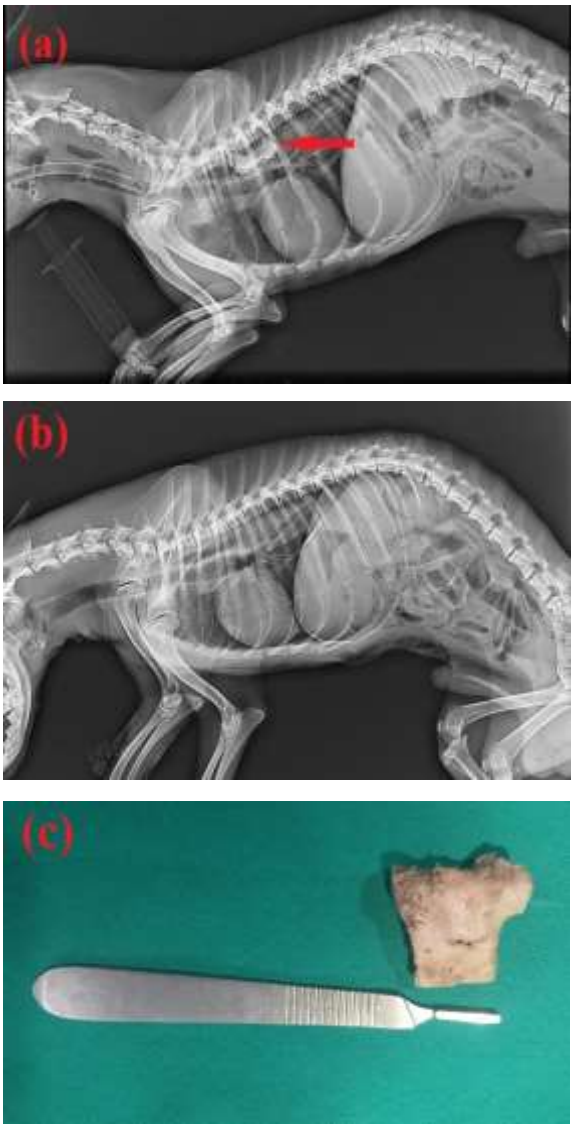

Figure 1: (a) A 4-year-old, intact, male Pomeranian dog with esophageal foreign body (red arrow). (b) Plain radiograph after removal of foreign body. (c) The extracted bone.

Case II

A 4.5-year-old, neutered, male Pomeranian dog (8.5 kg) was referred to the Veterinary Teaching Hospital of Shiraz University on 21 May 2023 with clinical signs of dysphagia, regurgitation, and salivation for 2 days. The owner stated that a foreign body (bone) was diagnosed in the esophagus in another clinic and was not removed by endoscopy performed there. Radiographic examination confirmed the presence of a foreign body with bone density in esophagus at the diaphragmatic hiatus with dimensions of 3.5 cm × 4.0 cm (Figure 2a). The fluid therapy was performed in the previous clinic, and the patient had no symptoms of dehydration when referred. Also CBC examination showed normal findings.

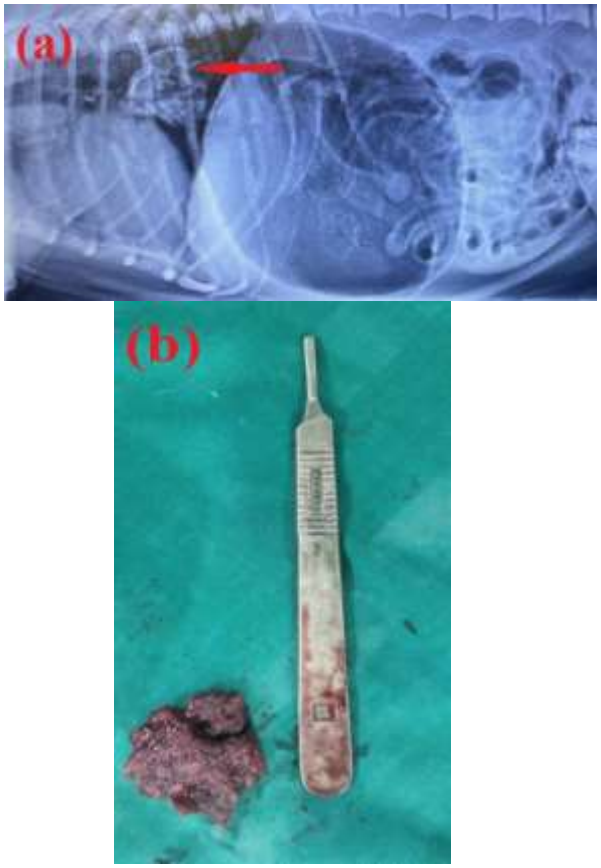

Figure 2: (a) A 4.5-year-old, neutered, male Pomeranian dog with esophageal foreign body (red arrow). (b) The extracted bone.

Surgical procedure

Prior to surgery, fluid therapy was carried out to treat the mild dehydration of the patients. The animals were sedated with a single dose of acepromazine (0.05 mg/kg, IM, Alfasan, The Netherlands). Then, the patients were catheterized and induced by propofol (4 mg/kg, IV, Beheshtan Darou Pharmaceutical Co., Iran). After induction, the patients were intubated in order to prevent the aspiration any material regurgitated from the esophagus. A stomach tube was used to advance the bone toward the stomach, but it failed in both cases; therefore, gastrotomy was selected as the course of treatment. The anesthesia was maintained by 1.5% isoflurane (Minrad International Inc., Orchard Park, USA) and a single dose of cefazolin (22 mg/kg, IV, Dana Pharmaceutical Co., Iran) was administered as prophylactic antibiotic therapy. Next, the patients were restrained in dorsal recumbency, and the surgical area (midline region) was shaved, scrubbed, and draped. During the surgery, vital signs including oxygen saturation (SpO_2), respiratory rate, and heart rate were constantly monitored and the patients received Ringer's Solution at 10 cc/kg/h (Shahid Ghazi Pharmaceutical Co., Tehran, Iran) and Fentanyl (5 µg/kg, slow IV, Caspian Tamin Co., Iran). A cranial midline incision (from umbilicus to xiphoid process) was made to access the stomach. After packing stomach with moist sterile gauze sponges, two stay sutures were placed on stomach. Gastrostomy was performed with an incision between the greater and lesser curvature of the stomach. The foreign bodies in both cases were then removed using a long fine-tips alligator forceps. First, stomach was sutured using a simple continuous pattern followed by a Cushing pattern using USP 2/0 Vicryl™ (Supabon, Supa Medical Devices Co., Iran). In this stage, the surgical gloves were changed with new ones, then abdominal wall (*linea alba*) and subcutaneous tissues were sutured in two individual layers using simple continuous pattern by USP 0 Vicryl™. At the end, skin was sutured using intradermal pattern by USP 2/0 Vicryl™.

Postoperative care included tramadol (1 mg/kg, P.O., q8h, Alborz Darou Pharmaceutical Co., Iran) for 3 days and cephalexin (20 mg/kg, P.O., q12h, Farabi Pharmaceutical Co., Iran) for 5 days. The owner was suggested to avoid feeding the dog orally for 24 hours, followed by a gradual introduction of a small amount of soft food. Sucralfate (500 mg per dog, P.O., q8h, Soha Pharma Co., Iran) was also administered to protect the esophageal mucosa. Both cases showed no complications after two weeks and were then fully recovered.

Discussion

Foreign body ingestion is a critical emergency condition that requires immediate medical/surgical intervention.

EFBs can be life-threatening if not treated, with the most common signs being regurgitation or increased vomiting (Davoodi et al, 2021; Luthi and Neiger, 1998). EFBs are commonly found in various species, including horses, cattle, cats, and dogs (Craig et al, 1990; Davoodi et al, 2021; Gomez et al, 2014; Haas, 2010). The most common type of EFB in veterinary medicine is bone (Luthi and Neiger, 1998).

EFBs can be usually trapped in the thoracic inlet, the base of the heart, and diaphragmatic hiatus, with the diaphragmatic hiatus being the most frequent site (62%) (Davoodi et al, 2021; Luthi and Neiger, 1998). Complete esophageal obstruction can cause ptyalorrhea and respiratory distress, while incomplete esophageal obstruction can cause anorexia and dysphasia (Haas, 2010). Small breeds like poodles, West Highland White Terriers, and Yorkshire Terriers are more likely to be affected (Deroy et al, 2015; Luthi and Neiger, 1998).

Diagnosing EFBs is crucial for prompt treatment. Oral examinations, palpation, passing stomach tubes, and endoscopy are the methods for detecting the presence of foreign bodies. Plain radiology is one of the other methods of reliable diagnosis that is useful to diagnose the presence, type, location, and dimensions of foreign body. Radiology can also help diagnose the aspiration, abdominal free air, and subcutaneous emphysema (Pfau, 2014). Double contrast radiology is also effective to diagnose the presence and type of foreign body (Haas, 2010).

Treatments include glucagon prescription (relaxes the smooth muscles of the esophagus), balloon catheter or Foley catheter technique (with or without fluoroscopy or endoscopy guide), flexible endoscopy (minimally invasive technique), laryngoscope or rigid endoscope (FBs located close to the pharynx), and various tools like Thygesens probing extractor, stomach tube, and covault hook etc (with or without endoscopy guide). However, there is always a risk of esophageal rupture/perforation when using these tools (Cangir et al, 2002; Pfau, 2014; Davoodi et al, 2021; Haas, 2010).

Foreign body treatment involves surgery, including esophagotomy (which can be performed in the cervical area or chest) and gastrotomy. But due to segmental blood supply, continuous peristaltic movements, and lack of serous layer, efforts have been made to avoid esophagotomy surgery as it has prolonged recovery and significant postoperative complications (Fossum, 2018). Instead, the foreign body is often directed into the stomach and extracted through a gastrotomy, which was done in the cases of the current study.

After foreign body extraction, minor lesions should be treated with broad-spectrum antibiotics and dietary restrictions. Esophagitis should be treated immediately with proton pump inhibitors and H_2-receptor antagonists, and sucralfate can provide a protective barrier against gastric acid (Luthi and Neiger, 1998). In general, no foreign body should be allowed to remain for more than 24 hours, and it should be removed within 6 to 12 hours since the time passes, the complications will increase (Pfau, 2014). Complications of this condition can be acute, including ulceration, esophagitis, and pneumothorax, and late, including fistulae and strictures (Davoodi et al, 2021). The prognosis can be good if intervention is done between 2 and 12 hours from the onset of symptoms. Foreign bodies that are not treated within 24 to 48 hours of obstruction have a much worse prognosis (Haas, 2010).

In conclusion, foreign body ingestion is an emergency condition with high priority to medical/surgical intervention. Endoscopy is an effective and non-invasive means of foreign bodies' diagnosis and treatment. If the foreign body cannot be removed via endoscopy, surgical intervention is required.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Corrective Osteotomy and Intramedullary Pinning in a Golden Eagle with Angular Malunion Fracture of Radius and Ulna

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Abstract

Some of avian orthopedic problems occur as a result of trauma (ceiling fan injuries, flying into a window or mirror, or getting stepped on); others occur from nutritional imbalances or as a result of genetic or developmental problems. An adult female golden eagle (*Aquila chrysaetos*) was presented to the clinic of faculty of veterinary medicine of Razi University (Kermanshah, Iran) with a history of being unable to fly. Physical examination revealed some abnormality in the left wing. Radiographic examination showed radius and ulna midshaft fracture angular malunion. The bird was prepared for aseptic surgery and so malunion part of the bone was cut. After correction and aligning of the bones, both ulna and radius were fixed with intramedullary pin. The bird was discharged without any complication. Case follow up showed an uneventful improvement and 6 weeks later the ulna pin was removed, but the radius pin was remained. Most of the orthopedic techniques developed for mammals can be applied to birds, taking into account the anatomical differences in avian species, and whether or not the bird can have flight restored. Fracture malunion in raptors can compromise muscle and tendon function and adversely affect normal activities that are essential for survival of these species. Malunion can be acutely corrected by osteotomy techniques, followed by bone fixation that provides sufficient stability to allow unimpeded healing with minimal soft tissue injury.

Keywords: Eagle, Intramedullary pin, Malunion, Osteotomy

Introduction

The Golden Eagle (Aquila chrysaetos) is one of the best birds of prey in the Northern Hemisphere. Like all eagles, golden eagle belongs to the eagle family. In Iran, this animal is found in many different parts of the country (Babaei et.al, 2022). Avian fractures heal in the same manner as those in mammals but the rate of bone heals is probably a little faster (Tully, 2002; Straw, 1984). It is most rapid in the smaller birds and one can detect signs of healing on X-ray plates within eight days (Ecams et al, 2007). Avian bones have a high calcium content, and a thin, brittle nature (Ritchie et al, 1994). As in mammals, excessive displacement of the fractured ends, movement and infection all retard healing (Ecams et al, 2007). Wing and leg fractures in birds are the most common problems (Ecams et al, 2007). Thirty percent of the fractures involve only the ulna, 60% involve the ulna and radius, and 10% only the radius (Forbes, 1998). These bones have little soft tissue support, the soft tissue being prone to desiccation. In birds having a fracture of either the ulna or the radius, cage

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rest alone (with or without support) is usually adequate, if there is little displacement. The bird should be able to move its wings but not extend or flap them fully, for a period of 2-3 weeks. (Forbes, 1998). Recommended surgical techniques used to repair ulnar fractures include the use of an intramedullary pin, external skeletal fixator tie-in or a type I external skeletal fixator (Sarker et al, 2022). Because of the relative mobility of the radius, external coaptation alone is not recommended for the management of radial fractures, which are best stabilized with an intramedullary pin (Ritchie et al, 1994). Viable and nonviable malunions can occur in birds. Malunions occur when the ends of fractured bones heal but not to each other and can produce bone shortening, angulation of the distal fragment, rotation of the proximal or distal fragment, development of joint pain, or cosmetic deformity (Roush, 1980). Stabilization requires removing necrotic debris, freshening the bone ends and compressing and rigidly stabilizing the fracture site (Roush, 1980). The case study describes, corrective osteotomy and intramedullary pinning in an eagle with angular malunion fracture of radius and ulna.

History

An adult female golden eagle (*Aquila chrysaetos*) (Figure 1) was presented to the clinic of faculty of veterinary medicine of Razi University (Kermanshah, Iran) with a history of being unable to fly. Physical examination revealed some abnormality in the left wing. Radiographic examination showed radius and ulna midshaft fracture angular malunion (Figure 2a). The bird was anesthetized with midazolam (Exir-Iran, Tehran, Iran) (1mg/kg) and ketamine (Alfasan, holland) (20mg/kg). The antebrachium feathers were plucked, and the surgical site was prepared routinely for Aseptic surgery. The bird was restrained in dorsal recumbency and the affected region was draped. An incision was made on craniomedial side of the antebrachium. A closing-wedge osteotomy was performed at the point of the greatest deformity of the ulna and radius by means of an oscillatory bone saw irrigated with 0.9% saline solution (Figure 3). After correction and aligning of the bones, the ulnar fracture was fixed with 2 mm Kirschner wire by retrograde intramedullary pinning method. The radius was fixed with an 1mm intramedullary pin via a shuttle method. The surgical site was vigorously irrigated with physiologic saline and muscles and subcutaneous tissues were sutured with simple continuous suture pattern using no. 3/0 catgut (Pezeskyaran, Tehran, Iran). The Skin was sutured with the same suture pattern using no. 2/0 nylon (Pezeskyaran, Tehran, Iran). Antibiotic therapy with enrofloxacin (Razak, Tehran, Iran) (20 mg/kg IM) was begun immediately after anesthetic induction and continued orally for 4 days. A figure-of-eight bandage was placed on the right wing, and the wing was bandaged to the body. The bandage was changed twice every five days and removed after 15 days. The owner of the bird was advised to keep the bird in confinement. Immediate postoperative radiographs showed correction of bone malalignment (Figure 2b). Although the recovery from the anesthesia was prolonged, but the bird was discharged without any complication. Case follow up showed an uneventful improvement and 6 weeks later the ulna pin was removed, but the radius pin was remained.

Figure 1: An adult female golden eagle (*Aquila Chrysaetos*) with angular malunion fracture of radius and ulna

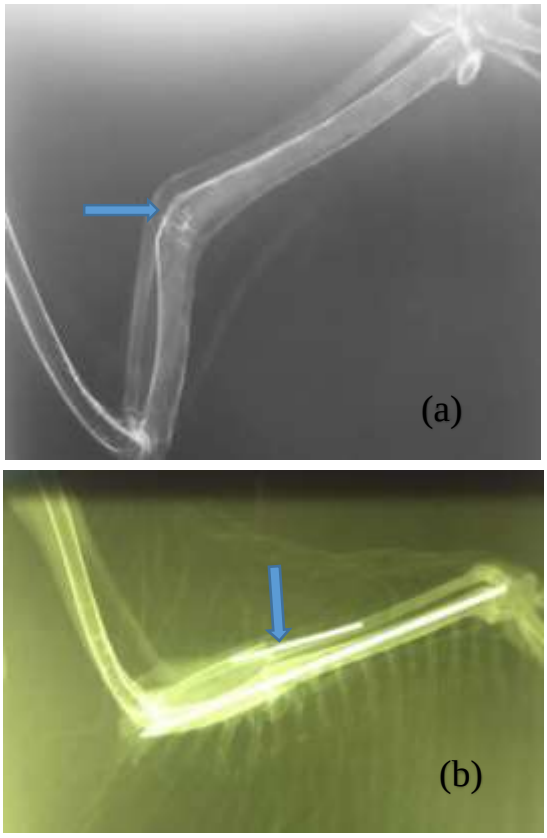

Figure 2: Radiographic images of the radius and ulna before(a) and after(b) corrective osteotomy

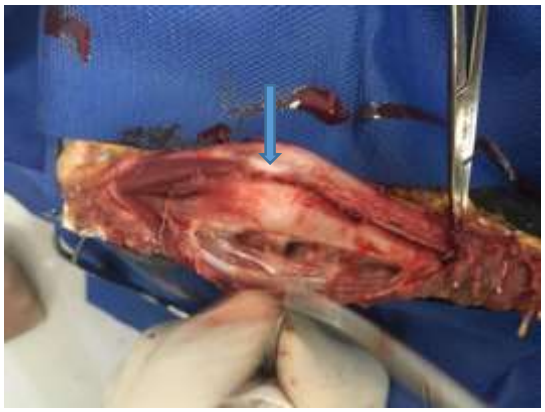

Figure 3: Intra-operative image of the malunion radius & ulna

Discussion and Conclusion

Fracture malunion in a raptor can compromise muscle and tendon function and adversely affect normal activities that are essential for survival in the wild. Several techniques of corrective osteotomies are described that should be used according to deformity, including transverse-opening wedge, closing wedge, reverse wedge, oblique, stairstep, transverse derotational; and dome (Ecams et al, 2007). Malunion can be acutely corrected by osteotomy techniques followed by bony fixation that provides sufficient stability to allow unimpeded healing with minimal soft-tissue injury (Rochat et al, 2005). Different types of methods such as external coaptation, external skeletal fixation, intramedullary pin alone or tie-in, intramedullary rod, circular ring fixators, bone plates, and interlocking nail, may be used to fixate the fracture or osteotomy site (Sarker et al, 2022). In the majority of cases, the most satisfactory results in terms of restoration of limb anatomy and function are achieved using internal fixation. In comparison to mammalian patients, bone plating has received scant attention for a variety of reasons, including small patient and bone size, cost, morbidity associated with placement and in the case of wild birds the need for follow-up surgeries to remove implants (Ecams et al, 2007). Intramedullary (IM) pins and external skeletal fixators (ESF) alone continue to be used with success as shown in a metacarpal fracture in a golden eagle (Aquila chrysaetos) and tarsometatarsal fracture in a Harris hawk (Parabuteo unicinctus) (Grier, 1973). The introduction of the ESF – IM pin "tie in" or "hybrid" fixator, however, has revolutionised the management of avian long bone fractures (Ritchie et al, 1994). Internal fixation may be used for fracture management in both medium and large birds in much the same manner as mammals. The major problem involves the lack of appropriate sizes of pins, screws and plates. Most of these were developed for human or small animal use. Consequently, their size and weight are prohibitive, except for the larger raptors. Other problems associated with internal fixation include providing adequate exposure, minimal sepsis and articulation insult (Fowler et al, 2003). Steinman pins may be used for retrograde pinning of the humerus but seem to be of more value in ulnar fractures when the radius is also fractured (Bush, 1977). In

about 50% of cases either the radius or the ulna is fractured, but not both. The method of repair of fractures of the radius and ulna will depend upon the integrity of the other bone of the pair. When only one bone is fractured it is wiser to leave the fractured bone alone. The normal one will help to splint it. Even if there is not perfect alignment of the healed fractured bone which does not matter, the bird will manage quite adequately and fly again. Strapping of the wing for 2–3 days only is required. However, if both radius and ulna are fractured some method of splinting will be required (Rupiper, 1993; Kubiak et al, 2011). The ulna is pinned in normograde fashion. Retrograde placement is contraindicated, as it risks exiting the pin at the olecranon and damaging the joint (Helmer et al, 2006). However, in selecting a method of treatment for birds, care must be taken with regard to device weight, fracture and/or osteotomy location, the absence of soft tissue support, pneumatic long bones, with large intramedullary spaces, brittle and thin cortical bone, and level of function expected after treatment (Ozsemir et al, 2021; MacCoy, 1992). Although repair of avian malunion fractures have been reported (Kuzma et al, 1991; Marlow et al, 1981; Mitchell et al, 2002; Rochat et al, 2005). the treatment is always challenging and each case should be evaluated individually. In this case, after correction and aligning of the bones, both ulna and radius were fixed. The bird was discharged without any complication. Case follow up showed an uneventful improvement and 6 weeks later the ulna pin was removed from the ulna, but the radius pin was remained.

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Conflict of Interest

The authors have no conflict of interest to declare.

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اثر جایگزینی پودر ماهی با پودر ضایعات طیور بر پارامترهای سرمی و هیستومورفولوژی کبد و کلیه (*Oreochromis niloticus*, Linnaeus ۱۷۵۸) در ماهی تیلاپیا نیل

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چکیده

امروزه *Oreochromis niloticus* به یکی از محبوب‌ترین ماهی‌ها در جهان تبدیل شده است. با توجه به افزایش قیمت پودر ماهی و محدودیت دسترسی به آن، منابع پروتئینی مختلف از جمله پودر ضایعات طیور می‌توانند جایگزین پودر ماهی در جیره آبزیان شوند. بنابراین این مطالعه به منظور بررسی اثر جایگزینی پودر ماهی با نسبت‌های مختلف پودر ضایعات طیور بر روی تغییرات ساختار بافت کبد و کلیه، عملکرد رشد و برخی پارامترهای سرمی *O. niloticus* انجام شد. برای این منظور، ۱۲۰ ماهی *O. niloticus* به طور تصادفی در چهار گروه کنترل، ۲۵، ۵۰ و ۱۰۰ درصد تقسیم و به مدت ۴۴ روز از پودر ضایعات طیور به جای پودر ماهی تغذیه شدند. در پایان دوره درمان، پارامترهای رشد، سرم خون، آنزیم‌های کبدی و هیستومورفولوژی کبد و کلیه ماهیان مورد بررسی قرار گرفت. نتایج نشان داد که آنزیم‌های کبدی در گروه‌های با جایگزینی بالاتر در مقایسه با تیمار شاهد به طور معنی‌داری بالا رفت که در بررسی‌های بافت‌شناسی بر روی کبد نیز بافت کبد با افزایش مقدار جیره‌های جایگزین ساختار طبیعی و عملکرد خود را از دست داده بودند و در سیتوپلاسم هپاتوسیت‌ها واکوئل‌های چربی تجمع یافته بود. میزان اوره پلاسما هم با افزایش مقدار جیره‌های جایگزین در بین گروه‌های با جایگزینی صعودی، اختلاف معنی‌داری نسبت به تیمار شاهد داشته که در بررسی‌های بافت‌شناسی بر روی کلیه نیز این تغییرات در ساختار توبول‌ها و گلومرول‌ها مشهود بود. به طور کلی، داده‌ها نشان می‌دهد که ۱۰۰ درصد پودر ضایعات طیور برای جایگزینی پودر ماهی در *O. niloticus* توصیه نمی‌شود، اما پودر ضایعات طیور تا سقف ۵۰ درصد می‌توانند جایگزین پودر ماهی برای جیره *O. niloticus* بدون تأثیر نامطلوب بر عملکرد رشد و پارامترهای بیوشیمیایی ماهی شوند. اما باز هم برای بررسی مقدار دقیق‌تر باید جایگزینی‌هایی با مقدار کم‌تر از ۵۰ و به فواصل کم‌تر انجام شود.

کلمات کلیدی: پودر ماهی، عملکرد رشد، تغییرات هیستومورفومتری، *Oreochromis niloticus*، پودر ضایعات طیور

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تعیین مولکولی گونه‌های فوزوباکتریوم در سگ‌های مبتلا به (یا بدون) ژنژیویت/ پریودونتیت در اهواز و تهران

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چکیده

پریودونتیت، یکی از شایع‌ترین بیماری‌ها، در حیوانات خانگی می‌باشد. باکتری‌های محوطه دهانی، نقش مهمی در ایجاد این عارضه دارند. گونه‌های فوزوباکتریوم، یکی از فاکتورهای مهم باکتریایی است که در پیشرفت پریودونتیت نقش مهمی دارند. هدف از انجام مطالعه حاضر، بررسی ارتباط بین حضور فوزوباکتریوم و تحت گونه‌های آن و ژن‌های حدت آن‌ها (fadA و لکوتوکسین)، در سگ‌های مناطق اهواز و تهران، با یا بدون ژنژیویت/ پریودونتیت می‌باشد. یکصد و پنجاه قلاده سگ (۷۵ سگ از اهواز و ۷۵ سگ از تهران)، بین ۱۱-۲ سال، که ۷۸ سگ نر و ۷۲ قلاده ماده بودند، در طی ۱۰ ماه نمونه‌برداری شدند. نژادهای عمده مورد مطالعه شامل ترییر سفید، پودل، پامرانین، شیتزو، یورکشایر ترییر، پاگ، اشپیتز، مالتیز و بقیه از نژادهای دیگر بودند. آن‌ها از غذای خانگی، خشک و مخلوط تغذیه شده بودند. ۲۰ قلاده سگ (۱۳/۳۳ درصد) لثه سالم ،۳۲ مورد، پریودنتیت درجه ۱ (۲۱/۳۳ درصد) ۴۷ قلاده دیگر، پریودونتیت درجه ۲ (۳۱/۳۳ درصد) و ۵۱ سگ، پریودونتیت درجه ۳ (۳۴ درصد) داشتند. ۲۷ مورد از ۱۵۰ نمونه مورد مطالعه، مبتلا به جنس فوزوباکتریوم بودند (۱۸ درصد؛ ۲۴/۱- ۱۱/۸ درصد= $CI_{\%95}$). فروانی نسبی این آلودگی در تهران و اهواز به ترتیب ۲۱/۳ (۳۰/۶-۱۲/۰= $CI_{\%95}$) و ۱۴/۶ (۲۲/۶-۶/۶ درصد= $CI_{\%95}$) بودند. بررسی ژن لکوتوکسین در ۱۸ نمونه *Fusobacterium necrophorum* نشان داد که ۱۱ نمونه (۶۱/۱۱ درصد) (۹ نمونه از تهران و ۲ نمونه از اهواز) دارای این ژن بودند و این تفاوت مشاهده شده در دو شهر، در حضور این ژن، از نظر آماری معنی‌دار نبود ($p\text{-value}=0.43;\ df=1;\ X^2=0.62$). از این ۲۶ نمونه، ۹ نمونه (۳۴/۶۱ درصد) دارای ژن حدت *fad*A بودند که ارتباط بین وجود ژن *fad*A و درجات پریودنتیت از نظر آماری معنی‌دار نبود ($p\text{-value}=0.41;\ df=1;\ X^2=0.68$). رگرسیون لجستیک چند متغیره نشان داد که سن، جنس، نژاد، پریودونتیت، منطقه و نوع غذا ۹۷/۶ درصد از تغییرات آلودگی را توجیه می‌کند و تنها جنسیت و پریودونتیت تأثیر معنی‌داری برمیزان آلودگی داشتند. حضور ژن *fad*A در *فوزوباکتریوم نوکلئاتوم*، جدا شده از پلاک‌های دندانی سگ‌های مبتلا به پریودنتیت و ژن لکوتوکسین در *فوزوباکتریوم نکروفروم* تحت گونه *نکروفروم*، در پریودونتیت واقع در مناطق مختلف تهران و اهواز معنی‌دار نبود. در قسمت نتیجه‌گیری، میزان شیوع فوزوباکتریوم در پریودنتیت درجه سه، ۱۶ درصد و در لثه‌های سالم ۲ درصد بودند.

کلمات کلیدی: بیماری‌های پریودونتال، *فوزوباکتریوم*، لکوتوکسین، ژن *fadA*، ژنژیویت، سگ

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مسمومیت تحت حاد تجربی با مونسین در بزها: یافته‌های خون‌شناسی و الکتروکاردیوگرافی

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چکیده

اثرات سمی مونسین، یک آنتی‌بیوتیک پلی‌اتری که غالبا به عنوان کوکسیدیواستات به کار می‌رود، در طیف وسیعی از حیوانات توصیف شده است. مطالعه حاضر برای بررسی چهره خون‌شناسی و الکتروکاردیوگرافیک مسمومیت تحت حاد با مونسین در بزها انجام شد. برای این منظور، مونسین با دز ۱۳mg/kg روزانه به مدت ۵ روز متوالی به ۷ رأس بز بالغ با لوله معدی تجویز شد. فراسنجه‌های خون‌شناسی شامل هماتوکریت، هموگلوبین، گلبول‌های سفید و شمارش تفریقی آن‌ها، پروتئین تام پلاسما و غلظت فیبرینوژن آن در روز قبل از تجویز مونسین و ۱۰ روز متوالی اندازه‌گیری شدند. الکتروکاردیوگرافی نیز روزانه به روش استاندارد اخذ شد. نتایج افزایش معنی‌دار هموگلوبین در روز ۱، گلبول‌های سفید در روز ۷، درصد نوتروفیل‌ها در روزهای ۵ و ۸، در صد لمفوسیت‌ها در روزهای ۵، ۸ و ۹ و درصد منوسیت‌ها در روزهای ۱ و ۳ را نشان داد. همچنین تعداد مطلق منوسیت‌ها در روزهای ۱، ۳ و ۶ و پروتئین تام پلاسما در روز ۱ افزایش معنی‌دار را نشان دادند. یافت‌های غالب در الکتروکاردیوگرافی دام‌های مورد آزمایش تاکی کاردی سینوسی، برادی کاردی سینوسی، آریتمی سینوسی، پایین افتادن قطعه S-T، ضربان‌های زودرس بطنی و تاکی کاردی بطنی بودند. بر اساس مطالعه حاضر مسمومیت تحت حاد با مونسین در بزها تغییرات اندکی در فراسنجه‌های خونی ایجاد می‌کند ولی اختلات الکتروکاردیوگرافی چندی را باعث می‌شود که می‌تواند به کاردیومیوپاتی توکسیک مربوط باشد.

کلمات کلیدی: مونسین، آنتی‌بیوتیک‌های پونوفر، بز، الکتروکاردیوگراف، آریتمی

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اثرات ال- تریپتوفان بر بیان ژن تریپتوفان هیدروکسیلاز دینسفالیک در جوجه‌های گوشتی تحت استرس گرمایی

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چکیده

سیستم مونوآمینرژیک مغز در شرایط استرس گرمایی تغییر می‌کند. تحقیقات نشان داده است که طیور تجاری حیواناتی بسیار حساس به استرس گرمایی هستند. از این رو، این مطالعه به بررسی اثرات ال تریپتوفان بر بیان ژن تریپتوفان هیدروکسیلاز ۱ و ۲ (TH1, TH2) دینسفالیک در جوجه گوشتی تحت استرس گرمایی به عنوان مدل حیوانی پرداخت. چهل و هشت جوجه گوشتی ۷ روزه به سه گروه تقسیم شدند. به جوجه‌ها ال-تریپتوفان (۲۵ و ۵۰ میلی‌گرم بر کیلوگرم) و نرمال سالین به صورت داخل صفاقی تزریق شد. سپس به مدت ۵ ساعت در معرض استرس حرارتی (۳۹ درجه سانتی‌گراد) قرار گرفتند. پس از ۵ ساعت درمان، پرندگان قبل از مرگ با ایزوفلوران بیهوش شدند. نمونه‌های مغز جهت ارزیابی بیان ژن گرفته شد. داده‌ها نشان داد که بیان ژن دینسفالیک TH1 و TH2 در شرایط تنش گرمایی کاهش یافته است. تجویز تریپتوفان با دوز ۵۰ میلی‌گرم بر کیلوگرم به طور قابل توجهی باعث افزایش سطح بیان TH1 و TH2 در جوجه‌های در معرض استرس گرمایی شد. می‌توان نتیجه گرفت که مسیر سروتونرژیک دینسفالیک ممکن است نقش مهمی در بهبود اثر تریپتوفان در شرایط استرس گرمایی داشته باشد.

کلمات کلیدی: تریپتوفان هیدروکسیلاز، جوجه گوشتی، دمای محیطی بالا، دینسفال

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تأثیر عصاره هیدروالکلی ریشه گون بر پارامترهای خونی و سلول‌های مغز استخوان پس از تجویز میتومایسین سی در موش‌های صحرایی نر

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چکیده

میتومایسین C (MMC) یک داروی ضد سرطان است، اما یک عارضه جانبی دارد و آن افسردگی مغز استخوان است. Astragalus membranaceus در طب سنتی چینی استفاده می‌شود. این گیاه دارای اثرات فارماکولوژیک مختلفی از جمله خون‌سازی، رگزایی و تحریک ایمنی است. این مطالعه اثرات عصاره هیدروالکلی ریشه گون را بر سلول‌های خونی و مغز استخوان پس از تجویز MMC نشان داد. بیست و هشت موش صحرایی نر نژاد اسپراگ- داولی (به وزن 250±30 گرم) به چهار گروه کنترل (بدون دارو و درمان)، میتومایسین (MMC، ۲ میلی‌گرم در کیلوگرم، داخل صفاقی)، عصاره ریشه گون (عصاره ریشه گون، ۵۰۰ میلی‌گرم در کیلوگرم به مدت ۱۴ روز) و درمان (تجویز میتومایسین ۲ میلی‌گرم در کیلوگرم، داخل صفاقی و درمان با عصاره ریشه گون ۵۰۰ میلی‌گرم در کیلوگرم به مدت ۱۴ روز) تقسیم شدند. پارامترهای هماتولوژیک (هماتوکریت، هموگلوبین، تعداد گلبول‌های قرمز (RBC)، شاخص‌های گلبول‌های قرمز، تعداد پلاکت‌ها، تعداد گلبول‌های سفید (WBC) و تشخیص افتراقی گلبول‌های سفید) و سلول‌های مغز استخوان (سری اریتروئید و میلوئید) اندازه‌گیری شدند. پس از دریافت MMC، پارامترهای خونی و سلول‌های مغز استخوان کاهش یافت. نتایج نشان داد که عصاره هیدروالکلی گون می‌تواند پارامترهای خونی شامل هماتوکریت، هموگلوبین، تعداد گلبول‌های قرمز، تعداد گلبول‌های سفید و پلاکت‌ها و همچنین سلول-های مغز استخوان را افزایش دهد. این مطالعه نشان داد که عصاره هیدروالکلی ریشه گون تأثیر مثبتی بر نشانه‌های کم خونی دارد.

کلمات کلیدی: ریشه گیاه گون، کم خونی، میتومیسین سی، مغزاستخوان

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مقایسه چهار روش ارزیابی درد پس از عمل در سگ‌های تحت عمل جراحی برداشت رحم و تخمدان

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چکیده

هدف از مطالعه حاضر مقایسه ۴ روش ارزیابی درد شامل مقیاس توصیفی ساده (SDS)، مقیاس آنالوگ بصری (VAS)، مقیاس اندازگیری درد مرکب گلاسکو (GCPS-SF) و مقیاس درد دانشگاه ملبورن (UMPS) برای ارزیابی درد پس از عمل در سگ‌هایی که تحت عمل جراحی اوواریوهیسترکتومی قرار گرفتند، بود. بیست و دو سگ نژاد بومی به سه گروه بلوک خط برش (Incisional, n=7)، بلوک صفحه عرضی شکمی (TAP, n=7) و بلوک غلاف مستقیم شکمی (RSB, n=8) تقسیم شدند. پس از دریافت پیش‌بیهوشی با آسپرومازین (۰/۰۵ mg/kg) و مورفین (۰/۵ mg/kg)، بیهوشی با پروپوفول القا (۴ mg/kg) و نگهداری (۰/۴ mg/kg/min) شد. هر سگ به طور تصادفی یکی از روش‌های بی‌دردی را دریافت و سپس تحت عمل جراحی قرار گرفت. بی‌دردی پس از عمل تا ۶ ساعت پس از جراحی با روش‌های فوق‌الذکر بررسی شد. نتایج نشان داد که با روش گلاسکو، امتیاز درد در ساعات ۴، ۵ و ۶ پس از جراحی در بلوک خط برش و RSB بالاتر از زمان صفر بود. در روش ملبورن در گروه RSB در ساعات ۲، ۳، ۴، ۵ و ۶ پس از جراحی امتیاز درد بالاتر از زمان صفر بود. در روش VAS، امتیاز درد در بلوک RSB، در ساعات ۴، ۵ و ۶ پس از جراحی بالاتر از زمان صفر بود. در روش SDS، امتیاز درد بلوک خطی و RSB در زمان‌های ۳، ۴، ۵ و ۶ پس از جراحی نسبت به زمان صفر بالاتر بود. نتیجه‌گیری شد که روش UMPS می‌تواند درد را زودتر و با حساسیت بیشتری نسبت به سه روش دیگر نشان داد. برای اثبات نتایج نیاز به مطالعات بیش‌تر است.

کلمات کلیدی: مقیاس اندازه‌گیری درد مرکب گلاسکو، مقیاس درد دانشگاه ملبورن، مقیاس آنالوگ بصری، مقیاس توصیفی ساده، اوواریوهیسترکتومی

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اهمیت ورم پستان‌های استرپتوکوکی در گاوهای شیری در استان چهارمحال و بختیاری

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چکیده

ورم‌پستان بیماری التهابی غده پستان است که توسط عوامل عفونی متعددی همچون باکتری‌ها، قارچ‌ها و ویروس‌ها ایجاد می‌شود. استرپتوکوک‌ها جز پاتوژن‌های اصلی ایجادکننده ورم پستان در سراسر جهان هستند که می‌توانند اشکال درمانگاهی و تحت درمانگاهی ورم پستان را ایجاد کنند. ورم پستان یکی از بیماری‌های اولیه گاوهای شیری است که به دلیل کاهش کمیت و کیفیت شیر، هزینه درمان و حذف زود هنگام گاوها، زیان‌های اقتصادی قابل توجهی را به همراه دارد. با توجه به وجود گاوداری‌های صنعتی و سنتی فراوان در استان چهارمحال و بختیاری و این مهم که ورم پستان شایع‌ترین بیماری در گاوداری‌های شیری است، این مطالعه با هدف شناسایی نقش استرپتوکوک‌ها در ورم‌پستان‌های تحت درمانگاهی در گاوهای شیری استان چهارمحال و بختیاری انجام شد. برای این منظور، ۱۳۴ نمونه شیر ورم‌پستان تحت درمانگاهی از ۸ گاوداری در استان چهارمحال و بختیاری بر اساس نتایج آزمایش کالیفرنیایی ورم‌پستان (CMT) جمع‌آوری و از نظر علل استرپتوکوکی ورم‌پستان غربال شدند. DNA نمونه‌های جمع‌آوری شده استخراج شد و با پرایمرهای اختصاصی *استرپتوکوکوس آگالاکتیه*، *استرپتوکوکوس یوبریس* و *استرپتوکوکوس دیسگالاکتیه* واکنش زنجیره‌ای پلیمراز انجام شد. از ۱۳۴ نمونه شیر ورم‌پستانی، ۱۰ نمونه (۷/۵درصد)، ۱۴ نمونه (۱۰/۴ درصد) و ۵ نمونه (۳/۷درصد) به ترتیب از نظر *استرپتوکوکوس آگالاکتیه*، *استرپتوکوکوس یوبریس* و *استرپتوکوکوس دیسگالاکتیه* مثبت بودند. نتایج این مطالعه نشان می‌دهد که ۲۱/۶ (۲۹:۱۳۴) درصد از ورم پستان‌های تحت درمانگاهی گاوهای شیری در منطقه مورد مطالعه قرار گرفته، می‌تواند ناشی از استرپتوکوک‌ها باشد. نتایج به دست آمده می‌تواند در راهبردهای مربوط به مدیریت و پیشگیری ورم پستان گاو در استان چهارمحال و بختیاری مورد استفاده قرار گیرد.

کلمات کلیدی: ورم پستان، *استرپتوکوکوس*، گاو، PCR، چهارمحال و بختیاری

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ارزیابی رادیوگرافی عوارض استخوانی در سگ‌های ارجاعی به بیمارستان دامپزشکی دانشگاه شهید چمران اهواز

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چکیده

عوارض استخوانی در حیوانات خانگی، درصد نسبتاً قابل توجهی از موارد ارجاعی به بیمارستان یا کلینیک را در هر منطقه شامل می‌شود. عمده مشکلات استخوانی در سگ‌های جوان در حال رشد و نژادهای بزرگ گزارش شده است؛ ضمن این که سگ‌های نژاد کوچک نیز به برخی دیگر از بیماری‌های استخوانی مستعد می‌باشند. عدم تعادل در جیره غذایی (به ویژه از نظر کلسیم و فسفر) یکی از عوامل مؤثر در ایجاد عوارض استخوانی محسوب می‌شود. هدف از انجام مطالعه حاضر، تعیین میزان شیوع و انواع ضایعات استخوانی نظیر شکستگی‌ها، عفونت‌ها، تومورها و دیگر عوارض اکتسابی استخوانی در اندام‌های حرکتی، سر و ستون مهره‌ها بود. مطالعه حاضر در طی ۱۱ سال و بر اساس رادیوگراف‌های تهیه شده در بیمارستان دامپزشکی دانشگاه شهید چمران اهواز انجام شد؛ در ادامه فاکتورهایی نظیر سن، جنس، نژاد و محل عارضه، جهت تعیین ارتباط با عوارض استخوانی نیز بررسی شدند. در این مطالعه، ضایعات استخوانی از قبیل شکستگی‌ها، استئومیلیت و استئوآرتریت، نئوپلازی‌ها، دررفتگی‌ها در سگ‌ها و دیگر عوارض نظیر پان‌اوستئایتیس، استئوکندروز و استئودیستروفی در حیوانات جوان تعیین شدند. نتایج، به صورت آمار توصیفی، ارائه شده‌اند. جمعاً از ۴۳۵۵ مورد ارجاعی به بخش رادیولوژی، ۱۰۵۴ مورد (۲۴/۲۰ درصد) مربوط به سگ‌ها بودند. از این تعداد، ۴۲۵ مورد (۴۰/۳۲ درصد) دچار عوارض استخوانی بودند که ۴۶/۵۹ و ۵۳/۴۱ درصد، به ترتیب مربوط به نژادهای بزرگ و کوچک بودند. عوارض استخوانی شامل شکستگی‌ها، دررفتگی‌ها و دیگر عوارض بودند. از مهم‌ترین این موارد می‌توان شکستگی رادیوس (۲۶/۷۱ درصد)، ران (۲۸/۳۴ درصد)، درشت‌نی (۲۲/۴۶ درصد) و زند زیرین (۲۷/۹۵ درصد) را نام برد. سن حیوانات مورد مطالعه، در محدوده ۲ ماهگی تا ۹ سالگی قرار داشتند. از لحاظ جنسیت، ۶۲/۳۵ درصد از سگ‌ها، نر و ۳۷/۶۵ درصد از آن‌ها ماده بودند. از لحاظ آماری، تفاوت معنی‌داری از لحاظ سن، بین بالغین (۵۱/۲۹ درصد) و نابالغین (۴۸/۷۱ درصد)، جنس، محل عارضه و نژاد (بزرگ و کوچک) مشاهده نگردید. در قسمت نتیجه‌گیری، بیش‌ترین شیوع عوارض استخوانی، به ترتیب شکستگی‌های استخوان ران (۲۸/۳۴ درصد) و زند زیرین (۲۷/۹۵ درصد) بودند. نتایج به دست آمده نشان داد که رادیوگرافی یک روش قابل اطمینان، برای تشخیص عوارض استخوانی و تعیین فراوانی آن‌ها در سگ‌ها می‌باشد.

کلمات کلیدی: رادیوگرافی، عوارض استخوانی، شکستگی، سگ، اهواز

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اثر بازسازی داربست کیتوزان و اسید هیالورونیک با و بدون سلول‌های بنیادی مزانشیمی بر بهبود زخم

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چکیده

این مطالعه با هدف بررسی اثر ترمیمی داربست اسید هیالورونیک/ کیتوزان (CHAS) با و بدون سلول‌های بنیادی مزانشیمی (MSCs) بر روند بهبود زخم در موش‌های صحرایی انجام شد. گروه‌های مختلف درمان زخم به شرح زیر بودند: بدون درمان یا کنترل (C)، درمان زخم با CHAS، پوشش زخم با CHAS همراه با سلول‌های بنیادی مزانشیمی. اثر ترمیم زخم با اندازه‌گیری مساحت ناحیه زخم در هر موش در روزهای ۳،۵،۹ و ۱۴ اندازه‌گیری شد. سپس برای ارزیابی هیستوپاتولوژیک در روزهای فوق، هر زخم و ۵ میلی‌متر از بافت نرمال پوست اطراف هر زخم جدا و فیکس شد. نتایج نشان داد که در روزهای سوم و پنجم پس از تشکیل زخم، سطح زخم باقی‌مانده در گروه CHAS به طور قابل توجهی کوچک‌تر از CHAS با سلول‌های بنیادی مزانشیمی بود، اما تفاوت معنی‌داری با گروه C مشاهده نشد. همچنین سطح زخم باقی مانده در روزهای نهم و چهاردهم در گروه‌های مورد مطالعه تفاوت معنی‌داری نشان نداد. با این حال، در روز ۱۴، میانگین ناحیه زخم در گروه CHAS با سلول‌های بنیادی مزانشیمی کوچک‌تر از دو گروه دیگر بود. بررسی‌های بافت‌شناسی محل زخم از نظر آرایش کلاژن، التهاب، تشکیل عروق، بافت گرانولاسیون و بازسازی اپیتلیال مورد مطالعه قرار گرفت. مطالعات از نظر آرایش کلاژن، تشکیل بافت گرانوله و تشکیل عروق نشان داد که در روز سوم، بین گروه‌ها تفاوت معنی‌داری وجود داشت، در حالی که تفاوت آماری معنی‌داری بین گروه‌ها از نظر التهاب و بازسازی اپیتلیال در روزهای مورد مطالعه مشاهده نشد. همه این نتایج نشان می‌دهد که تفاوت معنی‌داری بین گروه CHAS و گروه CHAS با سلول‌های بنیادی مزانشیمی و همچنین با گروه C وجود ندارد.

کلمات کلیدی: کیتوزان، هیالورونیک اسید، سلول‌های بنیادی مزانشیمی، ترمیم زخم

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اثرات آلفا پاینن بر استرس اکسیداتیو و پاسخ التهابی در زخم‌های حاد معده در موش‌های صحرایی

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چکیده

با وجود پیشرفت‌های درمانی فراوان، زخم معده همچنان شایع است. در مطالعات مختلف نشان داده شده است که ترکیبات طبیعی نقش مهمی در پیش‌گیری از زخم معده ایفا می‌کنند. هدف از این مطالعه بررسی اثر محافظتی آلفا پاینن در برابر زخم معده ناشی از اتانول در موش‌های صحرایی با ارزیابی تأثیر آن بر سایتوکاین‌های پیش التهابی و شاخص‌های استرس اکسیداتیو بود. موش‌های نر نژاد ویستار قبل از القا زخم معده با اتانول آلفا پاینن خوراکی (۵۰ و ۱۰۰ میلی‌گرم بر کیلوگرم) دریافت کردند و ضایعات مورفولوژیکی ناخالص، سطوح سیتوکین‌های پیش التهابی و شاخص‌های استرس اکسیداتیو در بافت‌های معده مورد ارزیابی قرار گرفتند. آلفا پاینن باعث کاهش ضایعات مورفولوژیکی در مقایسه با حیوانات گروه کنترل شد. در موش‌های صحرایی تیمار شده با اتانول، آلفا پاینن با دوزهای ۵۰ و ۱۰۰ میلی‌گرم بر کیلوگرم به واسطه کاهش فعالیت میلوپراکسیداز بافتی و سطوح مالون دی‌آلدئید استرس اکسیداتیو را کاهش داد. علاوه بر این، آلفا پاینن در هر دو دوز کاهش GSH و CAT ناشی از اتانول را بهبود بخشید. همچنین آلفا پاینن در هر دو دوز IL-1β و TNF-α را در مقایسه با گروه درمان نشده کاهش داد. آلفا پاینن ممکن است نقش درمانی مفیدی در آسیب معده ناشی از اتانول داشته باشد زیرا استرس اکسیداتیو و عوامل پیش التهابی را کاهش می‌دهد.

کلمات کلیدی: آلفا پاینن، سیتوکین‌های التهابی، پاسخ‌های اکسیداتیو، آسیب حاد معده

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برداشتن اجسام خارجی مری با گاستروتومی در دو قلاده سگ

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چکیده

یکی از جدی‌ترین موقعیت‌های اورژانسی در سگ‌ها، انسداد مری است. استخوان‌ها بیش‌ترین اجسام خارجی گزارش شده هستند که باعث انسداد می‌شوند. این مطالعه دو موقعیت مشابه را گزارش می‌کند که در آن‌ها، استخوان در مری گیر کرده بود. سگ‌ها علایمی مانند استفراغ (بدون محتویات)، سختی تنفسی، ترشح بزاق و عوق زدن را داشتند. در کلینیک‌های دامپزشکی قبلی، اندوسکوپی برای درآوردن استخوان از دهان انجام شده بود، اما برای هر دو بیمار ناموفق بود و هر دو به بیمارستان آموزشی دامپزشکی دانشگاه شیراز ارجاع شدند. رادیوگرافی ساده برای تأیید وجود جسم خارجی تکرار شد. در ابتدا تصمیم بر آن شد تا اجسام خارجی را به سمت معده پیش برده و جراحی گاستروتومی انجام شود. با توجه به این که هر دو جسم خارجی در مخاط مری قرار داشتند و مری نباید تا حد امکان برش بخورد، از پنس بلند با نوک ریز برای انتقال هر دو جسم خارجی از برش گاستروتومی استفاده شد. در هر دو بیمار، جسم خارجی بدون ایجاد خراش در مخاط، از مری خارج شدند. هیچ عارضه‌ای در هر دو بیمار طی دو هفته پیگیری نشان داده نشد.

کلمات کلیدی: مری، جسم خارجی، جراحی

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گزارش علمی

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استئوتومی اصلاحی و تثبیت شکستگی بدجوش خورده زاویه دار رادیوس و اولنا با پین داخل مدولاری در یک بهله عقاب طلایی

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چکیده

برخی از مشکلات ارتوپدی پرندگان در نتیجه ضربه (آسیب به وسیله پنکه سقفی، پرواز و برخورد با پنجره یا آینه، یا پا گذاشتن روی آن)، و برخی دیگر در اثر عدم تعادل تغذیه‌ای یا در نتیجه مشکلات ژنتیکی یا رشدی رخ می‌دهند. یک بهله عقاب طلایی ماده بالغ با سابقه ناتوانی در پرواز به کلینیک دانشکده دامپزشکی دانشگاه رازی (کرمانشاه، ایران) ارجاع شد. معاینه فیزیکی برخی ناهنجاری‌ها را در بال چپ نشان داد. در معاینه رادیوگرافی شکستگی بد جوش‌خورده زاویه‌ای در قسمت میانی بدنه استخوان رادیوس و اولنا مشخص شد. پرنده برای جراحی آسپتیک آماده شد و قسمتی از بدجوش‌خوردگی استخوان بریده شد. پس از اصلاح و تراز شدن استخوان‌ها، هر دو استخوان اولنا و رادیوس توسط پین داخل مدولاری ثابت شدند. پرنده بدون هیچ عارضه‌ای مرخص شد. پیگیری صورت گرفته بهبودی بدون مشکلی را نشان داد و ۶ هفته پس از جراحی، پین داخل مدولاری اولنا خارج گردید، اما پین داخل مدولاری رادیوس باقی ماند. بیش‌تر تکنیک‌های ارتوپدی مورد استفاده برای پستانداران را می‌توان با در نظر گرفتن تفاوت‌های آناتومیکی در گونه‌های مختلف پرندگان، و این که آیا پرنده می‌تواند دوباره پرواز کند یا نه، برای پرندگان به کار برد. بد جوش‌خوردگی شکستگی در پرندگان شکاری می‌تواند عملکرد ماهیچه‌ها و تاندون‌ها را به خطر بیندازد و بر فعالیت‌های عادی که برای بقای این گونه‌ها ضروری است، تأثیر منفی بگذارد. بدجوش-خوردگی استخوان را می‌توان با تکنیک‌های استئوتومی و به دنبال آن تثبیت استخوان که ثبات کافی را فراهم می‌کند تا امکان التیام بدون تداخل با حداقل آسیب بافت نرم فراهم شود، اصلاح نمود.

کلمات کلیدی: عقاب، پین داخل مدولاری، بدجوش‌خوردگی، استئوتومی

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