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JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Contents

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Evaluation of complete blood count in bitches with ovarian tumors: A preliminary study

Tuğba Çoban[1], Zeynep Günay Uçmak[2*], Mehmet Ragıp Kılıçarslan[2], Özge Erdoğan Bamaç[3]

1. Institute of Postgraduate Education, Istanbul University-Cerrahpaşa, Istanbul, Turkey. **2.** Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Istanbul University-Cerrahpaşa, Istanbul, Turkey. **3.** Department of Pathology, Faculty of Veterinary Medicine, Istanbul University-Cerrahpaşa, Istanbul, Turkey. Çoban, T. ORCID: 0000-0003-4129-7720, Günay Uçmak, Z. ORCID: 0000-0003-2530-1291, Kılıçarslan, M. R. ORCID: 0000-0003-3412-766X, Erdoğan Bamaç, Ö: ORCID: 0000-0002-0352-4841.

Research Article

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ABSTRACT

Ovarian tumors are rarely seen in bitches and they usually occur in older ages. The most common type of ovarian tumor is epithelial tumors. Hematological parameters are frequently affected by the presence of neoplasms. There are various types of anemia associated with neoplasms. In the present study; we investigated the relationship between complete blood count (CBC) and the presence of ovarian tumors in bitches. Seven bitches with ovarian tumors and 6 bitches with histologically healthy ovaries were selected for this study. Increased level of platelet (PLT) was determined in bitches with ovarian tumors and considered as a significant finding to estimate the cancer progression. Besides, red blood cells (RBC), hematocrit (HCT), and hemoglobin (HGB) levels were decreased in bitches with ovarian tumors, which might be associated with cancer related anemia. In conclusion, the evaluation of complete blood count in bitches with ovarian tumors can be important to estimate the hematological effect of tumor progression.

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Introduction

Ovarian tumors are rarely seen in bitches due to neutralization at early ages (Gomez-Laguna et al., 2008). Ovarian neoplasms occur in a wide age range in bitches, but granulosa cell tumors (GCT) and teratomas appear at a younger age than others (Diez-Bru et al., 1998). Four main categories are defined for ovarian neoplasms; epithelial, germ cell tumors, sex-cord stromal tumors and mesenchymal tumors. Epithelial tumors include papillary adenomas, papillary adenocarcinomas, rete adenomas and undifferentiated carcinomas. Epithelial tumors constitute 40 % to 50 % of all canine ovarian tumors (Bertazzolo et al., 2004). Papillary adenocarcinoma is a common type and often/frequently occurs bilaterally, similar to papillary adenoma (Nielsen et al., 1976). Germ cell tumors consist of dysgerminomas, ovarian teratomas and embryonal carcinomas. Sex-cord stromal tumors include granulosa cell tumors, luteomas and thecomatas (Bertazzolo et al., 2004). Luteoma is a less common tumor type that has limited reports in mares, cats and monkeys (Namazi et al., 2015). There is not much information about breed predisposition in dogs however Bulldog and Boxer breeds display the highest risk (Sforna et al., 2003).

The clinical observation in dogs with ovarian tumors is variable. Ovarian tumors can be detected by

*Corresponding Author: Zeynep Günay Uçmak
E-mail: vethekzeynep86@gmail.com

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chance during the sterilization process. The most common clinical manifestation is peritoneal or pleural effusion. Prolonged pro-oestrus or oestrous (prolonging/extended more than a month) may also be associated with ovarian tumors (McEntee, 2002). It has been reported that ovarian tumors mostly arise at the left ovary. The recommended treatment for dogs with ovarian tumors is operative intervention (Diez-Bru et al., 1998).

The most common paraneoplastic syndrome in veterinary oncology is anemia (Aydın et al., 2011). Moreover, the amount of PLT was found to be higher in dogs with mammary tumors (Günay Uçmak and Güvenç, 2019). Correspondingly, this study aims to investigate changes in complete blood count (CBC) in dogs with ovarian tumors.

Materials and Methods

Animals and study design: Ethics approval for the study was obtained from the unit Ethics Committee (number: 2020/28). Thirteen intact bitches were enrolled in the study. All bitches were presented to the clinic of Obstetrics and Gynaecology for ovariohysterectomy. They were gynaecologically examined with B-mode real-time ultrasonography. The bitches were allocated into two groups according to histopathological examination of the ovaries. Group OT consisted of seven bitches with ovarian tumor and group H consisted of six bitches with histologically healthy ovaries. The groups were designed regardless of breed variation.

B-mode real-time ultrasonography: All bitches were checked with B-mode real-time ultrasonography (MyLab 5-Vet ESAOTE®, Genova, Italy) using a microconvex probe of 6.5 MHz via transabdominal route.

Determining the stage of oestrus: The stage of oestrus was determined by vaginal cytology. The samples were collected using cotton swab. Smears were stained with Diff Quick method according to manufacturers' instructions (Hemacolor® stain; Merck, Darmstadt, Germany). Vaginal epithelial cells were evaluated using a light microscope (BAB-LAM, BAB, Turkey) at X400 magnification.

Hematological examination: Blood samples were collected from all bitches for CBC. After asepsis and antisepsis was provided, 2 ml blood samples were collected into etilendiamine tetraacetic acid (EDTA) tubes from vena cephana parva of each bitches. Red blood cell (RBC, M/µL), Hematocrit (HCT, %), Hemoglobin (HGB, g/dL), White blood cell (WBC, K/µL), Platelet (PLT, K/µL), Plateletcrit (PCT, %) were measured using hematology analyzer (ProCyte Dx, IDEXX Laboratories, USA).

Surgical intervention: The bitches were initially premedicated with atropine sulfate (0.03 mg/kg, sc) (Atropin®, Teknovet, Turkey). For induction of the anesthesia 1 % propofol (Lipuro®, Braun, England) was used at 4 mg/kg, iv and 3 % isoflurane (Forane liquid®, Abbott Laboratories, England) and 0.5-1 % oxygen combination was used to maintain the anesthesia. Median line was preferred for laparotomy incision. Absorbable suture materials (Monocryl No:0) were used for ligations and sutures in the ovariohysterectomy section.

Histopathological examination: Ovarian tissues were fixed in 10 % neutral buffered formalin, paraffin embedded, sectioned at 4 µm, and stained with hematoxylin and eosin (HE) for histopathological examination by light microscopy.

Statistical analysis: The SPSS 13.0 packet program was used for the statistical analyses. Levene's test was performed to determine the equality of variances. Statistical difference in terms of RBC, HCT, HGB, WBC, PLT and PCT between the groups was investigated by t-test. The mean values and standard errors of the related parameters were determined.

Results

The mean age of bitches in group OT and group H were 10.43 ± 1.87 and 6.17 ± 0.47 years; respectively. The mean age of the bitches with ovarian tumors were tended to be higher but failed to reach significance (P=0.06). All bitches in both groups were of different races. During the ultrasonography examination, enlargement of the affected ovaries was determined in bitches with granulosa cell tumor (GCT) (6.1x4.3 cm), luteoma (2.9x3.6 cm), papillary adenocarcinoma (1.9x1.8 cm) (Figure 1) while the other neoplastic ovaries in group OT could not be detected. The ovary sizes of the ovaries belong to the bitches in group H were in normal ranges. After ovariohysterectomy section was performed, the

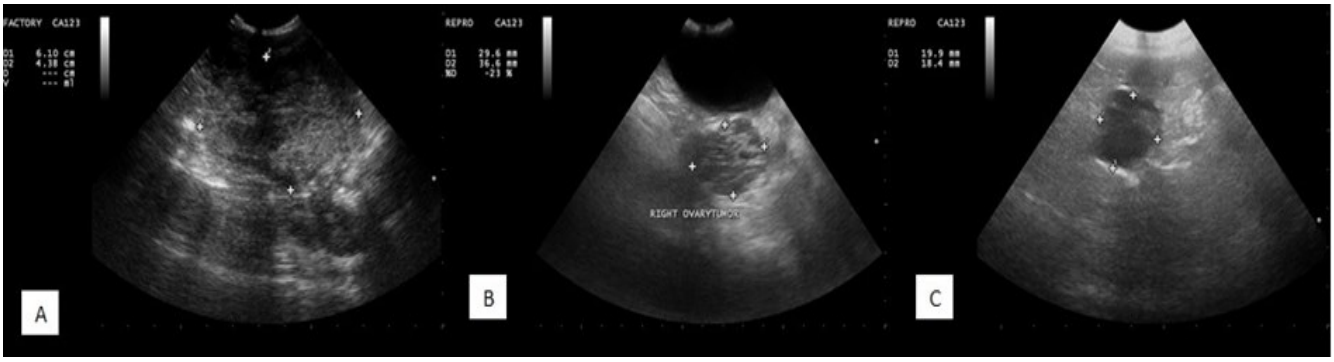

Figure 1. B- mode real time ultrasonography images in bitches with ovarian tumor
A: Granulosa cell tumor, B: Luteoma, C: Papillary adenocarcinoma

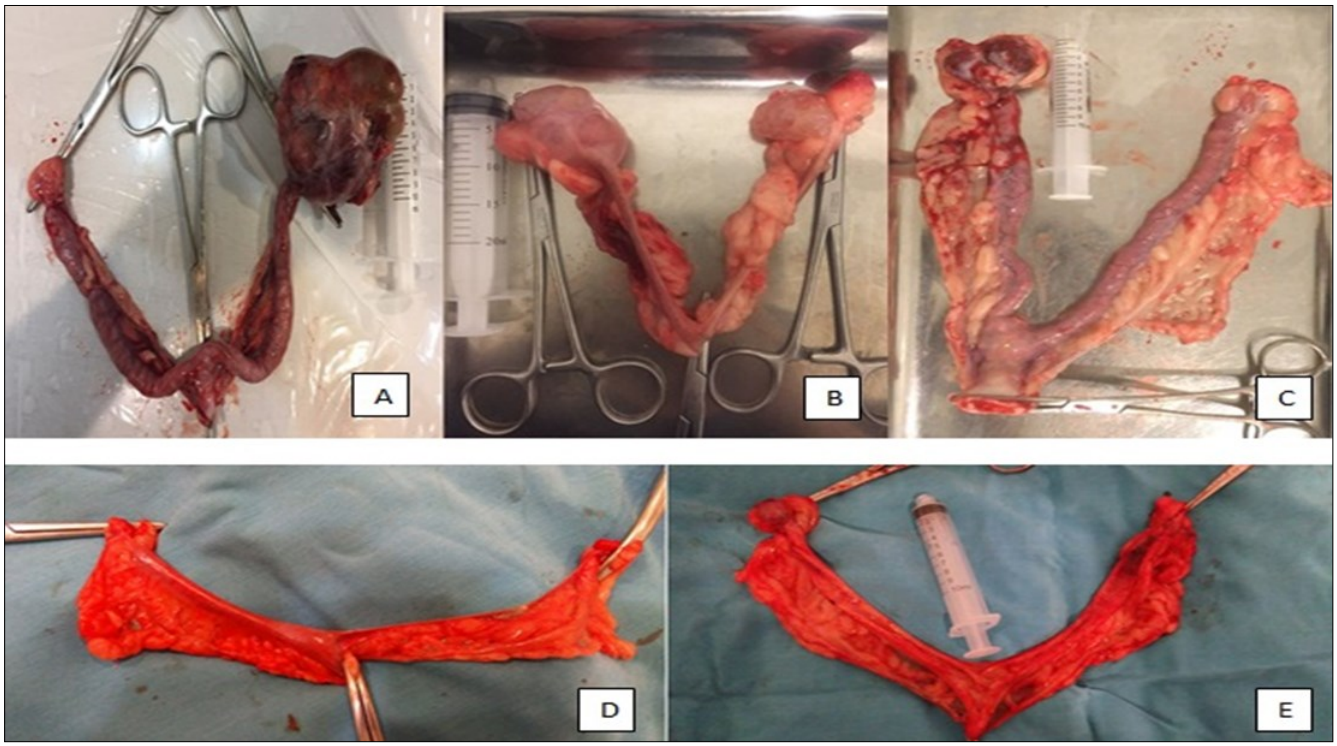

Figure 2. Macroscopic images of the genital tract with ovarian tumors after ovariohisterectomy section.
A: Granulosa cell tumor B: Luteoma C: Papillary adenocarcinoma D: Adenoma E: Luteoma

affected ovaries in some of the bitches could be visualized macroscopically (Figure 2). Histopathological examination revealed one GCT, two luteoma, one papillary adenocarcinoma, two adenomas and one cystic papillary adenoma in group OT (Figure 3). Also subserosal localized tumor metastases on cervix uteri and cystic glandular hyperplasia in uterine horns were determined in the bitch with GCT. Five of seven bitches with ovarian tumors were in the dioestrus stage. The bitch with GCT which had vaginal bleeding for two months, was in oestrus stage. Six bitches were enrolled in group H. The bitches in group H were in dioestrus (n=3) and anoestrus (n=3).

The mean values and standard errors of RBC, HCT, HGB, WBC, PLT and PCT values in group OT and group H and their significances were presented in Table 1.

Discussion

Canine ovarian tumors are usually observed in older ages and affected animals vary from 5 to 15 years old (Sforna et al., 2003). The average age of the bitches in group OT were consistent with previous reports.

The researchers (Arlt and Haimerl, 2016) reviewed that ovarian cysts and ovarian neoplasia in female dogs regarding epidemiologic, clinical and fertility aspects. Ultrasonography is one of the diagnostic methods to reveal the pathologies in gynaecology. Ultrasonographic appearances of ovarian tumors have variable echogenicity. Structures of papillary adenocarcinomas in ovaries are usually solid or solid with cystic components (Diez-Bru et al., 1998). One of the malignant ovarian tumors in the presented study was papillary adenocarcinoma which had an anechogenic display in the ultrasonographic

Table 1. Mean values and standard errors of complete blood count parameters and their significances related to groups

	RBC (1×10^6/µL)	HCT (%)	HGB (g/dL)	WBC (1×10^3/µL)	PLT (1×10^3/µL)	PCT (%)
Group OT	5.76 ± 0.41	36.5 ± 2.67	12.7 ± 0.96	23.9 ± 5.24	741.3 ± 83.11	0.64 ± 0.12
Group H	7.11 ± 0.26	44.9 ± 1.93	15.7 ± 0.64	18.2 ± 8.69	373.3 ± 43.07	0.36 ± 0.04
P values	<0.05	<0.05	<0.05	NS	<0.01	= 0.07

RBC: Red blood cell, HCT: Hematocrit, HGB: Hemoglobin, WBC: White blood cell, PLT: Platelet, PCT: Plateletcrit

examination due to the cystic structure, similar to the previous report (Diez-Bru et al., 1998). Even if GCT could occur in different animal species as queens (Günay Uçmak et al., 2018), mares (Kurban et al., 2019) and bitches, ultrasonographic images of GCTs appeared with similar characteristic. In line with the other researchers' reports (Günay Uçmak et al., 2018; Kurban et al., 2019), the ovary affected with GCT was massive, solid and multilobular structure that had a hypoechogenic display in transabdominal ultrasonography. Although the distant metastasized organ was different, similar to the researchers' statement (Günay Uçmak et al., 2018), GCT was metastasized in this research. A benign ovarian neoplasia that was visualized in ultrasonographic examination was diagnosed as luteoma following the histopathologic examination, in this study. Yılmaz et al. (2017) reported a case of unilateral luteoma in the ovary of a dog which was 13 cm in size. In ultrasonographically it appeared as a hypoechoic, round mass with homogeny architecture. Although contradictive results obtained in mass size, ultrasonographic data from to ovary with luteoma were similar to observation from Yılmaz et al. (2017).

In addition to the ultrasonographic examination, CBC and thoracic radiographs should be performed for the diagnosis of ovarian neoplasms (McEntee, 2002). Anemia is the most common paraneoplastic syndrome from hematological indicators of neoplasms (Aydin et al., 2011). Due to increased estradiol secretion in granulosa cell tumors, non-regenerative anemia improves in the affected bitch (Nak et al., 2012). Although the bitches with benign ovarian neoplasms had mild anemia, decreased RBC, HCT and HGB levels were presented in group OT. Both the bitch with GCT and the bitch with papillary adenocarcinoma had severe anemia.

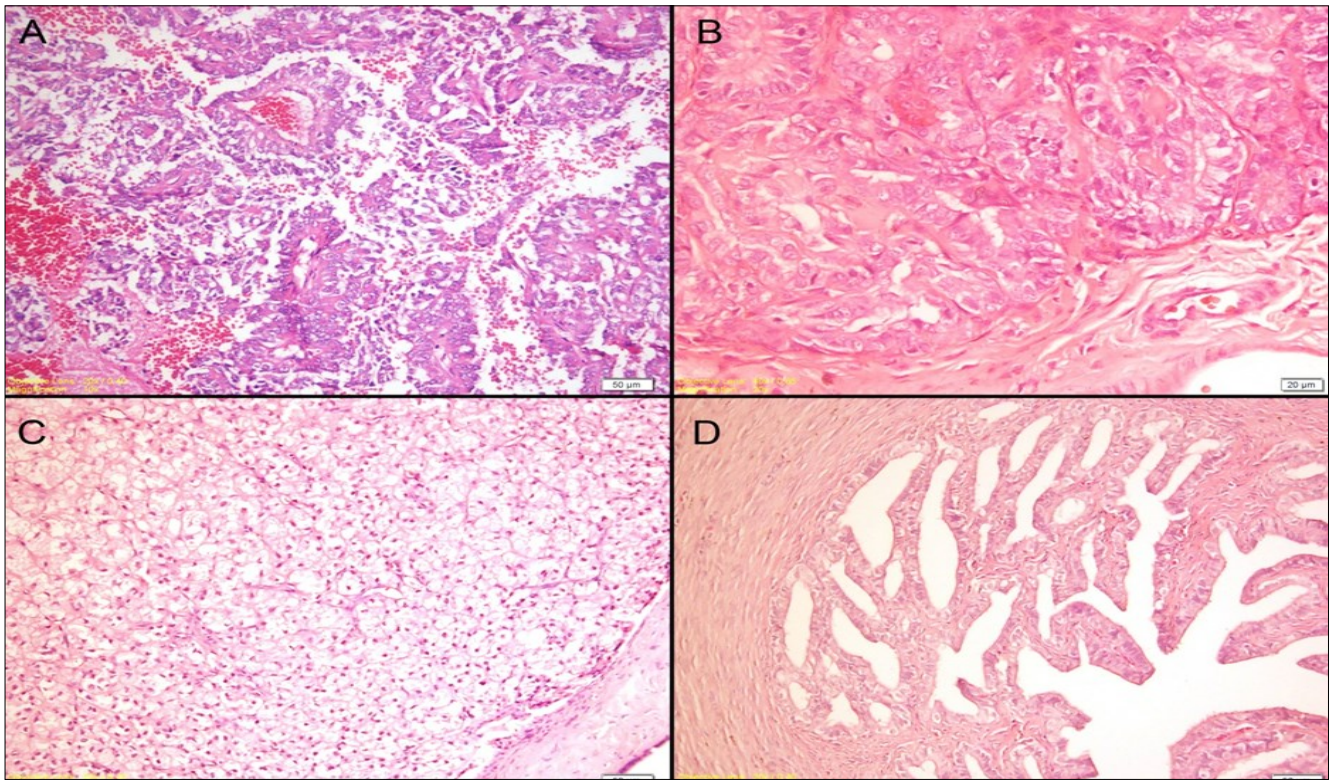

Figure 3. Histologic features of ovarian tumors. A: Granulosa cell tumor (HE, bar: 50 mm)
B: Papillary adenocarcinoma (HE, bar: 20 mm). C: Luteoma (HE, bar: 50 mm). D: Papillary adenoma (HE, bar: 50 mm).

The decline of RBC, HCT and HGB levels were supposed to be due to the malignant ovarian tumors. Oviedo-Penata et al. (2020) were determined normocytic normochromic anemia. The researchers (Oviedo-Penata et al., 2020) were reported that anemia was formed as a result of suppression of erythropoiesis due to the chronic condition in pyometra. As in the previous report, ovarian tumor (GCT) and pyometra existed together in one case of the presented study and the bitch had anemia. Consistent with the previous report (Oviedo-Penata et al., 2020), severe anemia was thought to exist due to the vaginal bleeding for two months that developed as a symptom of pyometra in the bitch with GCT. However, other reasons for normocytic and normochromic anemia are acute haemolysis, haemorrhagia, iron deficiency, chronic renal diseases, chronic inflammation and neoplasia (Arun 2013). In accordance with the previous statement, normocytic and normochromic anemia could exist due to presence of ovarian neoplasms in group OT.

Cancer progresses due to platelet activation and thrombotic complications (Li, 2016). A significant increase of PLT levels in group OT indicates the cancer progression, as noted in the previous report (Li, 2016). The tendency of higher PCT and significant rise of PLT in group OT suggest that PLT and PCT are related to cancer progression notwithstanding histopathological character of the tumor. Platelet adhesions to cancer cells strongly contribute to metastasis via a hematogenous way (Honn et al., 1992). In contrast with Honn et al. (1992), although only one case in group OT had distant metastasis, PLT levels were significantly higher than group H. However, group OT had both malignant and benign ovarian tumors. It was suggested that both malignant and benign ovarian tumors should be followed up in terms of distant metastasis.

In conclusion, the evaluation of complete blood count in bitches with ovarian tumor could be important to estimate the hematological effect of tumor progression.

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Evaluation of dietary supplementation of fenugreek seed (*Trigonella foenum-graecum* L.) as a growth promoter in broiler diet and its impacts on growth performance, carcass quality and cost effectiveness

Abdimajid Hassan Ali[1,2], Tahera Yeasmin[1], Yonis Abukar Mohamed [2,3], Abdiaziz Idiris Mohamud [2,4], and Pravin Mishra [5*]

1.Department of Dairy and Poultry Science, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University, Dinajpur. **2.**Faculty of Veterinary Science, Benadir University, Mogadishu, Somalia. **3.**Department of Pathology and Parasitology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University, Dinajpur. **4.**Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh. **5.**Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

Ali, A. H. ORCID: 0000-0001-8590-0179, Yeasmin, T. ORCID: 0000-0001-9372-8946, Mohamed, Y. A. ORCID: 0000-0002-5235-0921, Mohamud, A. I. ORCID: 0000-0002-8477-9183, Mishra, P. : ORCID: 0000-0002-8315-0463.

Research Article

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ABSTRACT

This study was carried out to evaluate the efficacy of Fenugreek seeds (*Trigonella foenum-graecum* L.) on overall performance of broiler. A total of 96-day old Cobb-500 chicks were randomly divided into four dietary treatment groups namely T_0, T_1, T_2 and T_3 having three replications in each treatment group. Brooded chicks were randomly separated into replications wise separate pen to rear up to 4 weeks. Each treatment group contains 24 birds (8 birds in each replication). Experimental birds in T_1, T_2 and 3 were provided fenugreek seeds meal with 0.5%, 1% and 1.5% of feed while T_0 was provided with standard feed and considered as control group. The results of this study were indicated that final live weight gain and feed efficiency of birds was significantly ($P<0.05$) higher in T_3 compared to T_2, and T_0 respectively. The result also indicated that feed efficiency was increased at dose rate of 1.5% fenugreek seeds meal in T_3 compared to T_2, T_1 and control T_0 group respectively. In case of meat yield parameters there was significant ($P<0.05$) difference among treatment groups except liver weight. The carcass weight was significantly ($P<0.05$) higher in T_3 group compared to the control group. The lowest feed cost was found in T_0 and highest profit in T_3 group. Based on the current study, it is concluded that fenugreek seed meal at a dose of 1.5% can be used as growth promoter for the production of broiler chicken.

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Introduction

The usage of antibiotics as a growth promoter is widely banned by European Union-wide in 2006 due to their side effects on animal health and their residues in meat for human health (EU-wide, 2005). Novel and beneficial feed additives including different dietary fibers with adequate amount promotes the growth performances and maintain their immune and gut health (Jha et al., 2020; Jha and Mishra, 2021). Fenugreek is a popular medicinal plant grown in nature, mostly in India, Nepal, Pakistan, and China. Fenugreek seeds are hypoglycemic, antibacterial, anti-inflammatory, antipyretic and antimicrobial

*Corresponding Author: Pravin Mishra
E-mail: mpravin470@gmail.com

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ingredentials seeds with multiple therapeutic impacts (Xue et al., 2007). This includes neurin, biotin, trimethylamine, and its effect on the nervous system appears to promote appetite (Al-Habori and Raman, 2002). It contains dietary proteins, carbohydrate, minerals and vitamins which are known to be a healthy source for humans as well as livestock (Michael and Kumawat, 2003).

The poultry is used as good source of meat and prepared money in crisis needs, thus giving food and economic securities to rural peoples mostly in developing countries (Mohamud et al., 2020). Broiler has a shorter life cycle and its production requires less capital compared to other meat producing animals. Since, the majority of the people irrespective of caste or religion prefer chicken meat (Mohamud et al., 2020), its demand is very high. Many illnesses such as coronary disease (Pan et al., 2012), type 2 diabetes (Pan et al., 2011), stroke (Kaluza et al., 2012), obesity (Vergnaud et al., 2010), some cancers (Ma and Chapman, 2009) and early mortality (Pan et al., 2012) have been shown to be directly linked with daily intake of red meats. As a result, global production and per capita intake of broiler meat in recent years have risen quickly (FAO, 2009).

Feed is a crucial component that influences the net return of the poultry business. In the broiler industry, feed costs are considered to be one of the greatest challenges, mainly in developing countries. It comprises around 60-80% of the total expense of poultry meat production (Thirumalaisamy et al., 2016). Protein supplementation is often essential to improve poultry performance, and this should be finished with respect to their requirements in addition to the balance of different nutrients available. The expansion of the poultry company depends to a large degree on the availability of sufficient and cost-effective high-quality feed to both farmers and consumers (Ravindran, 2013).

The research was therefore planned to forecast the impact of supplementation with Fenugreek seeds on growth efficiency, carcass characteristics, mortality, and also to assess the profitability of broiler diets.

Materials and methods

Chicken coops: Experiment shed was constructed with metal mesh and wooden materials having a compartment of housing for 8 birds. At first, the experimental house was washed and cleaned thoroughly using tap water. Ceiling, walls, and floor were thoroughly cleaned and subsequently disinfected with bleaching powder, and then the room was kept closed for two weeks. After that, the house was again disinfected with Virocid® solution 1ml/3liter water. At a same time, all feeders, drinkers and other necessary equipment were also properly cleaned, washed and disinfected with bleaching powder. After proper drying, the house was used for the birds rearing.

Source and preparation of Fenugreek seeds: Fenugreek seeds purchased from the commercial local markets of Dinajpur district in Bangladesh for incorporate in the diets of broiler chickens. Fenugreek seeds were washed with tap water and then sun dried under shade. Then the dried Fenugreek seeds were stored in sealed polyethene bags, and kept at room temperature until used in commercial diet.

Chickens: A total of ninety-six vigorous day-old Cobb-500 chicks were collected from Nourish Poultry and Hatchery Ltd., Bangladesh. The experiment was conducted for a time period of 28 days from 15 August to 12 September 2019 at Poultry Shed of Hajee Mohammad Danesh Science & Technology University, Dinajpur, Bangladesh.

Experimental design: The trial chicks were distributed randomly in four different treatment groups i.e. T_0: Only commercial diet (Control), T_1: Commercial diet + 0.5% Fenugreek seeds, T_2: Commercial diet + 1% Fenugreek seeds, T_3: Commercial diet + 1.5% Fenugreek seeds. Each treatment groups having 3 replicates of 8 chicks in each. The uniform and identical management practices were given to all groups.

Experimental diet: Ready feed was used for the experimental study. At first required amount of ready feed ingredients were weighted by digital weighing balance. Two phases (broiler-starter and broiler-grower) were used to separate the experimental duration. The broiler chicks were fed broiler starter for 0 and 14 days and broiler grower for 15 to 28 days of age. Composition of experimental diet is in Table 1.

Table 1. Calculated compositions of experimental diets

Nutrients	Amount (kg/100kg feed)	
	Starter (1-14 days)	Grower (15-28 days)
Crude protein (%)	22.0	21.00
Crude fiber (%)	3.00	3.00
Crude fat (%)	5.00	6.00
Lysine (%)	1.30	1.25
Methionine (%)	0.52	0.50
Calcium (%)	1.00	0.90
Phosphorus (%)	0.50	0.48
Moisture (%)	11.00	11.00
Metabolizable Energy, ME (kcal/kg)	3000	3100

Birds management: Initially, chicks were raised up to 7 days in the brooding house to adjust with the environmental condition and then housed on the floor and regularly handled like any other commercial broiler flock. Heating was provided by a single electric brooder, where the initial temperature was set at 35° C and decreased by 1°C per day to final temperature of 28°C at the end of experiment. Round plastic drinker and linear feeder were used at brooding period and later linear feeder was substituted by round plastic feeder. Water and feed were provided manually to the broiler as per the experimental schedule and per seven birds one drinker and feeder and were provided. During the experimental phase, all birds were subject to a continuous illumination of 23 hours and a dark phase of one hour per day. Electric bulb with 20 lux intensity was provided to the birds throughout the study. All broilers were vaccinated against New Castle (Ranikhet) Disease and Infectious Bursal (Gumboro) Disease. As a medication, in first week Gluco-C was used 50 g/L water. Water soluble vitamins and regular saline were also given for the first 3 days of brooding. Biosecurity and sanitation are strictly maintained throughout the experiment. Disinfectant was used to disinfect the shed premises regularly and foot bath was kept at the entrance of the shed area. The fumigation process was carried out before the birds arrived.

Clinical observation and recording of performances: All the birds inspected twice a day for any physical changes such as restlessness, lordosis, unusual gait, vices and depression as well as feeding. During the 28 days of experimental period, growth performance was evaluated. Before treatment, body weight was taken for each group of birds. Body weight and feed intake were documented weekly, and body gain and feed conversion were then assessed. During the entire study period, mortality was also documented.

Feed consumption: Feed consumption is the daily consumption of the feed. It has been measured on a daily basis for each treatment. The remainder of the feed was weight at the end of the day and was removed at the beginning of the day from the weight known of the feed. The total number of birds has been split. The commodity was divided by the number of birds in total. Feed intake = Feed intake in replication/ No. of birds in replication.

Body weight and gain (kg): At the beginning of the experimental trial, body weight was determined for all birds and it was performed daily at the beginning of the week at the same time. Live weight gain was determined by subtracting the live weight at the beginning of the week from the live body weight of the next week. Body Weight gain (kg) = Final weight – Initial weight.

Feed conversion ratio: Feed conversion ratio (FCR) was determined every week at the amount of feed intake per unit of body weight gain (average weekly feed consumption (g)/average weekly weight gain (g)). FCR = Feed intake (g) / Weight gain (g).

Evaluation of carcass characteristics: Prior to slaughtering, the birds were restricted to feed for 10 hours, but water was provided *ad libitum*. Two birds were randomly selected in each replication for slaughtering. The live weight of birds was taken individually before slaughtering. At the time of slaughtering, the birds were secured by holding both shanks with one hand and both wings with other hand by the help of an assistant to prevent struggling. Slaughtering was done by Halal method with sharp knife. After complete bleeding shank, head, and skin were removed. Finally, evisceration was done manually to separate liver, spleen, heart, gizzard, and meat yield.

The dressing percentage is based on the relationship between the weight of the dressed carcass and the weight of live birds after removal of things such as the hide and internal organs. The percentage of dressing can be determined by taking the carcass weight divided by the weight of live birds. Dressing percentage (%) = (Weight of the carcass (g) / Weight of live bird (g)) x 100.

Statistical analysis: The data from each procedure was collected and entered into the Excel datasheet and then exported for statistical analysis to SPSS, Version 22.0 (IBM Corp, 2013). All analyzed information were stated as Mean ± Standard Error (SE).

Results

Effect of fenugreek seeds on body weight: The effect of Fenugreek seed on body weight gain is shown in Table 2. The current research showed that there was no significant variation in initial body weight between the dietary groups ($P>0.05$), but final body weight and body weight gain were significantly differed ($P<0.05$) between the dietary groups.

Body weight gain in different dietary treatments during experimental periods, the differences were significant at $P<0.05$. The initial body weight was in T_0 (38.16±0.60), T_1 (39.20 ± 0.49), T_2 (39.26 ± 0.60), and T_3 (40.16 ± 0.57) respectively. At 7 days of age, the body weight was almost similar in all dietary groups. Significant different ($P<0.05$) were found at 14 days, 21 days and 28 days of age. The highest body weight gain was observed in treatment group T_3, followed by T_2, T_1 and T_0 respectively.

Table 2. Effect of dietary fenugreek seed meal supplementation on body weight of broiler chicken

		Body weight gain				
	Initial BW	7 d	14 d	21 d	28 d	1-28 d
T_0	38.16 ± 0.60	155.81 ± 2.02	185.57 ± 1.18^{a}	395.43 ± 1.79^{a}	475.44 ± 1.74^{a}	1250.41 ± 7.33^{a}
T_1	39.20 ± 0.49	156.42 ± 1.22	185.11 ± 19.01^{a}	414.26 ± 2.63^{b}	498.49 ± 1.42^{b}	1293.48 ± 24.77^{b}
T_2	39.26 ± 0.60	158.71 ± 0.89	212.88 ± 1.83^{ab}	431.81 ± 1.44^{c}	519.37 ± 4.87^{c}	1362.03 ± 9.63^{c}
T_3	40.16 ± 0.57	159.64 ± 0.89	230.46 ± 1.45^{b}	448.14 ± 1.56^{d}	539.28 ± 1.50^{d}	1417.68 ± 5.97^{d}
P value	NS	NS	*	*	*	*

a, b, c, d = means within a column without common superscripts differ significantly. BW = body weight. NS = non-significant; statistically significant difference are expressed as. * = P<0.05.

However, with the rise in age, there was a pattern of rising live weight (P<0.05). There was a tremendous increase of live weight for increasing Fenugreek seed at 14, 21, and 28 days of age in all treatment groups. The outcome of this study showed that 0.5%, 1% and 1.5% fenugreek seed supplementation of broiler diets resulted in increase in live weight than 0% fenugreek seed in broiler seeds. The broiler of T_3 and T_2 group was significantly higher in average of weekly live weight gain compared to T_1 and T_0. During the finisher and complete times, significant differences were detected in body weight gain.

Effect of fenugreek seed on feed consumption: Feed consumption (FC) values for day-old broiler supplemented with experimental diets are presented in Table 3. The highest values were recorded by birds fed with 1.5 percent fenugreek seed, but birds with 0.5 percent and 1 percent had the lowest values compared to the control group. Feed intake in different dietary treatments during experimental periods was almost statistically difference and the values were significant (P<0.05). The total feed intake was T_0 (1816.36±7.49), T_1 (1848.62±7.59), T_2 (1904.14±8.72) and T_3 (1928.03±5.94). So, inclusion of Fenugreek seeds in broiler diet resulted in increase in feed consumption at 7, 14, 21 and 28 days of age (P<0.05). It was found that at 0.5%, 1% and 1.5% dietary Fenugreek seeds group consumed the highest amount whereas lowest in control diet.

Effect of fenugreek seeds on feed conversion ratio: Weekly FCR of broilers on different dietary fenugreek seed differed (P<0.05) during 21 and 28 days of age. There are no significant differences were observed between control and treatment groups at the 7 and 14 days age. (Table 4)

Table 3. Effects of dietary fenugreek seed meal supplementation on feed intake in broiler chickens

	Feed intake (g)				
	7 d	14 d	21 d	28 d	1-28 d
T_0	160.48 ± 1.20^{a}	256.09 ± 1.14^{a}	601.05 ± 3.61^{a}	798.74 ± 1.54^{a}	1816.36 ± 7.49^{a}
T_1	159.55 ± 0.87^{a}	268.37 ± 1.76^{b}	613.10 ± 2.94^{b}	807.60 ± 2.02^{b}	1848.62 ± 7.59^{b}
T_2	165.06 ± .66^{b}	283.23 ± 2.10^{c}	630.44 ± 1.59^{c}	825.41 ± 3.37^{c}	1904.14 ± 8.72^{c}
T_3	170.81 ± .35^{c}	290.38 ± 1.03^{d}	636.35 ± 2.12^{c}	830.49 ± 1.44^{c}	1928.03 ± 5.94^{d}
P value	*	*	*	*	*

a, b, c, d = means within a column without common superscripts differ significantly. Statistically significant difference is expressed as * = P<0.05.

Table 4. Effects of dietary fenugreek seed meal supplementation on feed conversion ratio in broiler chickens

	FCR (%)				
	7 d	14 d	21 d	28 d	1-28 d
T_0	1.02 ± 0.02	1.38 ± 0.01	1.52 ± 0.01^{c}	1.67 ± 0.01^{c}	1.45 ± 1.02^{d}
T_1	1.02 ± 0.01	1.48 ± 0.17	1.48 ± 0.01^{b}	1.62 ± 0.01^{b}	1.42 ± 0.30^{c}
T_2	1.04 ± 0.01	1.33 ± 0.02	1.46 ± 0.01^{b}	1.59 ± 0.01^{b}	1.39 ± 0.90^{a}
T_3	1.07 ± 0.01	1.26 ± 0.01	1.42 ± 0.01^{a}	1.53 ± 0.01^{a}	1.36 ± 0.99^{b}
P value	NS	NS	*	*	*

a, b, c, d = means within a column without common superscripts differ significantly. NS = non-significant; statistically significant difference are expressed as * = P<0.05.

Table 5. Effects of dietary fenugreek seed meal supplementation on meat yield in broiler chickens

			Organs		
	Live weight	Carcass weight	Liver weight	Heart weight	Gizzard
T_0	1250.41 ± 7.33[a]	662.79 ± 1.39[a]	32.60 ± 1.08	4.32 ± 0.06[ab]	33.26 ± 0.70[a]
T_1	1293.48 ± 24.77[b]	719.19 ± 2.62[b]	33.42 ± 0.78	4.30 ± 0.02[a]	35.12 ± 0.55[a]
T_2	1362.03 ± 9.63[c]	778.31 ± 2.05[c]	32.18 ± 0.98	4.45 ± 0.03[b]	38.45 ± 0.88[b]
T_3	1417.68 ± 5.97[d]	850.67 ± 1.01[d]	34.26 ± 0.58	4.65 ± 0.02[c]	40.66 ± 0.45[c]
P value	*	*	NS	*	*

a, b, c, d = means within a column without common superscripts differ significantly. NS = non-significant. Statistically significant difference are expressed as *(P<0.05).

Effect of fenugreek seed on carcass quality and mortality of broiler: The effect of Fenugreek seeds on final live weight, dressed weight, heart, liver and gizzard of broiler is presented in Table 5. The highest final live weight was observed in T_3, followed by T_2, T_1 and the lowest T_0. The dressed weight was significantly higher in T_3 and T_2 group compared to 1 and T_0. The morality of broiler was seen only in T_0 i.e., 4.17%.

Cost-effectiveness of broiler production: The total rearing costs of broiler are kept under different treatment groups T_0, T_1, T_2 and T_3 was TK. 123,904, TK. 125,312, TK. 127,776, and TK. 128,832 respectively. Where, miscellaneous cost summed up TK. 8 per broiler, which included the estimated cost of electricity, labor, litter, and disinfectant. The average live weight/broiler in group T_0, T_1, T_2 and T_3 was 1.25 kg, 1.29 kg, 1.36 kg, and 1.41 kg respectively. The broiler was sold in live weight basis at the rate of Tk. 130/kg. The net profit/Kg live weight in the T_0, T_1, T_2 and T_3 group was found to be TK. 31.2, TK. 33.33, TK. 36.76, and TK. 39.71 respectively. The level of fenugreek seeds used in the ration exhibited their effect on the profit margin of the broiler. Cost of different groups of bird is shown in Table 6.

Discussion

Live weight: The increase in the live weight of broilers in the current experiment for the inclusion of fenugreek seeds in the diet correlates with the findings stated by Qureshi et al. (2015). They indicated that the addition of either whole, crushed or powder type of fenugreek seeds increased the live weight of broilers at different levels in broiler diets. The increase in the live weight of fenugreek seeds broilers may be due to the fact that fenugreek seeds contain essential fatty acids and high-quality proteins (Żuk-Gołaszewska and Wierzbowska, 2017) and has a stimulating impact on broiler villus height of the digestive system (Mamoun et al., 2014; Mahmood et al., 2015).

The findings obtained are consistent with those of El-Ghamry et al. (2004), who found the fenugreek additions at 1.5% level had significantly ($P < 0.05$) higher live body weight and body weight gain than those fed on control diet. Similarly, Morsy (1995) recorded that there was a substantial improvement in live body weight and body weight gain of Hubbard broiler chicks fed 500 g fenugreek per ton diet.

Table 6. Economics of broiler production kept under different treatment groups from day old chick to 28 days of age

Dietary groups with fenugreek seed (%)				
Parameters (TK)	T_O	T_1	T_2	T_3
Chick cost	26	26	26	26
Vaccine + medicine	10	10	10	10
Feed price/kg	44	44	44	44
Miscellaneous cost/ chick	8	8	8	8
Feed cost/ kg production	79904	81312	83776	84832
Total cost Tk. /broiler production	123904	125312	127776	128832
Average live weight (kg)/broiler	1.25	1.29	1.36	1.41
Sale price Tk./kg	130	130	130	130
Sale price /broiler	162.3	168.4	177	184.4
Net profit Tk./broiler	39	43	50	56
Profit/kg live weight	31.2	33.33	36.76	39.71

In term of organs, in our study liver weight changed non-significantly which is similar to the study of Abbas (2010) and the weight of heart and gizzard were changed which is opposite to the finding of Weerasingha and Atapattu (2013).

Feed consumption: Compared to the control group, birds fed 0.5%, 1% and 1.5% fenugreek showed the highest values, which may be due to the shift in feed taste, as stated by Stukie (1986), who suggested that birds have a sense of taste. Hernández et al. (2004) indicated that the change may be due to the presence of fatty acids or to the relaxing impact on the digestive system due to fenugreek diets. It is possible to assess the positive impact of fenugreek seed on feed intake on the basis of various perspectives that fenugreek has improved palatability as a natural feed additive and could be linked to carbohydrates and their main components, galactomannan, which stimulate the appetizing and digestive process. Michael and Kumawat (2003); Alloui et al. (2012) and are in agreement with this outcome. They stated that neurin, trimethylamine, and biotin are also found in fenugreek, which helps to stimulate appetite through their action on the nervous system.

Feed conversion ratio (FCR): The results of Abu-Dieyeh and Abu-Darwish (2008) agree with this finding. Fenugreek seed feed efficiency improvement may be linked to the production of gastrointestinal tissue morphological changes in broiler chick gut (Alloui et al., 2012; Amal et al., 2013; Weerasingha and Atapattu, 2013). Morphological variations in gastrointestinal tissues can be caused by differences in the microbial content of the intestines, including their metabolites (Xu et al., 2003). Yadav and Jha (2019) also mentioned in their study, gut microbiota and their metabolic products improve absorption and nutrient utilization in poultry.

Meat yield characteristics: Mamoun et al. (2014) reported that the 1% level supplementation of fenugreek seeds in the broiler chicken diet caused substantial improvements in the percentage of carcass and intestinal length. Due to the inclusion of fenugreek seeds in diet, a major impact on the digestive parts seen, increase in weight and length of intestines has been reported (Duru et al., 2013). However, Weerasingha and Atapattu (2013) reported that fenugreek seed supplementation had no significant impact on intestinal length per 100g of body weight when measured. In addition, Bhaisare et al. (2014) observed that dietary inclusion of Fenugreek seeds at 0.5% level in the eight-week diet of Nandanam Turkey poults resulted in substantial improvement in dressed weight ($P<0.05$) and attributed it to fenugreek's antimicrobial properties. Contact between digesta and mucosal epithelium might prolong the positive impact on intestinal morphology, which may be more efficient for nutrient absorption (Bogusławska-Tryk et al., 2012).

Conclusions

Based on the findings of the current experiment, it can be concluded that the seeds of fenugreek have a major impact on poultry production. The results of the study also indicate that the 1.5% dietary supplementation of fenugreek seeds has a high potential as a commercial application for broiler production efficiency. However, to clarify the active principle(s) of antimicrobial activity and other beneficial effects of fenugreek seeds, more research needs to be performed.

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Evaluation of the stimulatory and inhibitory effects of *Malva sylvestris* leaf extract on some beneficial and pathogenic bacteria from the colon

Ahu Demirtaş

Department of Physiology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, 15030, Burdur/TURKEY. Demirtaş A: ORCID: 0000-0003-2942-6243

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ABSTRACT

The aim of the present study was to evaluate the stimulatory and inhibitory effects of *Malva sylvestris* leaf extract on some selected beneficial and pathogenic bacteria from the colon to form a presupposition on its efficacy on intestinal health. The sensitivity of colon bacterial strains to *M. sylvestris* leaf extract was tested by a broth dilution method in the anaerobic cabinet. *Malva sylvestris* leaf extract stimulated the growth of *Bifidobacterium bifidum* from beneficial species starting from 0.06 mg/mL dose ($P<0.05$). The same stimulatory effect was observed for other beneficial species *Bifidobacterium infantis* and *Lactobacillus acidophilus* from 0.125 mg/mL dose ($P<0.05$) and that effect was more obvious for *B. infantis.* On the other hand, the extract did not have any effect on *Lactobacillus casei* up to 4 mg/mL dose. *Malva sylvestris* leaf extract also had a potential inhibitory activity against pathogenic *Escherichia coli*, *Clostridium perfringens*, and *Staphylococcus aureus* from 0.25, 2, and 4 mg/mL concentrations respectively ($P<0.05$). The dose of 8 mg/mL of the extract (MIC; minimal inhibitory concentration) completely inhibited *Fusobacterium nucleatum* ($P<0.05$), other enteropathogen, which is associated with colorectal cancer. It was concluded that *M. sylvestris* leaf extract at 0.06-8 mg/mL dose could have favorable effects on colon bacteria since the extract selectively promoted the most of the beneficial species' growth at this dose range while it had a potential inhibitory or inhibitory effect on pathogenic ones. Investigating the effects of *M. sylvestris* leaf extract on other colon bacteria and testing the *in vivo* effectiveness will contribute to a better understanding of its efficacy on colon microbiota and intestinal health.

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Introduction

Colon constitutes a complex community of bacteria, over 99% of which are anaerobic bacteria (Macfarlane and Macfarlane, 2003). Commensal or resident bacteria of the colon are considered as "beneficial" since they have remarkably crucial influences for the physiology, immunity, and susceptibility to diseases of the host (Kau et al., 2011). Consequently, disturbance of the colon microbiota can cause to expansion of pathogenic species and subsequent abnormal physiological states (Ahn et al., 1998). Recently, many studies have been focused on potential prebiotic and antibiotic effects of medicinal plants and plant metabolites on beneficial and pathogenic bacterial species from the colon microbiota (Phoem and Voravuthikunchai, 2012; Thapa et al., 2012; Goker and Demirtas, 2020).

Malva sylvestris, commonly known as mallow, is a plant native to Europe, Asia, and North Africa with dark green leaves and red to blue-purple flowers (Elsagh et al., 2015). This plant has a long history of

*Corresponding Author: Ahu Demirtaş
E-mail: ahu-demirtas@hotmail.com

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use in Mediterranean and European traditional medicine to treat inflammations, dermal infected wounds, bronchitis, and particularly digestive problems such as peptic ulcers, gastritis, enteritis, and colitis due to its high mucilage content and its colon cleansing properties (Gasparetto et al., 2012; Hamedi et al., 2015; Al-Rubaye et al., 2017). Aqueous extracts of aerial parts of *M. sylvestris* and also isolated polysaccharide of the plant were effective in preventing the inflammatory lesions of ulcerative colitis in the rats (Hamedi et al., 2015). The leaves have shown high effectiveness against swine constipation and in the treatment of mastitis in bovines when applied in enemas or compresses (Uncini-Manganelli et al., 2001). Phytochemical analyses of the *M. sylvestris* leaves have also relieved the presence of a wide range of phytochemical groups with antimicrobial properties such as flavonoids, alkaloids, phenolic acids, quinones, tannins, saponins, steroids, terpenoides, carotenoids, and unsaturated fatty acids (Barros et al., 2010; Dowek et al., 2020). There are reports about antibacterial activity of the extracts of *M. sylvestris* leaf on plant (Razavi et al., 2010), oral (Vahabi et al., 2019), and wound pathogens (Zare et al., 2012) and on some clinical isolates (Azadpour et al., 2016). However the literature is scarce regarding the effects of *M. sylvestris* leaf extract on colon bacteria especially on beneficial ones. Therefore, the aim of the present study was to evaluate the stimulatory and inhibitory effects of *M. sylvestris* leaf extract on some selected beneficial and pathogenic bacteria from the colon.

Materials and Methods

***Malva sylvestris* leaf extract:** The extract was provided by Kale Naturel Herbal Products Company, Ltd., Balikesir, Turkey. As specified by the manufacturer, *M. sylvestris* leaves were air dried, ground into 0 to 200 µm large particles and screened. Powdered plant leaves used for extraction in 80% ethanol (1/10, w/v) at 30°C for 4-5 h and then filtered. The extract was concentrated to 1/5 of its volume with a rotary vacuum evaporator at 35°C for 8 h. The drying process of the extract was performed using a laboratory-scale spray dryer. Afterwards, dry extracts liquefied in the mixer at an adequate ratio.

Microorganisms and growth conditions: The assay was carried out using the following bacterial species: *Bifidobacterium bifidum* ATCC 29521, *Bifidobacterium longum* subsp. *infantis* ATCC 15697, *Lactobacillus acidophilus* ATCC 4356, and *Lactobacillus casei* ATCC 393 as beneficial bacterial species. *Staphylococcus aureus* subsp. *aureus* ATCC 12600, *Escherichia coli* ATCC 11775, *Clostridium perfringens* ATCC 13124, and *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586 as pathogenic bacterial species. The cultivation medium was Mann Rogosa Sharpe (MRS) broth for *B. infantis, L. acidophilus*, and *L. casei*; MRS broth with 0.05% cysteine (MRS-C) for *B. bifidum*; Luria–Bertani (LB) medium for *E. coli*; tryptic soy broth (TSB) for *S. aureus*; and liquid form of medium 2 (Hobson, 1969) for *C. perfringens* and *F. nucleatum*. Medium 2 was prepared anaerobically according to Hobson (1969) with only slight modification. Trypticase peptone was used instead of casitone in medium 2 (Table 1). Ruminal fluid which was used as a component of the medium 2 brought from the slaughterhouse, mixed, and filtered through three layers of cheesecloth to partition into liquid and solid (digesta) fractions. The liquid fraction was centrifuged at 15000 rpm, and the clear supernatant was used as a component of the medium (Table 1). All strains were grown at 37°C for 24 h under an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 in an anaerobic cabinet (Whitley DG250, Don Whitley, West Yorkshire, UK).

Table 1. Composition of medium 2 (for 100 mL)

Component	
Trypticase peptone (BD 211921 Bacto™)	1.0 g
Yeast extract (Sigma Y1625)	0.25 g
Mineral solution 1	15 mL
Mineral solution 2	15 mL
Clarified rumen fluid	20 mL
Resazurin (Sigma R7017)	0.0001 g
Sodium lactate (70% w/v)	1.0 g
Glucose	0.2 g
Maltose	0.2 g
Cellobiose (Sigma 22150)	0.2 g
Cysteine HCl (Sigma C7880)	0.05 g
$NaHCO_3$ (Sigma S5761)	0.4 g
Deionized water	to 100 mL

Mineral solution 1 - 3 g/L K_2HPO_4 (Sigma P3786); Mineral solution 2 - 3 g/L KH_2PO_4 (Sigma P9791), 6 g/L $(NH_4)_2SO_4$ (Sigma A4915), 6 g/L NaCl (Sigma S7653), 0.6 g/L $MgSO_4 \cdot 7H_2O$ (Sigma 230391), and 0.6 g/L $CaCl_2$ (Sigma C1016).

Sensitivity of bacterial species to *M. sylvestris* leaf extract: The sensitivity of colonic bacterial strains to *M. sylvestris* leaf extract was tested by a broth dilution method (CLSI, 2016) in the anaerobic cabinet. A stock solution was prepared by dissolving the plant extract in 50% ethanol. For broth microdilution, 20 µl of an overnight bacterial culture was transferred to wells of a 96-well plate (Flat bottom, Corning 3599) that

already containing 200 µl of two-fold serially diluted *M. sylvestris* leaf extract in the bacterial strain specific growth media. Final concentrations of extract were kept at the ranges of 0.015-8 mg/mL. Each strain was tested in triplicate wells. Plates were incubated for 24 h at 37°C in the anaerobic cabinet. Bacterial growth was detected with a microplate reader at 600 nm (Epoch, BioTek, USA). The minimal inhibitory concentration (MIC) was the lowest concentration of the extract that allowed no visible growth. A significantly lower OD600 value compared to control dose (0 mg/mL) was accepted as potential inhibitory activity (Ko et al., 2018) while significantly higher value was accepted as stimulatory effect (Das et al., 2015).

Statistical analyses: Statistical analysis was carried out by the use of one-way ANOVA followed by Dunnett's test. Each well of a 96-well plate was an experimental unit. A value of P<0.05 was taken to indicate a significant difference.

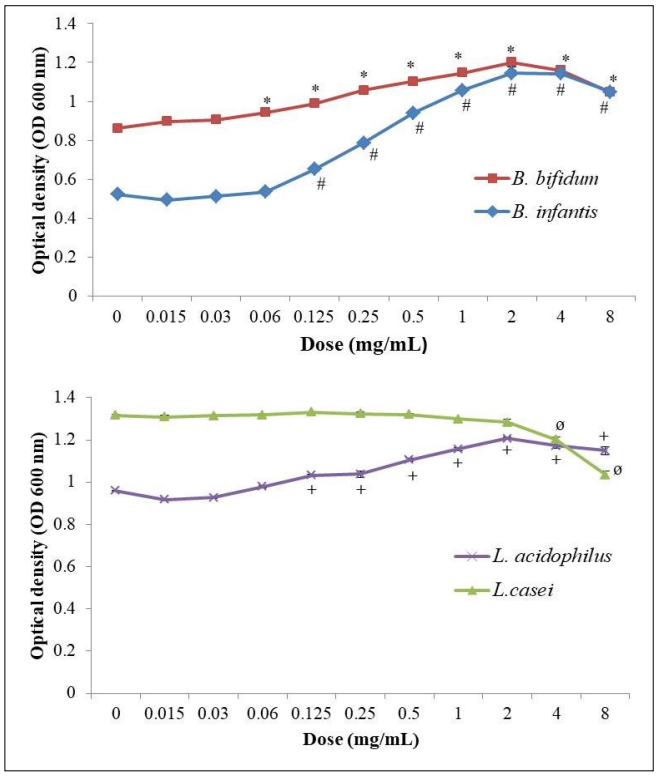

Figure 1. Effects of *M. sylvestris* leaf extract on beneficial colon bacteria. The results represent the mean ± standard error. *P<0.05, extract treated culture vs *B. bifidum* control; #P<0.05, extract treated culture vs *B. infantis* control; +P<0.05, extract treated culture vs *L. acidophilus* control; and øP<0.05, extract treated culture vs *L. casei* control. Control level was 0 mg/mL of the extract.

Results

Effects of *M. sylvestris* leaf extract on colon bacteria are presented in Figure 1 and Figure 2. *Malva sylvestris* leaf extract did not have inhibitory effect on

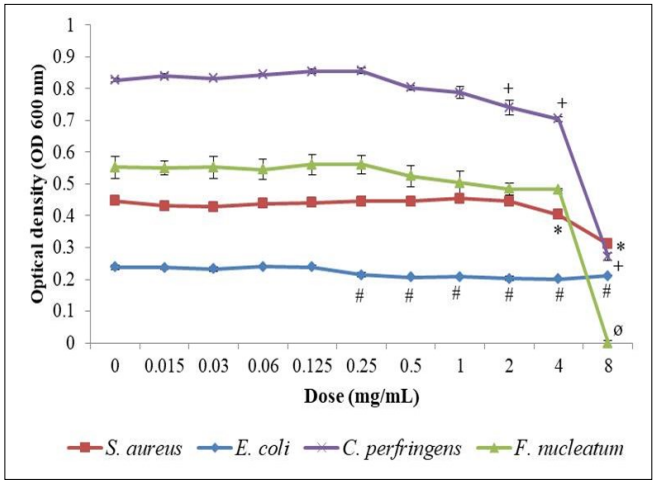

Figure 2. Effects of *M. sylvestris* leaf extract on pathogenic colon bacteria. The results represent the mean ± standard error. *P<0.05, extract treated culture vs *S. aureus* control; #P<0.05, extract treated culture vs *E. coli* control; +P<0.05, extract treated culture vs *C. perfringens* control; and øP<0.05, extract treated culture vs *F. nucleatum* control. Control level was 0 mg/mL of the extract.

bifidobacteria species and *L. acidophilus*. Besides, the extract stimulated the growth of *B. bifidum* starting from 0.06 mg/mL dose (P<0.05). The same stimulatory effect was observed for *B. infantis* and *L. acidophilus* from 0.125 mg/mL dose (P<0.05) and that effect was more obvious for *B. infantis*. The extract did not have any effect on *L. casei* up to 4 mg/mL dose, however showed a potential inhibitory activity from that dose (P<0.05). *Malva sylvestris* leaf extract also had a potential inhibitory activity against *E. coli*, *C. perfringens*, and *S. aureus* from 0.25, 2, and 4 mg/mL concentrations respectively (P<0.05). The extract, on the other hand, completely inhibited *F. nucleatum* at 8 mg/mL (MIC) (P<0.05).

Discussion

Many species of bacteria have adapted to grow in the colonic lumen with concentrations up to 10^{11} or 10^{12} cells/g of luminal contents (Guarner and Malagelada, 2003). Of these bacterial groups, the bifidobacteria is one of the most important genera in the human and animal intestinal tract with its role in the fermentation of the complex carbohydrates (Crociani et al., 1994), producing vitamins, enhancing immunity, and inhibiting invasion of potential pathogens (Shen et al., 2011).

Malva sylvestris leaf extract promoted the growth of *Bifidobacterium* species in a dose-dependent manner in the present study. The density of *B. infantis* nearly doubled in the high-dose groups. Same stimulatory effect was observed for *L. acidophilus* which is the other beneficial bacteria of the colon, which provides health-promoting effects (Shen et al.,

2011). *Malva sylvestris* is rich in polysaccharides such as mucilages, which are particularly responsible for the therapeutic effects of the plant in the gastrointestinal disorders (Gasparetto et al., 2011). It is reported that the mucilage content of *M. sylvestris* leaves is 17.2% which is 2.3 times higher than in roots and flowers (Karawya et al., 1971). The mucilages consist mainly of glucuronic acid, galacturonic acid, galactose, rhamnose, glucose, sucrose, fructose, and trehalose, but uronic acid, fucose, mannose, arabinose, xylose, raffinose, and 2''-O-a-(4-O-methyl-a -d-glucuronosyl)- xylotriose have also been found (Gasparetto et al., 2011). All these sugars are harvested from mucin throughout the gastrointestinal tract by saccharolytic members of the colon microbiota such as bacteroides, *bifidobacteria,* and *lactobacilli* genera (Shen et al., 2011; Bäumler and Sperandio, 2016). These sugars are crucial for the metabolism of colon bacteria as vital carbon sources. Crociani et al. (1994) showed that 96% of *B. bifidum* strains are the only consumers of porcine gastric mucin among 290 bifidobacteria strains tested, and *B. infantis* was the only species that fermented D-glucuronic acid. Consequently, sugars in the mucilage content of the *M. sylvestris* leaf extract could be responsible from the stimulatory effects on the growth of beneficial bacteria except *L. casei* in the present study.

Escherichia coli, *C. perfringens*, and *S. aureus* can often cause foodborne infections and they are associated with gastroenteritis (Ørskov and Ørskov, 1992; Rajkovic, 2014). *Malva sylvestris* leaf extract exhibited a potential inhibitory activity against *E. coli*, *C. perfringens*, and *S. aureus* from 0.25, 2, and 4 mg/ mL concentrations respectively, however it did not inhibit these bacteria completely. Dowek et al. (2020) reported that methanolic extract of *M. sylvestris* leaves showed potential antimicrobial activities against *S. aureus,* a clinical isolate, as that recorded in this study and the zone of inhibition for *S. aureus* was almost 47.2% of the zone for positive control antibiotics. The rate of antibacterial effect was 30.6% relative to negative control for the highest dose in our study. Extract of *M. sylvestris* leaves also did not have an inhibitory activity on *S. aureus* (ATCC 25923) in another study (Azadpour et al., 2016). The extract prepared from the root, leaves, and flowers of *M. sylvestris* at 7.5 mg/mL dose exhibited antibacterial effect of 50% of positive control against a clinical isolate of *S. aureus* while the maximum antibacterial activity was recorded at 15 mg/mL that was almost twice the highest dose (8 mg/mL) used in this study (Walter et al., 2011). The findings about weak antibacterial activity of *M. sylvestris* leaf extract on *E. coli* in the present study are also in accordance with the results of the studies in which *E. coli* strains - without intestinal isolate- was little affected by the methanolic extracts of *M. sylvestris* leaves (Dowek et al., 2020) and resistant to the methanolic extract of *M. sylvestris* aerial parts (Dulger and Gonuz, 2004). On the other hand, there is no report about the effects of *M. sylvestris* extract on *C. perfringens*. However, 1 mg/mL of the extract from the leaves of *M. parviflora*, Egyptian mallow, had a growth inhibition percentage of less than 1% relative to negative control on the same strain of *C. perfringens* used in this study (Omar et al., 2006). The inhibition percentage of the extract on *C. perfringens* was 4.8% relative to negative control in the present study (without statistically significant difference). However, the inhibition percentage of the extract increased to 67.1% at the highest dose of 8 mg/mL.

The other enteropathogen, *F. nucleatum*, is obviously associated with colorectal cancer and promotes the development of colorectal neoplasms (Shang and Liu, 2018). The growth of *F. nucleatum* was inhibited completely by 8 mg/mL of *M. sylvestris* leaf extract in the present study. The ethanolic extract of *M. sylvestris* leaves was also reported to inhibit the same strain of *F. nucleatum* at 1 mg/mL concentration in a previous study (Benso et al., 2015). *Malva sylvestris* leaves were purchased from a local farmer in the northeast Brazil in that study. Phytochemical studies revealed that leaves of *M. sylvestris* are rich in flavonoids and phenolic acids that dominated by luteolin and chlorogenic acid (Terninko et al., 2016) whose antimicrobial effects were reported (Lou et al., 2011; Qian et al., 2020). However, composition of the extracts can vary according to collection location, climatic conditions, soil characteristics, possible differences in the plant genotypes, harvest time, handling types, storage conditions, and extraction method (Fidan et al., 2019). Therefore, the difference between inhibitory concentrations in this and above study (Benso et. al., 2015) might be due to one or more of these factors.

Conclusion

As a conclusion, *M. sylvestris* leaf extract at 0.06-8 mg/mL dose could have favorable effects on colon bacteria since the extract selectively promoted the most of the beneficial species' growth at this dose range while it had a potential inhibitory or inhibitory effect on pathogenic ones. Investigating the effects of *M. sylvestris* leaf extract on other colon bacteria and

testing the *in vivo* effectiveness will contribute to a better understanding of its efficacy on colon microbiota and intestinal health.

Conflict of interest

Author has no conflict of interest to declare.

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The effect of coenzyme Q10 on blood plasma nitric oxide and total antioxidant capacity levels in hypothyroidism-induced rats

Cemşah Yazıcı[1], Tufan Keçeci[2], Durmuş Hatipoğlu[2]

1. Special Trakya Hospital, 22030, Edirne, Turkey. **2.** Selcuk University Faculty of Veterinary Medicine Department of Physiology, 42003, Konya, Turkey. Yazıcı, C. ORCID: 0000-0001-9177-9299, Keçeci, T. ORCID: 0000-0002-6479-3025, Hatipoğlu, D. ORCID: 0000-0003-3790-7821

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ABSTRACT

In this study, the effect of coenzyme Q10 (CoQ10) on nitric oxide (NO) and total antioxidant (TAS) capacity in rats for which experimentally hypothyroidism was induced through PTU was investigated. A total of 32 healthy male Wistar Albino rats weighing 300-350g, approximately 12 weeks old, were used as animal material in the study. . Rats were divided into 4 experimental groups as control (K), Coenzyme Q10 (C), Hypothyroidism (H), and Coenzyme Q10 + Hypothyroidism (CH). During the trial period of three weeks, 3mg CoQ10 (10mg/kg/day) was dissolved in 0.3 ml of maize oil and intraperitoneally administered for each animal in group C. In group H, PTU has added to drinking water daily at a weight/volume (w/v) ratio of %0.05. In the HC group, coenzyme Q10 was administered intraperitoneally and PTU was administered with drinking water at a rate of %0.05. TT4, TT3, and TSH levels were determined in serum samples and NO and TAS levels in plasma samples. In the present study; the highest plasma NO level among the groups was determined in group H ($p<0.05$) and there was no significant difference between other groups (H, C, HC) ($p>0.05$). The plasma TAS value of group H was found to be significantly higher than the same value in the K, C and HC groups ($p<0.05$). The plasma TAS level in group C had no difference from the same value in the HC group ($p>0.05$), although it was higher than the same value of group K ($p<0.05$). As a result, it was found to cause oxidative stress in hypothyroidism-induced rats with a particular increase in plasma NO levels, and CoQ10 was found to be effective in normalizing the increased plasma NO level due to hypothyroidism.

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Introduction

One-third of the world's population lives in the region where iodine deficiency is present, and its effects on the neurological development of fetuses and children in cases where iodine deficiency is severe are well known (Zimmermann, 2009). Moreover, the possible effects of iodine deficiency during pregnancy on the cognitive development of fetus have also been increasingly recognized in recent years (Bath et al., 2013). Changes in food and agricultural practices since the 1950s have caused iodine deficiency to come back to the agenda even in countries where iodine was previously believed to be sufficient, including some developed countries (Taylor et al., 2014). Hypothyroidism, also known as a condition in which the thyroid gland is unable to produce as many hormones as necessary, can be caused by the thyroid gland (primary hypothyroidism), the pituitary gland (secondary hypothyroidism), or the hypothalamus

*Corresponding Author: Cemşah Yazıcı
E-mail: cemsahyazici@gmail.com

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(tertiary hypothyroidism) (Schmid et al., 2006). The most common cause of primary hypothyroidism is severe iodine deficiency (Taylor et al., 2018).

The thyroid hormones, thyroxine (T4) and triiodothyronine (T3) play a critical role in growth and development and regulate the basic metabolic processes that affect almost every organ system in adults (Biondi & Cooper, 2019). Thyroid hormones affect metabolic processes, especially due to changes in ATP metabolism. ATP production, along with other related processes including apoptosis triggering, redox signalling, and intracellular Ca+2 regulation, is primarily driven by mitochondria (Scheffler, 2011). In this respect, hypothyroidism is known to reduce oxygen consumption and promote low metabolism, which causes impairments in hemodynamic, heart and kidney function, as well as lipid metabolism (Franco et al., 2011). Whether T3 and T4 regulate the expression of various membrane-associated respiratory enzymes and metabolite transporters in mitochondria is still controversial.(Paradies et al., 1994; Scheffler, 2011; Schönfeld et al., 1997). Hypothyroidism also affects the expression of mitochondrial proteins from the respiratory chain and decreases coenzyme Q10 (CoQ10) levels, as well as the antioxidant capacity of mitochondria (Fernández-Vizarra et al., 2008; Venditti et al., 2003).

CoQ10 is a fat-soluble vitamin-like compound that can be found in any cell, acting as a coenzyme in enzymatic reactions that occur during energy production in cells (Stocker, 2007). CoQ10, also known as 'Ubiquinone', is found in all tissues in the body, even if its amount is variable, and has a role in all oxidative reactions (Saini, 2011). CoQ10 plays an important role in electron transport and ATP synthesis in mitochondria. There may also be a decrease in the amount of CoQ10, as oxidative damage may occur in patients with both hyperthyroidism and hypothyroidism (Mancini et al., 2011). CoQ10 plays a very important role in the body since it is involved in ATP synthesis and is essential for the health of every tissue and cell in the body. It also has an important antioxidant function (Santoro, 2020).

Considering that hypothyroidism is a serious problem in our country and throughout the world in the case of experimentally hypothyroidism-induced rats, the extent to which CoQ10 will affect blood plasma thyroid hormone levels and levels of nitric oxide and total antioxidant capacity parameters planned to be investigated to contribute to the relevant information.

Materials and Methods

Animal Material: In the study, a total of 32 healthy male Wistar Albino rats, approximately 12 weeks old, weighing 300-350 g, obtained from S.Ü Experimental Medicine Research and Administration Center were used. In the experiment, which included a 10-day adaptation and 3-week main study period, the rats were provided with suitable living conditions in the form of 22 + -2ºC room temperature, \ 50% + -10 relative humidity and 12/12 night and daylight period. In the study, the average amount of water that rats can drink daily (average 50 ml/day/rat) was determined and their water was refreshed daily. The animals were fed with standard rat feed ad libitum.

During the research period, rats hosted in 8 separate cages and 4 in each cage were divided into 4 trial groups: control (K), coenzyme Q10 (C), hypothyroidism (H) and Coenzyme Q10+ hypothyroidism (CH).

Control Group (K): no administration was made to the animals in this group, and during the study, their feed was given as ad libitum, while daily drinking water was given in a determined amount.

Coenzyme Q10 Group (C): rats in this group were administered intraperitoneally during the trial, dissolving in 0.3 ml of maize oil, approximately 3mg CoQ10 (TCI, C1971) (10mg/kg/day) per animal according to their alive weight (Singh et al., 2000).

Hypothyroidism Group (H): 6-N-propyl-2-thiouracil (PTU) (brand; SIGMA p3755) was added to drinking water daily at a rate of \%0.05 weight/volume (w/v) during the trial to induce hypothyroidism in rats (Das & Chainy, 2001).

Hypothyroidism + Coenzyme Q10 Group (HC): animals in this group were intraperitoneally administered coenzyme Q10 dissolved in maize oil in the amount of 10mg/kg/day during the trial.%0.05 PTU was added to drinking water.

At the end of the administration period, blood was taken separately and in sufficient quantities from rats in groups to EDTA and serum tubes with cardiac puncture under general anaesthesia performed with 70 mg/kg ketamine + 5 mg/kg Rompun. Blood samples were centrifuged at +4 ºC at 3500 rpm to obtain plasma (Hettich Rotina 35 R). Serum and plasma samples were stored at -20 °C until they were analyzed. The obtained serum samples of total thyroxine, total triiodothyronine, Thyroid Stimulating Hormone (TSH); Nitric Oxide (NO) and Total Antioxidant Capacity (TAC) levels were determined from plasma samples. The lives of the blood-drawn animals were ended by cervical dislocation, which was

The research was approved by the animal experiments Ethics Committee of Selcuk University Experimental Medicine Practice and Research Center on 28.12.2018 with decision no.2018-49.

From the serum samples taken, TSH, TT4 and total TT3 levels were measured by Kemuliminescence measurement method in Abbot Architect i2000 analyzer, Abbott kits were used for measurements Plasma nitric oxide (Cayman, 780001) and total antioxidant capacity (Tas, Red Assay Diagnostics®) levels of animals in trial groups were determined by the spectrophotometric method by reading absorbency values under commercial kit prospectuses using Biotek brand LX800 model Elisa device and Cayman brand test kits (Messerah et al., 2011) (Messarah et al. 2010).

Statistical analysis of the data obtained at the end of the research SPSS 18.0 (SPSS, Inc. Chicago, IL, USA) was performed using. Variance analysis was performed to determine the importance of the differences between the trial groups, and Duncan multiple comparison tests were performed for posthoc analyses.

Results

In the study, serum thyroid-stimulating hormone (TSH), total thyroxine (TT4) and triiodothyronine levels (TT3) and plasma nitric oxide (NO) and total antioxidant capacity (TAS) values were determined in all four groups are shown in Table 1.

Considering the TSH, TT4 and TT3 values in the study, it is seen that PTU administration causes hypothyroidism in the hypothyroid (H) and hypothyroid + coenzyme Q10 (HC) groups. Serum TSH levels in the H and HC groups were higher ($p < 0.05$) than the control (K) and coenzyme Q10 (C) groups (Table 1), and serum TT4 and TT3 values, which are the main thyroid hormones affecting tissues, were both H and TT3. The lower amount of rats in HC groups compared to other groups confirms this (Table 1).

The highest mean serum TT4 and TT3 levels recorded among the trial groups in the study were observed in Group K, which was significant ($p<0.05$) compared to those of other groups (C, H and HC). While there was no difference between the H and HC groups in terms of these values ($p>0.05$), serum TT4 and TT3 levels in Group C were lower than in Group K ($p<0.05$) and higher than in the H and HC groups ($p<0.05$) (Table 1).

The highest mean serum recorded among the trial groups in the study was observed in group K, which was significantly higher ($p<0.05$) than those of other groups (C, H and HC). While there was no difference between H and HC groups in terms of these values ($p>0.05$), serum TT4 and TT3 levels in group C were lower ($p<0.05$) than in group K, and higher than in H and HC groups ($p<0.05$) (Table 1).

When plasma NO levels were taken into account, it was found that the highest value among groups was in group H with hypothyroidism ($p<0.05$), while plasma NO levels did not differ significantly among other groups (H, C, HC) ($p>0.05$) (Table1).

In the study, it was determined that the plasma TAS value of group H was considerably higher than the same value in the K, C and HC groups ($p<0.05$). The plasma TAS level in group C was recorded as showing no significant difference ($p>0.05$) than in the HC group, although the K group had higher amounts ($p<0.05$) than the same value (Table 1).

Discussion

Since thyroid hormones affect the metabolic activities of a living thing, the development and growth of tissues, and the rate at which nutrients are used to provide energy, (Mortezaee et al., 2019; Pascual & Aranda, 2012), when the secretion of the thyroid hormones decreases, the functions of almost all systems in the body are affected, metabolic activities are disrupted and hypothyroidism occurs (Hall & Hall, 2020).

Table 1. Serum means TSH, TT_4, TT_3 levels and NO and TAS values for all groups.

	Control	Hypothyroidism	Coenzyme Q10	Hypothyroidism + CoQ10
TSH (µIU/ml)	$2.45 \pm 0,055^{b}$	23.28 ± 2.17^{a}	1.80 ± 0.42^{b}	18.82 ± 1.33^{a}
TT_4 (µg/dl)	2.41 ± 0.07^{a}	0.26 ± 0.14^{c}	2.08 ± 0.12^{b}	0.12 ± 0.01^{c}
TT_3 (ng/ml)	0.33 ± 0.18^{a}	0.02 ± 0.003^{c}	0.20 ± 0.05^{b}	0.02 ± 0.01^{c}
NO (µM)	51.5 ± 3.62^{b}	87.1 ± 6.24^{a}	57.5 ± 6.93^{b}	64.0 ± 4.14^{b}
TAS (mmol/l)	9.47 ± 0.85^{c}	15.87 ± 0.68^{a}	12.91 ± 1.33^{b}	10.42 ± 0.94^{bc}

CoQ10 = coenzyme Q10, TSH =thyroid-stimulating hormone, TT_4 = total thyroxine, TT_3 = triiodothyronine levels, NO = nitric oxide, TAS = total antioxidant capacity .

In experimental studies conducted to investigate the effects of hypothyroidism, various anti-thyroid agents that inhibit thyroid hormone synthesis are used. Among these, one of the most widely used is propylthiouracil (PTU). PTU acts by inhibiting the activation of iodine and its binding to tyrosine by inhibiting the tyrosine peroxidase enzyme in the thyroid gland, by preventing the binding of monoiodotyrosine and diiodotyrosine to each other, and by inhibiting the conversion of T4 to T3 with deiodinase inhibition in the periphery. (Cooper et al., 1983). In this study, PTU was administered to rats in the H and HC groups, and it was determined that PTU administration induced hypothyroidism in animals in these groups (Table 1).

According to the findings obtained in the study, the increase in serum TSH level ($p<0.05$), decreased TT4 and TT3 levels ($p<0.05$) in the H and HC groups administered in PTU compared to groups K and C experimentally shows that hypothyroidism was induced.

CoQ10 is a powerful antioxidant in all cells, with electron carrier properties in the electron transport chain. It is synthesized in the body under normal physiological conditions and the amount is sufficient for the body. But various diseases, ageing and degenerative processes can lead to an insufficient amount of CoQ10 synthesized in the body(Bhagavan et al., 2007; Quiles et al., 2020; Sawashita et al., 2020). Therefore, its increasing use as a food supplement is becoming more and more common and is the subject of scientific research in this direction (Gholnari et al., 2018; Jorat et al., 2019; Lee et al., 2012).

CoQ10 can also be used frequently in some endocrinological disorders (Mancini et al., 2011). Since thyroid hormones have common biosynthesis pathways through CoQ10 and tyrosine amino acid, it is common for tight interactions between the hormones of the endocrine glands in question and CoQ10 (Sayıner & Kısmalı, 2016). Metabolic stress caused by a slowing basal metabolic rate in the case of hypothyroidism and an increased basal metabolic rate in the case of hyperthyroidism affects all systems of the body (Venditti & Di Meo, 2006). In cases where metabolic stress and free radical production increased, CoQ10 was found to decrease. (Bhagavan & Chopra, 2006). Therefore, concerning thyroid health, it is thought that CoQ10 supplementation in a controlled manner may be beneficial in people with both hyperthyroidism and hypothyroidism (Saini, 2011).

Different findings in various studies on the effect of CoQ10 on hypothyroidism are notable (Mancini et al., 1989; Mancini et al., 2011; Ogura et al., 1980; Pandolfi et al., 1994; Resch et al., 2002; Saini, 2011). Mancini et al. (1989) report that CoQ10 levels in the blood show a significant inverse relationship with thyroid hormone levels in patients with hyper or hypothyroidism. In another study carried out by the same researchers (Mancini et al. 2011), they noted that due to oxidative damage in patients with both hyperthyroidism and hypothyroidism, there may be a decrease in the amount of CoQ10 that plays an important role in electron transport and ATP synthesis in mitochondria. In comparison to people with hyperthyroidism and hypothyroidism, there are also studies indicating that although serum CoQ10 levels in hyperthyroidism are lower than euthyroidism and hypothyroid subjects, there is no significant difference in hypothyroidism than euthyroidism which reports that the decrease in coq10 level of hyperthyroidism is more than hypothyroidism, on the other hand, it has been emphasized that oxidative stress increases in both hyperthyroidism and hypothyroidism, and that negative changes in the enzymatic and non-enzymatic antioxidant system can have a significant impact (Resch et al., 2002)

In this study, the serum TSH, TT4 and TT3 levels of the C group given CoQ10 did not show a significant difference compared to the control group ($p> 0.05$), the serum TSH level in the HC group given CoQ10 with PTU was higher than the control group ($p <0.05$), The low levels of TT4 and TT3 ($p <0.05$) suggested that CoQ10 was not effective in correcting serum TSH, TT4 and TT3 levels due to hypothyroidism under the conditions in this study (Table 1).

The most important production site of free oxygen radicals in cells is mitochondria (Brown & Borutaite, 2012; Di Meo et al., 2016), therefore, it is inevitable that increases and decreases in levels of thyroid hormones affect the production of free oxygen radicals (Chainy & Sahoo, 2020). Because thyroid hormones make significant changes in the activity and number of certain respiratory chain components in the mitochondria in tissues (Guerrero et al., 1999)significant changes in the oxidant and antioxidant systems of the body occur in both hyperthyroidism and hypothyroidism (Hosseini-Zijoud et al., 2016; Mancini et al., 2011; Resch et al., 2002) Increased metabolic rate with the effect of thyroid hormones accelerates electron transport in mitochondria, which increases superoxide production. Superoxide radicals lead to the formation of many other reactive oxygen species (Freinbichler et al., 2011). Thus, the acceleration of all metabolic events in hyperthyroidism leads to lipid peroxidation, increasing

oxidative metabolism (Joshi et al., 2018; Venediktova et al., 2020). In hypothyroidism, since the metabolic rate slows down, oxidative byproducts are also expected to decrease (Joshi et al., 2018; Pereira et al., 1994), in contrast, there are also studies showing increased oxidative stress (Costantini et al., 1998; Yilmaz et al., 2003). In case of hypothyroidism, oxidized lipoproteins in the hydrolysis of lipid peroxide which serum paraoxonase (PON-1) activity recorded when the decrease occurred, hypothyroidism observed in lipid peroxidation, serum PON-1 enzyme activity and an increase in the reduction of LDL cholesterol to undergo oxidation quickly discussed it with (Sarandol et al., 2006). In this study, compared to the control group, the high plasma NO level ($p<0.05$), which is one of the oxidative stress indicators in hypothyroidism induced rats, supports this view (Table 1). Similarly, Verma et al. (2013) in a study conducted in humans, did not record that serum NO levels increased in those with hypothyroidism compared to controls (Verma et al., 2013). In contrast, plasma NO levels are not affected in the case of hypothyroidism (Hermenegildo et al., 2002). These different findings among some studies may be due to differences in tissue and organ sensitivity, measurement methods, animal species and administration methods (Messerah et al., 2011).

CoQ10, which joins the mitochondrial respiratory system as an antioxidant, protects cells and tissues from the harmful effects of free radicals (Cooke et al., 2008). It performs this function by acting as a coenzyme of three mitochondrial enzymes (complex I, II, III) (Littarru & Tiano, 2010). Furthermore, the quinol form of CoQ10 plays a potential antioxidant role by directly suppressing free radicals in the inner membrane of the mitochondria or by reducing the α - tocopherol radical (Kwong et al., 2002). Paunović et al (2017) report that CoQ10 administration strengthens erythrocyte antioxidant capacity by clearing ROS of the toxic effects of cadmium and interrupting lipid peroxidation (Paunović et al., 2017). Gholami et al (2018) CoQ10 supplementation in women with Type 2 diabetes, adiponectin concentrations and the level of MDA including the fall of adiponectin/leptin ratio were effective in increasing observed (Gholami et al., 2018). In Moazen et al (2015), they noted that the concentration of MDA, a marker of oxidative stress, decreased with the administration of CoQ10 (Moazen et al., 2015). Although the plasma NO level of the HC group is dramatically lower than the H Group ($p<0.05$), it can be concluded that CoQ10 administered to hypothyroidism-induced animals is effective in correcting the changing no level due to hypothyroidism (Table 1). In parallel with AL-Megrin et al (2020) declarations that the administration of CoQ10 in rats increases the body's antioxidant capacity (AL-Megrin et al., 2020), in this study, the plasma TAS of Group C given CoQ10 was found to be higher than that of the control group ($p<0.05$). At the same time, the presence of plasma TAS levels in hypothyroidism-induced rats in Group H in the study in higher amounts than rats in groups K, C and HC ($p<0.05$) may indicate that the body's total antioxidant capacity may have increased due to increased oxidative stress in hypothyroidism (Table 1).

Conclusion and Recommendations

There are many studies with different results regarding the effect of hypothyroidism on the oxidant and antioxidant system and the effect of CoQ10 on the correction of oxidative damage that may occur in hypothyroidism. It is thought that different data, which can be found even in the same tissue and parameters in the literature, may depend on the species, age of the animals, and the different administration methods used (addition to drinking water, intraperitoneal or subcutaneous injections, different anti-thyroid drugs, dosage regimen and duration). Therefore, the relationship between hypothyroidism and oxidative stress and the effect of CoQ10 needs to be studied in more comprehensive research.

In this study, plasma NO and TAS parameters examined within the framework of research opportunities also presented information in harmony with some, although they differed from some literature data. Especially when plasma NO and TAS values are taken into consideration, the results obtained indicate that although oxidative stress increases in hypothyroidism, the addition of CoQ10 may be an effective agent in eliminating oxidative stress formed as an antioxidant.

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Conflict of interest

Author has no conflict of interest to declare.

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Post-operative stifle range of motion in dogs with medial patellar luxation undergoing either trochlear wedge recession or trochlear block recession surgery

Radka S. Garnoeva

Department of Veterinary Surgery, Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, Bulgaria. ORCID ID: 0000-0002-3373-3468

Research Article

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ABSTRACT

The aim of this study was to evaluate post-operative range of motion of stifle joints in dogs affected with medial patellar luxation in order to compare two surgical techniques: trochlear wedge recession vs. trochlear block recession. The study was done with 67 joints from 54 small breed dogs (21 Mini-Pinschers, 16 Chihuahuas, 11 Pomeranians and 6 Yorkshire terriers). From these, 31 joints underwent wedge recession surgery and the other 36: block recession surgery. In wedge recession surgery, trochlear groove deepening was done by removal of a V-shaped wedge, whereas in block recession, the wedge was rectangular. Block recession is suggested to provide a deeper and wider trochlea with preservation of the major part of the articular cartilage. The results suggested that both trochleoplasty techniques resulted in reduction of joint range of motion and improvement of limb alignment at the first post-operative month.

Keywords: medial patellar luxation, dogs, goniometry, block recession, wedge recession

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Introduction

Medial patellar luxation is a common hereditary orthopedic condition in small breeds of dogs (Wangdee et al., 2013). The evaluation of bone deformities by radiography is important for decision making about the operative technique (Tomlinson et al., 2007). The aim of the surgical correction is to provide stability of the patella and to restore the normal quadriceps muscle mechanism. Surgery should not be postponed due to the risk from aggravation of tissue damage and bone deformities (Wangdee et al., 2008; Wangdee & Torwattanachai, 2010). Results from comparative assessment of the superiority of wedge and block recession of the trochlear groove are contradictory (Linney et al., 2011; Fujii et al., 2013; Wangdee et al., 2013). The depth of the trochlea is essential for the stability of the patella (Talcott et al., 2000).

Older cases of MPL are most prone to development of secondary osteoarthritis with progressive joint cartilage erosion and bone lesions (Edge-Hughes & Nicholson, 2007). Early signs of occurring degenerative joint disease are pain and reduced joint range of motion (ROM).

*Corresponding Author: Radka S. Garnoeva
E-mail: dr.garnoeva@abv.bg;

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The latter could be due to contracture of soft tissues caudal to the joint or to irreversible epiphyseal damage, especially in young animals. Such pathological alterations could affect post-operative stifle joint ROM and compromise surgery outcome (Olmstead, 1995).

The aim of this study was to compare post-operative range of motion of stifle joints in dogs with medial patellar luxation corrected by two surgical techniques: wedge recession and block recession of femoral trochlea.

Materials and Methods

Comparative studies were carried out for evaluation of two surgical techniques for treatment of canine MPL – trochlear wedge recession and trochlear block recession of femoral trochlea in 54 dogs (67 joints) with MPL grade II and III in four small breeds (21 Mini-Pinschers, 11 Pomeranians, 16 Chihuahuas and 6 Yorkshire Terriers). Forty-one dogs underwent surgery of one stifle only, while in 13 both stifles were operated. None of patients had data for preceding traumatic injury. The study included only dogs with grade II MPL (41 joints) and grade III MPL (26 joints). Out of them, 36 joints were submitted to block recession surgery, and 31: to wedge recession surgery. All interventions were done by the same surgeon.

Stifle joint ROM values were measured pre-operatively and 1 month after the surgery with a transparent plastic goniometer. Prior to measurements, dogs were anaesthetized as followed: s.c. premedication with 0.02 mg/kg atropine sulfate (Atropinum sulfuricum, Sopharma, Sofia – Bulgaria) and induction of anaesthesia 15 min later with 7.5 mg/kg i.v. tiletamine hydrochloride and zolazepam hydrochloride (Zoletil® 50, Virbac, France). For measurements, patients were in lateral recumbency with studied limb exposed. The centre of the goniometer was placed in the center of stifle joint rotation determined previously by flexion and extension of the joint. The stationary arm of the device was placed along the longitudinal axis of the femur determined by the line connecting trochanter major and the centre of lateral femoral epicondyle, while the lever was aligned on the line joining the lateral maleollus and proximal part of the tibia, caudally to the tibial crest (Figure 1). The angles of full

Figure 1. Measurement of full flexion (left) and full extension (right) angles

flexion and full extension of the joint were measured in degrees.

The non-parametric Mann-Whitney test was used to determine the differences between healthy joints and joints affected by various MPL grades. Values are presented as median [range]. Differences were considered significant at $p<0.05$.

Results

Before the surgery, median ROM of affected knees was 97°, and one month after block recession surgery, it decreased to 95.5° (p=0.09; Figure 2). The same tendency was observed in joints that underwent trochlear wedge recession surgery (Figure 3) – higher preoperative ROM (98°), which decreased to 91° at the first post-operative month (p=0.0079).

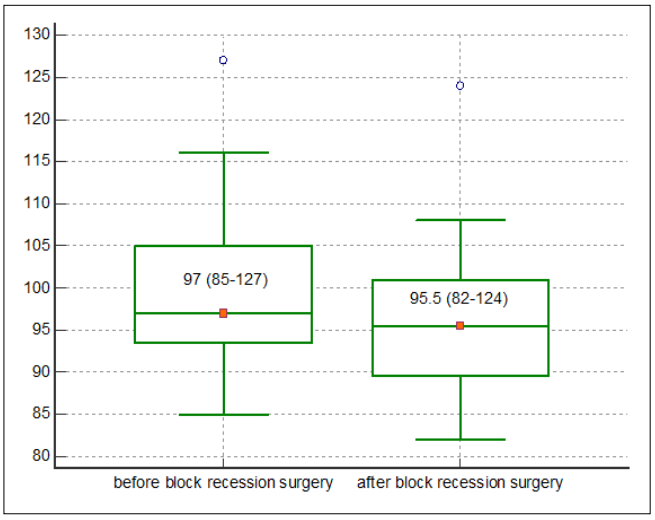

Figure 2. Stifle joint range of motion (degrees) in 36 patients undergoing block recession surgery – prior to and one month after the intervention. Values are presented as median (minimum-maximum range).

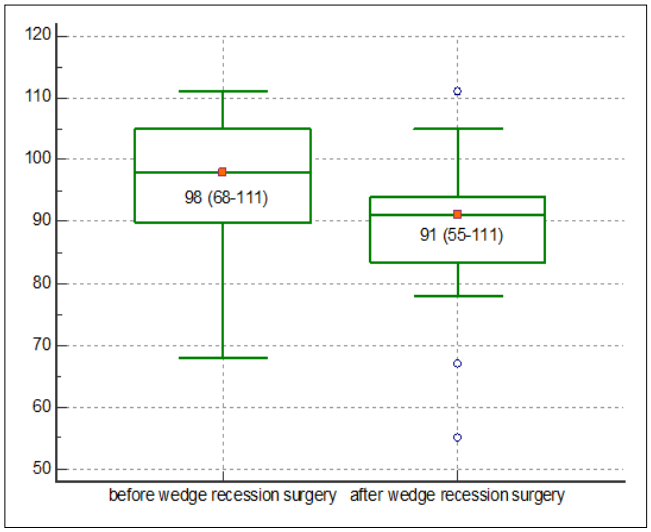

Figure 3. Stifle joint range of motion (degrees) in 31 patients undergoing wedge recession surgery – prior to and one month after the intervention. Values are presented as median (minimum-maximum range).

Discussion

Goniometry in dogs could be performed either in standing position or in lateral recumbency. Due to pain and discomfort in affected joints during their flexion and extension (Thomas et al., 2006) this procedure was performed in our patients in lateral recumbency after general anaesthesia to eliminate pain.

Dog breeds differ considerably by their body structure, body shape and live weight. Therefore, flexion and extension angles of carpal, elbow, stifle and hip joints are quite different. Metric and goniometric studies are mainly performed in large canine breeds (hounds, Golden retriever, Labrador retriever, German shepherd, Kangal, Border Collie, Irish wolfhound etc.) for creation of reference values (Jaeger et al., 2002; Benson et al., 2004; Thomas et al., 2006; Hasbach, 2007; Ates et al., 2011; Hady et al. 2015). Other researcher teams (Ates et al., 2011) have compared between-sex goniometric parameters of thoracic and pelvic limb joints within breeds yet did not found out any statistically significant difference. Our study was done on small dog breeds which are more commonly affected by medial patellar luxation.

It should be noted that different dog breeds have extremely variable body shape, structure and body weight. This is reflected in angles of full flexion and full extension of joints whose reference values have to be breed-specific (Mann et al., 1988; Benson et al., 2004). In mini-dachshunds, goniometric exam is even almost impossible due to the shape of limbs and excessive muscle mass (Thomovsky et al., 2016).

Luxation of patella alters the biomechanical properties of the stifle with resulting changes in full flexion and full extension angles (Thomas et al., 2006). Therefore clinical goniometry is an essential part of clinical examination of such patients (Petazzoni & Jaeger, 2008). The available information about joint's range of motion after orthopedic surgery is scarce (Daems et al., 2009).

In our study, ROM of stifle joint immediately prior to surgery was higher. This, in our opinion, could

be attributed to higher full extension angle due to contracted m. quadriceps femoris which at the same time, did not allow full flexion of the joint, e.g. higher flexion angle.

After both surgical interventions, stifle ROM decreased, but this change was statistically significant only after wedge recession surgery. At present, we could not provide a logical explanation of this fact. Radiography in mediolateral and craniocaudal views did not establish any changes that could be responsible because possible changes are in soft tissues and not in bones, hence are not visible on radiographs.

It was reported that following block recession surgery, recovery of limb function was faster and what is more, signs of degenerative joint disease were less severe (Johnson et al., 2001). In our survey, similar findings were also recorded perhaps due to preservation of the major part of joint cartilage after block recession surgery of trochlea.

Conclusion

In dogs with grade II and III medial patellar luxation, both applied trochleoplasty techniques resulted in decreased range of motion of operated joints, which was preoperatively increased by m. quadriceps femoris contracture. By the end of the first post-operative month, dogs demonstrated fairly good use of the operated limb as the reduced joint range of motion improved limb's clinical state and alignment.

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Efficaciousness of Sterne 34F-2 strain of *Bacillus anthracis* vaccine in cattle for anthrax control program in Bangladesh

Shahjahan Ali Sarker, Ahosanul Haque Shahid, and K. H. M. Nazmul Hussain Nazir*

Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.
Sarker S. A. ORCID: 0000-0001-7480-3576 ; Shahid A. H.: ORCID: 0000-0002-0281-4644; K. H. M. Nazmul Hussain Nazir ORCID: 0000-0002-3893-754X

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ABSTRACT

Live spore Sterne 34F-2 strain of *Bacillus anthracis* vaccine is being used to control anthrax disease in Bangladesh. After produced this vaccine in Livestock Research Institute (LRI) under the Department of Livestock Services (DLS), it is distributed at the farmers level through district and Upazila Livestock Offices. In these distribution pathways, the vaccine has been transported and stored for a few days in each station. The present study was carried out to evaluate the humoral immune response of the anthrax vaccine and to measure the impact of existing transportation and storage systems on immunity status. For that a total of 60 cattle were randomly selected , divided into three groups and used the vaccines collected from three distribution points. Serum samples were collected before and after the 1st month, 4th month, 7th 10th, and 13th month of vaccination respectively the anthrax antibody level in blood were monitored. The optical density was converted to ELISA units (EU) and used to express the antibody level in the vaccinated animals. It was significantly increased above the protection level (1.00) for a year. Before vaccination, the average ELISA unit of serum sample was 0.18± 0.01, after vaccination it was raised above the protective level (1.00) within one month and continued up to a year. In the chi-square test (95% confidence level), there was no significant difference ($p<.05$) ELISA unit among the three groups that means no impact on vaccine distribution points on the immunity level of the studied animal. The Sterne 34F-2 strain *Bacillus anthracis* vaccine has been found to be efficacious to protect animals from anthrax in the rural areas and no significant impact on immune response due to existing transportation and storage facilities.

Keywords: Sterne 34F-2, *Bacillus anthracis*, ELISA, IgG, community household cattle

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Introduction

Among the various infectious diseases that cause a hundred to thousands of death of livestock animals in Bangladesh, Anthrax is one of them. This disease is caused by *Bacillus anthracis*; a spore-forming gram-positive bacterium that can survive in soil as dormant for decades (Saile & Koehler, 2006; Hugh-Jones & Blackburn, 2009; Silvestri et al., 2015). It is considered a serious disease of livestock because it usually strikes suddenly with livestock showing few signs of illness before dying and has zoonotic importance (Shiferaw,

*Corresponding Author: K. H. M. Nazmul Hussain Nazir
E-mail: nazir@bau.edu.bd

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2004; Beyer & Turnbull, 2009; Thapa et al., 2014). Usually, herbivores (cattle, sheep, and goats) become infected when more than a specific quantity of spores enter the body with contaminated feedstuff in contaminated pastureland during grazing. Certain environmental factors such as drought, flooding rain, and soil tillage can all increase the risk of an anthrax outbreak in an area. On the other hand, weakly acidic loamy type soil (pH 6.38±0.15) with low organic matter like carbon and calcium level has positively influenced on anthrax incidence in animals. A human can become infected through direct or indirect contact with a sick animal only (Shafazand, et al., 1999; Chakraborty et al., 2012; Coffin et al., 2015; Islam et al., 2018). The preventive vaccination of livestock can manage the risk of anthrax infection. The anthrax vaccine, produced in Livestock Research Institute (LRI), Mohakhali, Bangladesh is a live spore vaccine. The master seed of this vaccine employed for this vaccine is Sterne 34F-2, which originated from Australia. It is effective immunologically to produce enough immunity to protect livestock against anthrax disease that was studied by Dipti et al., 2013 and Hasan et al., 2015. The morphological and immunological study was conducted on farmed goats at the Department of Pathology, Bangladesh Agricultural University during the period from 2012-2013 (Dipti et al., 2013), and another efficacy study of this vaccine was conducted in a commercial farmed cattle named Lal Teer livestock Limited Mymensingh during the period from April 2013 to April 2014 (Hassan et al., 2015). The vaccine was proved to be effective in farmed goat and cattle, raising anti anthrax antibody and activates antibody production above the reference value at day 7 of post-vaccination and that continues over one year (Dipti et al., 2013; Roy et al., 2013; Hassan et al., 2015). Bangladesh has 24 million cattle, 26 million goats, 3.5 million sheep, and 1.5 million buffaloes (DLS, 2019) that has been playing a vital role in the socio-economic development but more than 80% are reared under the traditional farming system (Huque et al., 2017; Saadullah, 2002). After production of anthrax vaccine at Livestock Research Institute (LRI), Bangladesh, it distributed to District Livestock offices, and then District Livestock offices send it to the Upazila Livestock Offices. This is the vaccine distribution system of Department of Livestock (DLS), Bangladesh. Cattle farmers receive vaccines for their livestock immunization through Upazila Livestock Offices. Usually, it takes more than months to collect the vaccine and they seldom follow the standard of storage (stored below temperature 8° C) and transport protocol (must maintain cool chain) due to lack of facilities or other adversity like ice melt at vaccine carriers, use insufficient ice, traffic jams, uninterrupted power supply of refrigerator, etc. that sometime noticed by general peoples. So the farmers sometimes have suspicion on the quality of the vaccine. Most of the previous studies were conducted on crossbred cattle in organized dairy farms that maintained a minimum standard of feeding management under farming condition but at the rural community level, there has a wide range of diversity among bred, age, weight, feeding husbandry, and other management factors (Reuveny et al., 2001; Xu & Frucht, 2007; Dipti et al., 2013; Roy et al., 2013; Hassan et al., 2015). So, the present study was carried out to evaluate the humoral immune response of anthrax vaccine among the cattle of community households and to measure the impact of existing transportation and storage systems on the immunity status of anthrax immunized cattle using the Enzyme-Linked Immunosorbent Assay (ELISA) technique.

Materials and Methods

Study area: The study was conducted in the Kamarkhandha Upazila of Sirajganj district, north-western Bangladesh, located in between 24°18' and 24°27' north latitudes and in between 89°35' and 89° 42' east longitudes (Figure 1) where "An integrated approach to establish an anthrax-free model area in Bangladesh" project was running from July 2018 to June 2020. This research was funded by the Ministry of Education (MoE), Government of the People's Republic of Bangladesh, Project No: 2018/501/MoE.

Study group: For this study, a total of 60 cattle of different ages, breed, and sex from 60 farmers (1 from each farmer) were randomly selected from 3 separate parts of the studied Upazila. Most of them were between the ages of 1 to 3 years, bull and heifer calves that were not vaccinated before or not likely to become pregnant during the study period, were chosen. The selected cattle were divided into 3 groups each group comprised of 20 cattle. Of 3 groups of cattle, group 1(T1): vaccine collected from Livestock Research Institute, Group 2(T2): vaccine collected from District Livestock Office (Sirajganj), and Group 3 (T3): vaccine collected from Upazila Livestock Office (Kamarkhandha) were used.

Vaccination: The selected cattle were vaccinated subcutaneously two times, one just after the first blood sample collection and another were 10 months later of the first vaccination. The dose of each time was 1ml per cattle (each ml has approximately 1×107 attenuated live spores of *Bacillus anthracis*) and other measures were taken as per the manufacturer's instruction. Commercial name of this vaccine was

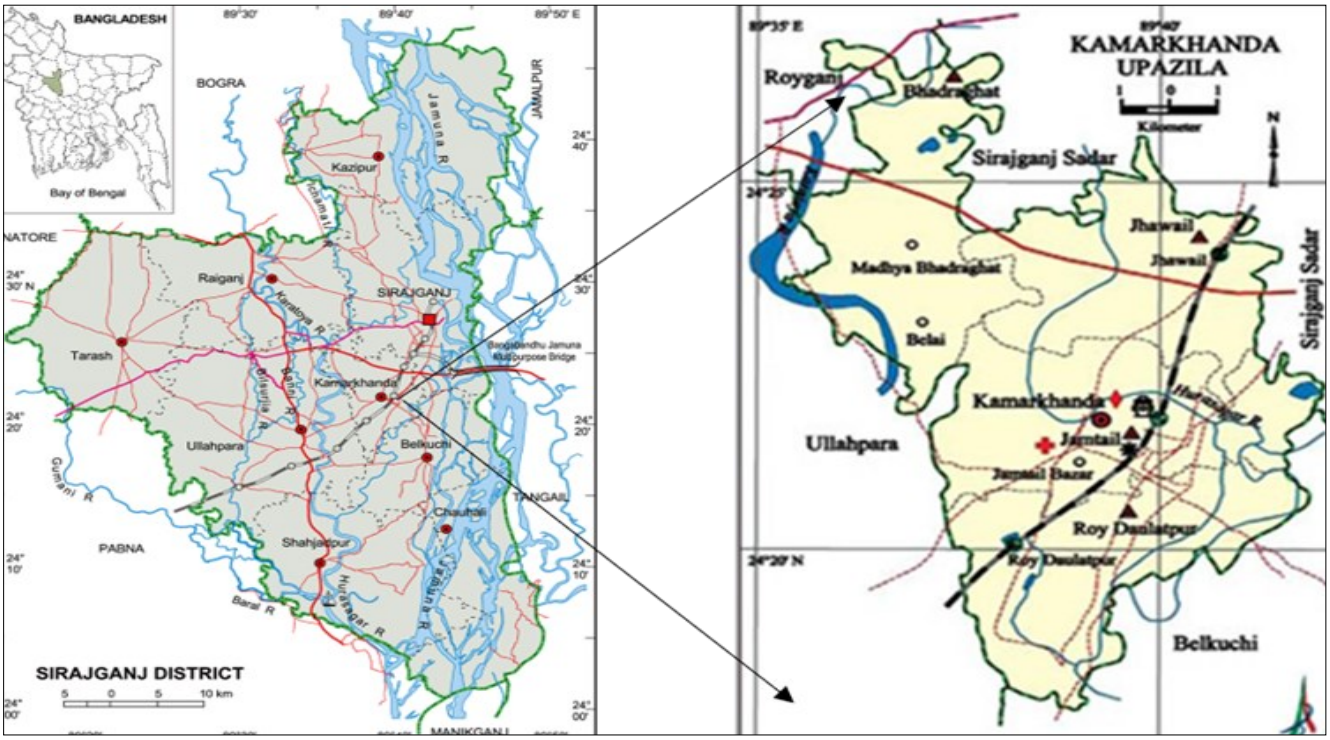

Figure 1. Location of the study area

Torka® vaccine. 5 ml blood sample from each animal was collected 6 times from all animals. After the collection of blood samples, serum was separated in sterilized epindrop tube and finally it was dispatched to the laboratory at the Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh maintaining a cold chain, according to standard protocol for ELISA. Serum separation and transportation methods were followed as Hassan et al., (2015)Serological analysis: The antibody level of serum samples was determined by the Enzyme-Linked Immunosorbent Assay (ELISA) to follow according to the manufacturer's assay protocol [Nori® Bovine Anthrax Receptor 1 (ANTXR1) ELISA Kit, Cat. # GRC 112080, Genorise Scientific Inc, USA]. This kit was for the quantification of anthrax receptors in cattle. This was a quick ELISA assay that reduces time to 50% compared to the conventional method, and the entire assay only takes 3 hours. This assay employs the quantitative sandwich enzyme immunoassay technique and uses biotin-streptavidin chemistry to improve the performance of the assays. An antibody specific for bovine of anthrax receptor was pre-coated onto a microplate. Standards and samples were pipetted into the wells and anthrax receptor was bound by the immobilized antibody. After washing away any unbound substances, a detection antibody specific for bovine of anthrax receptor was added to the wells. Following a wash to remove any unbound antibody reagent, a detection reagent was added. After intensive wash, a substrate solution was added to the wells and color develops in proportion to the amount of anthrax receptor bound in the initial step. The color development was stopped and the intensity of the color was measured (Genorise, INC).

Data analysis: All the data of serum samples were recorded to excel spreadsheets and again categorized into 3 groups based on vaccine collection sites as T1 (Vaccine collected from Livestock Research Institute (LRI), Mohakhali, Dhaka, Bangladesh), T2 (Vaccine collected from District Livestock Office, Sirajganj), and T3 (Vaccine collected from Upazila Livestock office, Kamarkhandha, Sirajganj). Data were summarized into Microsoft Excel 7 (Microsoft Corporation, Redmond, WA, USA) spreadsheet and statistically analyzed using Epi-Info 3.5.3 (CDC, Atlanta, USA). Descriptive analysis was performed, and results expressed in frequencies and proportions. Categorical response variables were presented as proportions and their associations determined by chi-square tests and one-way analysis of variance (ANOVA).

Results

The ELISA values of IgG against anthrax vaccine were determined by Cutoff mode with wavelengths 405, 630nm. Before vaccination, the average ELISA value of serum sample was 0.18± 0.01, after vaccination with Sterne 34F-2 strain of *Bacillus anthracis* vaccine it was increased above the protective level (1.00) within one

Table 1. ELISA value of serum samples collected form the cattle vaccinated with F-34 strain of *Bacillus anthracis* vaccine

Time	Before vac.	Month-1	Month-4	Month-7	Month-10	Month-13	P- Value
ELISA Value	0.18 ± 0.01	2.16 ± 0.06	1.78 ± 0.06	1.61 ± 0.05	1.35 ± 0.04	2.34 ± 0.08	0.000

The data represent the mean value and ± standard error. Before vac. = Before vaccination

month and continued up to the next vaccine schedule of next year. After the second dose (booster dose) of the vaccine, it further increased up to 2.34±0.08. Over time, the average ELISA value was statistically significant ($p<.05$) in 95% confidence intervals. The detailed findings are shown in Table 1-2 and Figure 2.

On the other hand, vaccines were categorized into three different groups based on their collection place. The ELISA value of the serum sample was divided into three groups as per the vaccine collection place. After analysis of the group-wise ELISA value, it was confirmed that the p-value was 0.98 which means there was no significant difference ($p<.05$) ELISA value among the three groups. The details findings are shown in Table 3. and Figure 3.

Discussion

In Bangladesh DLS under the Ministry of Fisheries and Livestock produces anthrax vaccine through LRI approximately 4.0 million doses of anthrax vaccine annually. However, the quantity of vaccines is not sufficient to immunize all susceptible animal species in Bangladesh. Due to a lack of resources like a shortage of manpower along with other facilities like a dedicated vaccine transportation system, proper cool chain are the constraints of a vaccination program (Sarker et al., 2013; Mondol and Yamage, 2014; Rahman et al., 2014). The main objective of this study was to evaluate the efficacy of the 34F-2 strain of the *Bacillus anthracis* vaccine at the farmers' level. This strain has been used in different counties of the world to prevent anthrax disease in animals (Turnbull, 1998; Siamudaala et al., 2006; Moazeni et al., 2007; Laxmi et al., 2016). The result of this study in the form of elevation of IgG anthrax vaccination in cattle was also confirmed by other researchers in Bangladesh (Dipti et al., 2013; Roy et al., 2013; Hassan et al., 2015). Moreover, individual variation of the immune response was noticed within the same species of different animals due to their biochemical and physiological differences (Glass et al., 1990; Koolhaas, 2008; Karim et al., 2010). The bovine T cell proliferation response was depending upon the major histocompatibility complex (MHC) class II (Petroff et al., 1997) for that reason the same dose of vaccine does not elicit the same amount of immune response that was also seen in this study. However, this finding is very insignificant therefore it could not affect our major finding. The successful development of protective immunity against anthrax vaccines in an animal requires a potent vaccine (Brey, 2005). In the 1930s, Sterne developed live, attenuated strains of *Bacillus anthracis*, which is still being used worldwide for immunization of domesticated animals against anthrax (Swartz, 2001). Further, vaccine response in household rearing cattle has been obtained in this study was corroborated by other researchers in Bangladesh (Dipti et al., 2013; Roy et al., 2013; Hassan et al., 2015). However, their study was conducted in commercially farmed animals.

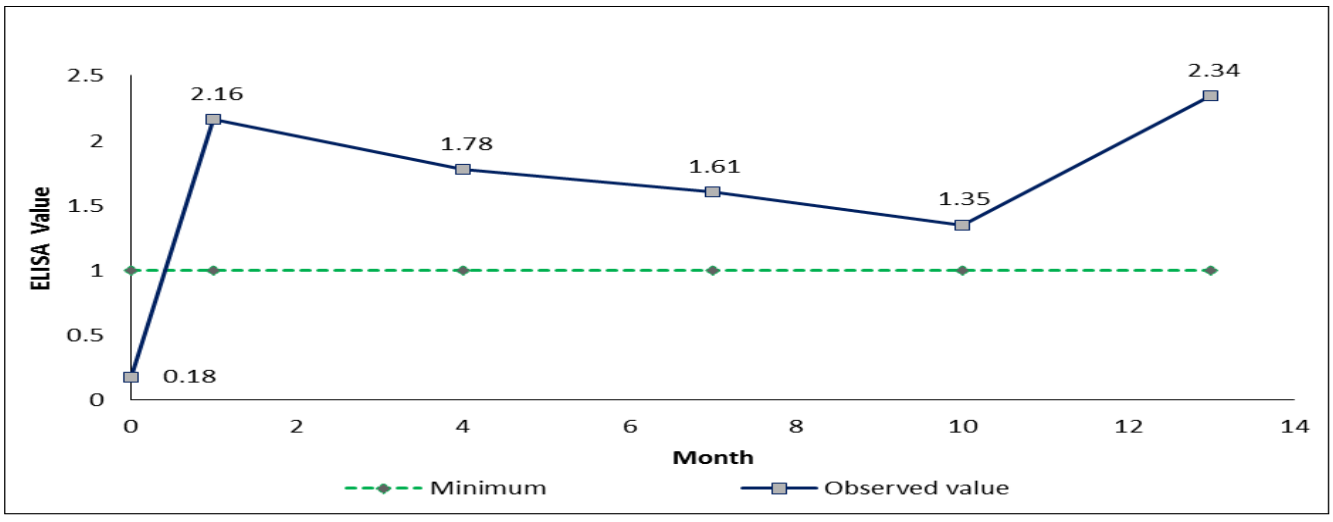

Figure 2. Antibody titer level of Sterne 34F-2 strain of *Bacillus anthracis* vaccine over time

Table 2. Groupwise ELISA value of serum samples according to collected Sterne 34F-2 strain of *Bacillus anthracis* vaccine from different place

Time	Before vac.			Month-1			Month-4			Month-7			Month-10			Month-13		
ELISA values	T 1	T2	T3	$T1^1$	$T2^2$	$T3^3$	$T 1^1$	$T2^2$	$T3^3$	$T 1^1$	$T2^2$	$T3^3$	$T 1^1$	$T2^2$	$T3^3$	$T 1^1$	$T2^2$	$T3^3$
	0.16	0.11	0.02	2.14	2.9	2.12	1.53	1.2	1.82	1.47	1.2	2.52	1.11	1	1.59	2.34	1.57	1.84
	0.17	0.12	0.18	2.75	1.5	2.36	1.5	1.73	1.86	1.37	1.96	1.36	1.01	1.63	1.56	2.56	1.85	2.36
	0.15	0.13	0.5	2.56	1.42	2.46	1.52	1.53	1.76	1.4	1.64	1.06	1.2	1.43	2.46	2.66	1.66	3.18
	0.23	0.17	0.37	2.36	1.37	2.34	1.49	1.67	1.23	1.37	1.97	1.19	1.26	1.57	2.04	2.56	1.84	2.81
	0.23	0.16	0.38	3.15	2.38	2.57	1.95	1.58	1.97	1.78	1.78	1.37	1.68	1.48	1.57	3.12	1.74	2.35
	0.3	0.15	0.12	1.79	1.54	2.56	1.56	1.74	1.39	1.45	1.94	1.36	1.22	1.64	1.06	1.92	1.89	2.13
	0.11	0.16	0.21	1.45	2.53	2.34	1.45	1.63	1.54	1.33	1.73	1.74	1.22	1.53	1.04	1.68	1.79	2.11
	0.12	0.26	0.16	1.68	1.46	1.23	1.5	1.86	1.73	1.42	1.26	1.23	1.22	1.76	1.23	1.58	2.12	2.19
	0.1	0.4	0.2	1.84	2.1	2.45	1.68	1.78	2.55	1.5	1.5	1.65	1.3	1.13	1.18	1.72	2.7	2.16
	0.15	0.5	0.1	1.65	2.23	3.56	1.6	1.43	2.13	1.3	1.63	2.09	1.2	1.19	1.07	1.75	2.93	2.87
	0.11	0.12	0.11	1.84	2.11	3.1	1.45	2.31	1.2	1.32	2.51	1.3	1.11	1.56	1.16	2.11	2.43	2.7
	0.08	0.11	0.1	3.14	1.28	1.23	2.5	1.88	1.33	2.1	1.48	1.03	1.5	1.68	1.33	3.33	1.99	2.13
	0.11	0.11	0.1	3.12	2.39	1.35	2.41	1.89	1.15	2.11	1.39	1.05	1.5	1.69	1.45	3.12	2	2.25
	0.21	0.15	0.13	3.01	2.37	1.25	2.88	2.27	1.65	2.34	1.27	2.05	1.96	1.07	0.95	3.22	2.42	1.78
	0.12	0.19	0.13	2.96	2.21	1.24	2.15	2.31	1.94	1.98	1.41	1.64	1.44	1.11	1.14	3.11	2.5	2.51
	0.15	0.23	0.15	2.45	2.34	1.23	2.03	1.54	2.13	1.98	1.74	2.03	1.4	1.34	1.33	2.49	2.77	2.58
	0.14	0.22	0.1	3.12	2.89	2.68	2.45	1.99	2.38	1.45	1.39	2.08	1.21	1.09	1.58	3.5	3.21	2.92
	0.21	0.41	0.21	1.45	3.1	1.98	1.23	1.45	2.78	1.11	1.3	2.58	1.01	1.3	1.29	1.85	3.61	2.11
	0.22	0.03	0.15	1.24	2.92	2.23	1.22	1.23	1.43	1.11	1.12	1.63	1	1.02	1.53	1.22	3.05	2.58
	0.14	0.03	0.23	1.04	1.57	2.12	1.12	2.17	2.22	1.21	1.77	2.32	1.1	1.31	1.32	1.26	2.2	1.59
Mean	0.16	0.19	0.18	2.24	2.13	2.12	1.76	1.76	1.81	1.56	1.60	1.66	1.28	1.38	1.39	2.36	2.31	2.36
SD	0.05	0.12	0.11	0.69	0.57	0.66	0.48	0.32	0.45	0.35	0.33	0.48	0.23	0.25	0.35	0.70	0.56	0.40
SE	0.14	0.36	0.79	0.47	0.33	0.45	0.38	0.29	0.33	0.29	0.30	0.30	0.22	0.25	0.28	0.46	0.45	0.29
P-value	0.67			0.82			0.92			0.79			0.43			0.96		
Remarks	NS			NS			NS			NS			NS			NS		

Legends: before Vac. : Befre Vaccination; SD:Stand Deviation; SE:stand Error; NS: Statistically not significant 1: Vaccine collected from Livestock Research Institute 2: Vaccine collected from District Livestock Office (Sirajganj), 3:Upazla Livestock Office (Kamarkand)

In this study, we found a minimum level of antibody titer elevation at the first blood sample (before vaccination), may due to got maternal antibodies from their mother (Turnbull et al., 1992; Lembo et al.,2011) or naturally got the infection through a low infective dose (WHO, 2008) since the study location is considered to be anthrax endemic area of Bangladesh (Islam et al., 2018).

Conclusion

Bangladesh Livestock Research Institute, Mohakhali, Dhaka produced Sterne 34F-2 strain of Bacillus anthracis vaccine is quite effective to produce sufficient immune responses. It also raised adequate immunity of the animal's body against anthrax for a year and there was no impact on the immune response due to the existing transportation and storage system. Further study may compare this strain with other strains of the *Bacillus anthracis* vaccine at the community level is demanding as a future endeavor.

Table 3. Comparative ELISA value of serum samples for the vaccine of three different places.

Vaccine collected form	Time of collection					P-value
	1st month	4th month	7th month	10th month	13th month	
Vaccine collected from Livestock Research Institute	2.24 ± 0.69	1.76 ± 0.48	1.56 ± 0.35	1.28 ± 0.23	2.36 ± 0.70	0,98
Vaccine collected from District Livestock Office (Sirajganj)	2.13 ± 0.57	1.76 ± 0.32	1.60 ± 0.33	1.38 ± 0.25	2.31 ± 0.56	
Upazila Livestock Office (Kamarkand)	2.12 ± 0.66	1.81 ± 0.45	1.66 ± 0.48	1.39 ± 0.35	2.36 ± 0.40	

Remarks: No significant difference exists between the three groups (p-value is larger than 0.05)

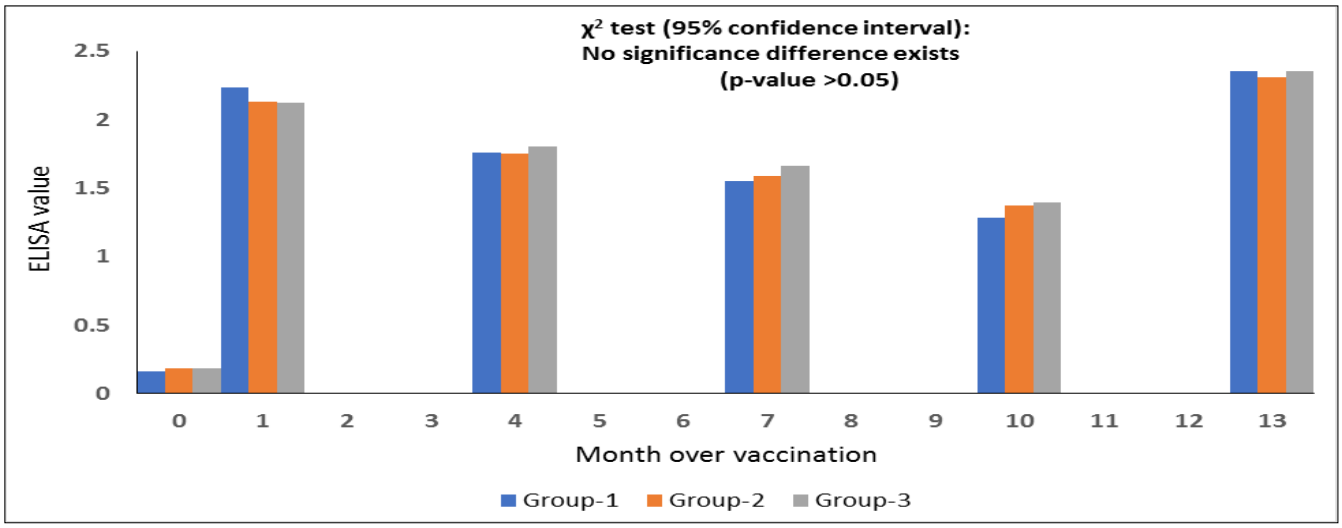

Figure 3. Compare of ELISA value between three groups (Group-1: Vaccine collected from Livestock Research Institute, Group-2: Vaccine collected from District Livestock Office and Group-3: Vaccine collected from Upazila Livestock office)

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Conflicts of Interest

The authors declare no conflict of interest.

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Current invasive and non-invasive biomarkers in canine mammary tumors

Aslıhan BAYKAL[1,*] **Özge TURNA** [1]

1. Istanbul University-Cerrahpaşa, Veterinary Medicine, Department of Obstetrics and Gynaecology, 34320, Istanbul, Turkey. Baykal A: ORCID: 0000-0002-2107-1874, Turna Ö: ORCID: 0000-0002-7638-0519.

Review Article

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ABSTRACT

In bitches, the incidence of mammary tumors is determined at %50 of all neoplasms. Most of the cases are malignant and tend to metastasize. Because of this, early diagnosis has an important value. Due to the molecular and clinical similarities of canine mammary tumors to human breast cancers, human breast cancer markers are also detectable in cases of canine mammary tumors. Generally, biomarkers provide information about criteria such as clinical diagnosis, early diagnosis, prognosis, and determination of the treatment protocol. When choosing the appropriate biomarker, it should be taken into account that it is easy to apply, has a positive correlation with tumor specificity and malignancy, and whether gives precise information about prognosis. Due to the heterogeneous nature of canine mammary tumors, an ideal biomarker has not determined yet. However, new studies have revealed the existence of many biomarkers. In this review it is given to qualified and current biomarkers can be used in dogs, including invasive and non-invasive mammary tumor biomarkers.

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Introduction

Mammary tumors are among the frequent neoplasms in bitches (Lana et al., 2007). Malign tumors constitute approximately 40-50% of the cases (Sorenmo et al., 2003). Besides, 50% of the malign masses tend to metastasis (Sleeckx et al., 2011). The studies conducted report that the most frequent mammary tumor types in dogs are mixed tumors (67%), adenoma and adenocarcinoma (32%) (Casalli et al., 2012). In its etiology, particularly steroid hormones play a role as well as the genetic, breed predisposition, nutrition, and environmental factors (Sorenmo et al., 2003; Chang et al., 2009; Beauvais et al., 2012). Although mammary tumors are detected in very old animals, it inclines to occur between the ages of 8-10 years (Sorenmo et al., 2003). Another reason for the increased incidence of mammary tumors is the time of neutering. While the risk for an animal to develop mammary tumor associated to prepubertal neutering is 0.5%, the risk increases in the subsequent cycles (Sorenmo et al., 2003). Besides, frequent phantom pregnancy/ false pregnancy or administration of exogenous progesterone also increase the risk of mammary tumors (Benavente et al., 2016).

Following the removal of the tumor by surgical incision, prognostic assessment is based on clinical grading, size of the tumor, spread to lymph nodes, existence of metastasis, and lymphatic and vascular invasion. Histopathologic examination is conducted according to the criteria determined by World Health Organization (Perez Alenza et al., 2000; Sorenmo et al., 2003; Ferreira et al., 2009; Santos et al., 2013; Tavasoly et al.,

*Corresponding Author: Aslıhan Baykal
E-mail: aslihan.baykal@istanbul.edu.tr

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2013). Typically, chemotherapy or radiotherapy is preferred for malignant masses based on the result of histopathology. However, the failure to determine the efficiency of the treatment protocols produces various restrictions such as toxic effect and high costs. Similar to humans, it is reported that the efficiency of the treatment in dogs may be limited due to the micrometastasis which have already been occurred at the treatment stage (Rogers, 1993). This emphasizes the importance of early diagnosis. Ki-67, CEA (Carcinoembryonic Antigen), VEGF (Vascular Endothelial Growth Factor) may be given as examples to diagnostic biomarkers. Although there is no sufficient information in the literature regarding the accuracy of the biomarkers used in veterinary medicine, the studies on this matter have increased recently. Human breast neoplasm cases resemble the canine mammary tumors in molecular and clinical aspects. As they have similarities in immunohistochemical properties, it is concluded that the same biomarkers may be used (Pena et al., 2014). Yet, the heterogeneous morphology of canine mammary tumor poses a challenge in the selection of the most suitable biomarker (Gama et al., 2008). The purpose of this review is to include the current invasive and non-invasive biomarkers proven to be used in canine mammary tumors.

A. Invasive Biomarkers

1. miRNA

miRNA, known as short non-coding RNA, is available in eucaryotes such as animals and plants. It has an important role in the regulation of gene expression at post-transcription level. Besides, miRNAs participate in the regulation of cellular growth, proliferation, differentiation, and apoptosis (O'day et al., 2010). Studies indicate that detecting the miRNA in circulation might be an important diagnostic tool in tumor cases. In addition to diagnosis and prognosis, miRNAs are also used in the selection of the efficient chemotherapy and considered as an effective biomarker for treatment. Irrespective of the histological type, miRNA profile of canine mammary tumors is expressed at different levels based on the malignity, type, and metastasis factory of the tumor (Bulkowska et al., 2017). While some miRNAs are upregulated in cancer cases, others show down-regulation; and this indicates that miRNAs may move as oncogene or tumor suppressor genes (Iorio et al., 2005). They are used both as tissue and serum biomarkers (Kaszak et al., 2018). Despite the numerous studies on miRNA in human medicine, the number of studies in veterinary medicine is limited. Bulkowska et al. (2017) reported that 9 out of 10 miRNA expressed in humans were similarly expressed in canine mammary tumors. According to researchers, miR-15a and miR-16 show down-regulation in ductal carcinoma samples, and miR-181b, miR-21, miR29b and miRlet-7f show up-regulation in tubular papillary carcinomas. Similar studies prove the existence of miRNA's mentioned related to human breast neoplasms in canine mammary tumors (Boggs et al., 2008; Chou et al., 2013; Lutful Kabir et al., 2015; Sahabi et al., 2018).

2. Ki-67

One of the well-established biomarkers in canine mammary tumors and an important one in the assessment of proliferation and apoptosis is Ki-67 (Zuccari et al., 2004). In addition to these, Ki-67 demonstrated positive correlation with parameters such as metastasis and survival (Pena et al., 1998). The increase in Ki-67 expression is typically associated with the size of the tumor, presence of ulcers, poor clinical picture such as inflammation, invasion towards surrounding tissues and involvement of lymph nodes (Lohr et al., 1997; Nowak et al., 2015; Rodrigues et al., 2016). Ki-67 may only be detected during the cell cycle on chromosomes, and it may not be detected in cells outside of the division phase (G0) (Scholzen and Gerdes, 2000; Endl et al., 2001). The availability of Ki-67 expression during the cell cycle and the non-availability of this expression at resting phase enable it to be the cell proliferation biomarker considered as an indication of neoplastic changes (Neumann et al., 2016). Another study reviewed the determination of Ki-67 antigen in canine mammary tumors using Diff-Quick method (Choi et al., 2009). It was concluded that Ki-67 antigen might be detected using Diff-Quick method when histopathology is not possible. Obtaining significant results through

cytological methods allowed Ki-67 to be one of the biomarkers that may be used for early diagnosis.

3. PCNA (Proliferating Cell Nuclear Antigen)

Besides being a proliferation biomarker, the concentration of PCNA fluctuates throughout the cell cycle, and a significant increase occurs in S phase which is one of the interphase stages (Bravo and Celis, 1980; Celis and Celis, 1985). PCNA plays a role in the nucleic acid metabolism, and it is responsible for essential DNA replication, cell cycle control, and RNA transcription (Juríková et al., 2016). In dogs with mammary tumors, PCNA expression was reported to be positively correlated with tumor size, ulceration, lymph node metastasis and degree of malignity, and that, accordingly, higher expressions might be associated with low survival and poor prognosis (Carvalho et al., 2016). However, PCNA expression may also be stimulated by cytokines. Therefore, it is not suitable to be used as the sole specific biomarker for cancer cases. Thus, it should be used combined with another biomarker (Kazsak et al., 2018).

4. Protein p53

p53 gene participates in DNA repair, regulation of cell cycle and apoptosis (Lee et al., 2004). In addition to these, it has tumor suppressing property, but it gains oncogenic property by accumulating in tumor tissue due to gene mutation during tumor development (Gasco et al., 2002). The studies conducted indicate that increased expressions are associated with the degree of malignity and low survival. (Lee et al., 2004; Łopuszyński et al., 2010).

As detected in human breast neoplasm cases, it was concluded that the existence of necrosis is associated with the increase in p53 expression (Kato et al., 2002; Dolka et al., 2016). A study reported that p53 positive mammary tumors are more frequent in large dog breeds, particularly in Boxers; this gene mutation may be genetic; and therefore, genetic susceptibility should be considered during assessments (Veldhoen et al., 1999). As in human medicine, increased expressions of p53 gene are important in veterinary medicine in terms of prognosis, and therefore, the use of the mentioned biomarker should be supported by future studies.

5. E-cadherin

E-cadherin is a member of cadherins known to have an important role in the regulation of cell-cell adhesion in epithelial tissues (Takeichi et al., 1991). E-cadherin is expressed on basolateral surfaces of epithelial cells and at the contact points of cells (Boller et al., 1985). In addition to tissue morphogenesis and differentiation, it participates in processes such as cell shape, proliferation. E-cadherin is located in mammary gland which is one of the epithelial tissues, and it ensures functional and structural integrity of the gland (Andrews et al., 2012). Low expressions occur in transition from epithelial origin to mesenchymal origin, and this reduction in expression is associated with the progress of the case towards malignity (Chan et al., 2015).

Transition from epithelial to mesenchymal origin causes the invasion and spread of tumor cells and occurs when the epithelial cells gain mesenchymal properties (Baranwal et al., 2009; Chan et al., 2015).

Immunohistochemical analysis exhibited that E-cadherin membrane expression reduces in canine mammary carcinoma (Asproni et al., 2015). This result was associated with aggressive clinical features observed in canine mammary carcinomas such as high mitotic index, large tumor size, infiltration, invasion, and lymph node metastasis (Brunetti et al., 2003; Matos et al., 2006; Gama et al., 2008). Besides, it is agreed that E-cadherins have an effect on tumor suppression. In tumors with partial or total loss of E-cadherin expression, higher infiltration and lymph node involvement were observed (Heimann et al., 2000). Reduced E-cadherin expression in canine mammary tumors were found to be associated with poor prognosis (Matos et al., 2006). It was also presented that low or negative E-cadherin expression in canine mammary tumors is a marker associated with aggressive properties exhibited by the tumor (Varallo et al., 2019).

6. CEA (Carcinoembryonic antigen)

Carcinoembryonic antigen (CEA) is produced by the cells located in gastrointestinal mucosa (Almeida et al., 2007). It participates in

intercellular adhesion (von Kleist et al., 1995). Increased expression occurs in breast, lung, and rectum adenocarcinoma cases (Souza et al., 2002). In human breast neoplasms, it is used in combination with CA 15-3 biomarker (Ledecky et al., 2013). Besides, European Group on Tumor Markers recommends using CEA and CA15-3 levels together in the early detection of disease progression and in the assessment of the treatment follow-up in breast cancer (Molina et al., 2005). On the other hand, regarding the use of CA 15-3 and CA together, American Society of Clinical Oncology suggests that the use of these biomarkers in monitoring, staging and routine surveillance of the disease after primary treatment would not lead to accurate data (Harris et al., 2007; Cardoso et al., 2008). While CEA may be detected in canine mammary tumor tissue, it is found at higher levels in tumor tissues compared to healthy tissues (Campos et al., 2012; Ledecky et al., 2013). There is no sufficient literature in veterinary medicine on this matter, and new research should be conducted to determine its usability in disease follow-up and other roles in the assessment of disease.

7. CA 15-3 (Carbohydrate antigen)

Carbohydrate antigen (CA 15-3) is a product of mucin 1 (MUC1) gene, and it is used as a biomarker which is expressed in high levels in adenocarcinomas and which may subsequently be detected in circulation (Perey et al., 1992; Hayes et al., 2012). Malignant changes that occur in the tissue and the changes in the structure of the relevant cells lead CA 15-3 to increase in the circulation, and from this aspect, CA 15-3 is accepted as an essential biomarker which may be used in detecting mammary gland carcinoma (Kausitz et al., 2003). During the transformation to malignity, MUC1 acts like an anti-adhesive molecule and causes the malign cells separate from each other; this increases the invasion and metastatic potential in tumor cells (Kaszak et al., 2018). Manuali et al. (2012) reviewed the correlation between the expression of CA 15-3 in cell line and tissue and the histological staging of tumor in canine mammary tumors. At the end of the trial, it was presented that CA 15-3 was expressed at higher levels at grade I compared to grade II and grade III carcinomas. This finding suggests that CA 15-3 is associated with parameters such as poor clinical picture, poor prognosis, tumor size, ulceration, and inflammation.

8. VEGF

Vascular endothelial growth factor (VEGF) is a protein that stimulates the migration and proliferation of vascular endothelial cells which have an important role in angiogenesis (Restucci et al., 2002). VEGF is produced by neoplastic cells, macrophages, and plasma cells, and it also stimulates the growth of neoplastic cells (Lui et al., 1995). It is typically used for the assessment of angiogenesis in human medicine. VEGF synthesis in many malignant tumor cases is associated with metastasis (Takahashi et al., 1995). VEGF expression is highest in tumors with significant necrosis, and this is known to occur associated with hypoxia in necrotic areas (Shweiki et al., 1992). This is because hypoxia has an effect of increasing the transcription of VEGF coding gene (Ikeda et al., 1995). The studies on canine mammary tumor present that high VEGF expressions are associated with poor prognosis and low survival (Queiroga et al., 2011). Similar to human medicine, its combined or individual use is indicated to be important for early diagnosis also in veterinary medicine (Lawicki et al., 2016; Zajkowska et al., 2016).

9. EGFR (HER-1)

Epidermal growth factor receptor (EGFR) is a transmembrane protein and participates in the regulation of morphogenesis, proliferation, migration, and adhesion in cells (Yarden and Sliwkowski, 2001). It is proven that EGFR expression occurs in aggressive canine mammary tumors (Gama et al., 2009). High EGFR expressions are also associated with stimulated angiogenesis and poor prognosis (Carvalho et al., 2013; Queiroga et al., 2017). Guimaraes et al. (2014) studied whether there was a relation between Cox-2 and EGFR in canine mammary tumors. As both parameters studied had positive correlation with each other, they concluded that EGFR and Cox-2 inhibitors may be a treatment option in canine mammary tumors. The studies conducted indicate that EGFR may be used in

clinical picture assessment and the inhibitors may be used in treatment protocol.

10. HER-2

Human epidermal growth factor receptor (HER-2) is one of the important biomarkers in mammary tumor cases. HER-2 regulates the events such as tumor growth, survival, and differentiation (Kazsak et al., 2018). Studies suggest that increased expressions of HER-2 protein in canine mammary tumors indicate poor prognosis (Dutra et al., 2004). On the other hand, in some studies, it was determined that the patient had longer survival time in malignant mammary tumors with high HER-2 expression (Lüftner et al., 2003). Supporting this finding, a study conducted by Hsu et al. (2009) analyzed the survival time with HER-2 expression. When the survival time were reviewed at the end of one year in trial, it was concluded that dogs with HER-2 positive had longer survival time. Accordingly, it was identified that HER-2 is not suitable to use directly in determining development and malignity. However, the area of use of this biomarker should be supported with new studies as there are opposite findings.

11. PR/ER

Estrogens bind to estrogen receptors (ER) in healthy canine mammary gland. When malignant masses were compared with benign masses or normal tissue in terms of ER expression, it was observed that the expression was low in malignant tumors (Canadas-Sousa et al., 2019). In addition to this, reduced ER expression is associated with large size of the mass and lymph node involvement, and therefore, ER is accepted as a marker with prognostic importance (Nieto et al., 2000; Las Mulas et al., 2005). Progesterone has severe effects on the growth and differentiation of normal mammary gland and malignant mammary epithelial growth (Queiroga et al., 2015). In a study, the expression levels of steroid hormones in serum and tissue were analyzed and at the end, an increased expression in malignant tumors was detected (Queiroga et al., 2015). There are studies that support the opposite of these findings. In a study where the progesterone receptor (PR) expression was reviewed a gradual reduction in PR expression was observed in hyperplastic, benign, and malign masses, respectively (Millanta et al., 2005).

Unlike the chemotherapy protocol administered to human breast tumor cases, the use of hormones in canine mammary tumors is not widespread due to the side effects. There is a single study which reviewed the potential therapeutic effect of tamoxifen and determined the safe dosage range (Tavares et al., 2010). However, the side effects of tamoxifen used in dogs with estrogenic effect such as pyometra should be taken into account (Morris et al., 1993).

12. Oxytocin

Oxytocin is essentially synthesized in hypothalamus and secreted by neurohypophysis and participates in different biological functions. (Zingg and Laporte, 2003). In the mammary gland, oxytocin primarily ensures lactation with the contraction of the myoepithelial cells (Geddes, 2007). Recently, the effect of oxytocin hormone on human breast neoplasm has been revealed (Cassoni et al., 2004). But the role of oxytocin receptors in dogs in the formation of tumors in mammary tissue has not yet been clarified. In a study conducted in 2012, the existence of oxytocin receptor expression on canine mammary tumor cell line was determined (Bergman, 2012), and the subsequent study revealed that oxytocin might have antiproliferative effect on cell line (Benavente et al., 2016). In another study by Benavente et al. (2019), the existence of oxytocin receptor expression was detected in all samples. When classified based on the histological grades, the oxytocin receptor expression among malign tumors was found significantly high in Grade III compared to Grade I and Grade II. In fibroadenoma, adenoma and benign mixed tumor group, it was shown that the expression in samples with fibroadenoma was higher, and there was not a significant difference between solid carcinoma, carcinosarcoma and tubulopapillar carcinoma samples. As a result, benign tumors have higher oxytocin receptor expression than malign tumors. When ER and ER malign mammary tumors were compared, the oxytocin receptor expression in ER tumors was found significantly higher, and no relation was detection between ER tumors and oxytocin

receptor expression. Finally, it was suggested that oxytocin receptor expression is not associated with tumor size, clinic phase or lymph node involvement. Based on this study, it may be proposed that oxytocin receptor expression may be used as cellular differentiation biomarker. The change in oxytocin receptor expression based on malignity in canine mammary tumors should be supported with new studies.

13. Heat-scock proteins

13.1. αB-crystallin

Heat-shock proteins is a group of proteins that increase with the exposure of cells to high temperature. Other stress factors except for high temperature may also cause an increase in these proteins. It participates in correct folding of the proteins or ensuring the detachment of wrong folded proteins from each other. The substances that assist the correct folding of proteins in the cell are called 'molecular chaperones' (Ellis et al., 1991; Deocaris et al., 2006; Chmielewska-Krzesinska et al., 2019). Many tumor cells produce high levels of chaperones. Heat-shock proteins released out of the cell participate in the spread of tumor cells to surrounding or farther tissues. belonging to small heat-shock proteins family, ATP inhibits the wrong folding and accumulation of proteins as an independent molecular chaperone (Bakthisaran et al., 2015). Besides, it supports the oncogenic transformation and participates in carcinogenesis at the transition from epithelial origin to mesenchymal origin in form of anoikis resistance, angiogenesis, and organ specific colonization (Malin et al., 2016). In a study by Chmielewska-Krzesinska et al. (2019), the usability of gene expression as a circulating tumor cell biomarker was reviewed. Circulating tumor cells are those which may be detected in blood in epithelial tumors produced from the primary focus or its metastasis (Plaks et al., 2013). Therefore, the use of circulating tumor cells as biomarkers may be important in determining the cells likely to form metastasis. So, the 'circulating tumor cells (CTCs)' may be recommended as biomarker for canine mammary tumors (da Costa et al., 2011). In the majority of the cases, gene expression was detected higher in tumor tissue than healthy tissue. Besides, increased expression of is associated with aggressive tumor characteristics and poor clinical picture (Goplen et al., 2010; Malin et al., 2014; Shi et al., 2016). In another study, it is indicated that CRY AB and CK17 expressions occurred 90% together in canine mammary adenoma and carcinoma, and therefore, these two genes might be referred to jointly (Chmielewska-Krzesinska et al., 2019). According to this, while CRY AB and CK17 increased by 70% in adenoma group, this increase was found to be 50% in carcinoma group. It was concluded that might be considered as a circulating tumor cells biomarker, but it might not be used as a universal biomarker. Due to the difference between the data and the lack of further literature regarding the given biomarker, there should be more studies related to the use of gene expression as a biomarker in veterinary medicine.

13.2. HSP90B1

Hsp90 is a member of heat-shock proteins family and plays an important role in mammary tumors. Hsp90B1 gene is expressed at high levels on many cancer cell lines and associated with aggressive growth and invasion (Lee et al., 2014). It is also associated with increased expression, distant metastasis, and decreased survival time on mammary cancer cell line (Cawthorn et al., 2012). Grp94 (Glucose regulated protein) is the endoplasmic reticulum paralogue coded by Hsp90B1 gene (Sunil Kumar et al., 2017). Carcinogenesis causes nutrition and hypoxic stress in cells and damages the protein mechanism. The wrong folded or unfolded proteins due to the damaged protein mechanism accumulate in endoplasmic reticulum lumen. Eventually, Grp94 expression increases to protect cell homeostasis. Besides, Grp94 participates in the living, proliferation, and invasion of cancer tumors (Scheuner et al., 2001; Nami et al., 2016). In a study, Hsp90B1 mRNA expression was compared in healthy and tumorous mammary tissues (Sunil Kumar et al., 2017). In this context, Hsp90B1 mRNA expression in tumorous tissues in form of carcinosarcoma, complex carcinoma, fibrosarcoma and simple carcinoma was higher than healthy tissues. In tumorous tissues, only simple carcinoma occurred significantly lower

compared to other tumor subtypes. Data obtained suggest that Grp94 is a promising biomarker in the assessment of prognosis although there are no sufficient sources regarding the correlation in serum concentration.

14. Interleukins

14.1. Interleukin-6 and interleukin-10

As the tumor growth and progression is controlled by the immune system, the importance of availability of cytokines in the occurrence of these events is proven in various studies (Lin and Karin, 2007; Fernandes et al., 2015). While certain cytokines participate in the mechanisms causing the growth and progression of the tumor, some involve in the mechanisms inhibiting the tumor growth (Nicolini et al., 2006; Newman and Gonzalez-Perez, et al. 2014). Interleukin-6 (IL-6) is a pro-inflammatory cytokine, and tumor cells play a role in its production (Ben-Baruch, 2003; Kim et al., 2010). Besides, IL-6 supports tumor growth and progression as it has numerous effects on tumor cell proliferation, patient's survival time and formation of metastasis (Nicolini et al., 2006). Studies on canine mammary tumor associate high levels of IL-6 in serum with poor prognosis (Estrela-Lima et al., 2016; Martins et al., 2016). Interleukin 10 (IL-10) is an anti-inflammatory cytokine and inhibits the pro-inflammatory cytokine production. It is also considered that IL-10 has an anti-tumor activity and supports tumor growth (Changkija et al., 2012). Merendione et al. (1996) reviewed increased IL-10 concentration and concluded that this increase is in correlation with advanced clinical findings. Estrela-Lima et al. (2016) addressed the change of IL-10 concentration in dogs with metastatic mammary tumor. As a result, they determined that IL-10 concentration was higher in the existence of metastasis compared to non-metastatic cases. In another study supporting the mentioned study, it was concluded that IL-10 concentrations were higher in inflammatory adenocarcinoma cases compared to non-inflammatory adenocarcinoma cases (De Andres et al., 2013). In light of these information, a study on healthy plasmas and plasmas wit mammary tumors in dogs investigated the usability of IL-6 and IL-10 as a biomarker in canine mammary tumors (Szczubial et al., 2018). It was detected that plasma IL-6 concentration in dogs with mammary tumor was higher compared to healthy dogs, and it was higher in Grade 3 tumors then Grade 1 tumors. IL-10 was detected at significantly higher levels only in malign tumors. The higher concentrations of IL-6 in Grade 3 tumors indicate that the mentioned cytokine has a positive correlation with the increase in the malignity grade of the tumor, and therefore, it may be used as a potential diagnostic and prognostic biomarker. The increase in IL-10 concentration suggests that it may assist in the assessment of the malign form.

14.2. İnterleukin-35

Interleukin-35 (IL-35) is a member of interleukin-12 family (Olson et al., 2013). It is also a cytokine which ensures the secretion of Treg cells and participates in inhibiting the functions and proliferation of T cells (Collison et al., 2010). Treg cells suppress the immune system cells through various mechanisms (Carvalho et al., 2019). It was proven that IL-35 is associated with tumor growth pathogenesis, progression of malignity, immunosuppression, and poor prognosis (Zeng et al., 2013; Liao et al., 2014; Hamidinia et al., 2015; Zhang et al., 2015). It was determined that IL-35 expression is higher in human mammary tumor tissues (Hamidinia et al., 2015; Hao et al., 2018). In another study, it was determined that the ratio of IL-23 and IL-35 (IL-23:IL-35) in the circulation decreases with the increase in the expressions of ki-67, p53 and EGFR, which are in the same family, in the human breast neoplasm tissue. According to this, it was concluded that IL-35 might be an important marker in the prognosis and progression of the disease (Hamidinia et al., 2015; Hao et al., 2018). When the literature on the given cytokine is reviewed, there is no sufficient literature in veterinary medicine. Carvalho et al. (2019) proved that high levels of IL -35 expressions are significantly associated with ulceration, formation of necrosis, histological grade of tumor, increased mitotic index, spread to lymph nodes, and aggressive tumor proliferation.

15. COX-2 (Cyclooxygenase-2)

Cox-2 participates in the formation of prostaglandins

mediating events such as cellular proliferation, apoptosis, angiogenesis by taking part in arachidonic metabolism during tumorigenesis (Grosch et al., 2006). The studies conducted suggest that Cox-2 expression is associated with lymph node involvement and distant tissue metastasis (Lavalle et al., 2009; Queiroga et al., 2010). In canine mammary carcinomas, Cox-2 and ki-67 expressions are associated with poor prognosis and short survival time (Araújo et al., 2016). Besides, expression of Cox-2 and HER-2 in positive correlation indicates poor prognosis (Millanta et al., 2006). In these tumor cases where Cox-2 expression existence is observed, the use of piroxicam and meloxicam of Cox-2 inhibitors helps in the treatment by inhibiting angiogenesis and cellular proliferation (Knottenbelt et al., 2006). It is known that the use of piroxicam particularly in inflammatory carcinoma cases is very effective (Hugo et al., 2015). As a result, in addition to the use of Cox-2 expression as a good marker, its inhibitors have a great share in treatment.

16. BRCA1/BRCA2 (Breast Cancer Type 1 and Type 2 Susceptibility Protein)

Mutations in BRCA1 and BRCA2 genes are known to play a role in the growth of canine mammary tumors. In addition to its use as a biomarker, its use in the target treatment is another research subject. BRCA1 gene is observed in malignant tumors and BRCA2 gene is observed in both malignant tumors and benign tumors (Nieto et al., 2003; Rivera et al., 2009). In a study where BRCA2 mRNA levels were analyzed, reduced BRCA2 expression was detected in tumorous tissue when compared to healthy mammary tissue (Yoshikawa et al., 2015). On the other hand, Ripoli Lüder et al. (2016) compared healthy tissues, benign and malignant tissues, and no significant difference was detected between them. Due to the contradicting results in veterinary oncology, the mentioned gene mutations are still open to research. Its usability as a biomarker should be studied by future genome studies.

17. Caspase-3

Caspases are members of cysteine protease family, and they are one of the major effectors of apoptosis (Zapata et al., 1998; Degterev et al., 2003). Apoptosis begins with the extrinsic and intrinsic stimulation of caspases in mammals (Reed, 2000). Once caspases are activated, they may break up many cellular targets such as cytoskeleton proteins, oncoproteins and DNA repair system proteins. Active caspase-3 is particularly responsible for breakdown the DNA repair system. As it occurs in the cytoplasm of apoptotic cells, the assessment of active caspase-3 allows detecting the early stage of apoptosis (Hadjiloucas et al., 2001). Besides, caspase-3 is accepted as an important indicator of morphological and biochemical changes associated with apoptosis (Degterev et al., 2003). In a study, caspase-3 positive cells were detected at lower levels in malignant mammary tumors (Rodrigues et al., 2015). It also is noted that there is a statistically significant relation between mitotic index which confirms the disrupted balance between proliferation and apoptosis in canine mammary tumors (Vakkala et al., 1999; Huang et al., 2015; Dolka et al., 2016). Recently, caspase-3, which is a protein associated with apoptosis, has been used as a potential biomarker in human breast tumors (Kymionis et al., 2001; Yildirim et al., 2014). Therefore, as a result of the similarities between human breast tissues and canine mammary tissues, the use of caspase-3 as a biomarker in veterinary oncology should be clarified through future studies.

B. Non invasive biomarkers

1. Urine

1.1. Spesific urine metabolites

Valko-Rokytovska et al. (2020), unlike invasive approaches, paved the way for the use of non-invasive biomarkers and detected the levels of tyrosine (TYR), tryptophan (TRP) and their metabolites from urine collected through palpation from healthy patients and patients with mammary tumor after 12-hour fasting. The imbalances in TYR and TRP and their metabolites occur as a result of cancer, neurologic and inflammatory disorders (Oto et al., 2008; Wiggins et al., 2015; Heng et al., 2016). Serotonin (5-HT) which is one of the TRP metabolites is a mitogenic factor for cancer cells and participates in the autocrine cycle of growth factors. Besides, it

contributes to the proliferation in aggressive tumors such as lung, bladder, and breast tumors (Jose et al., 2017). In addition to 5-HT, 5-hydroxyindol acetic acid (5-HIAA), which is another metabolite, participates in the follow-up of disease progression and the assessment of treatment response (Nam et al., 2009). Kynurenic acid (KYNA) is known to have a dual role by allowing malignant cells escape the immune system agents or having an anti-proliferative effect on cancer cells (Sagan et al., 2015). 3,4-dihydroxy-L-phenylalanine (L-DOPA) metabolizes into epinephrine and norepinephrine as a result of decarboxylation reaction (Valko-Rokytovska et al., 2020). Epinephrine participates in the formation of immunosuppressive microenvironment in mammary gland tumors in humans as well as some cancer types (Muthuswamy et al., 2017). At the end of the experiment, the values of epinephrine, L-DOPA, 5 -HIAA, indoxyl sulphate (IS), 5-HT, KYNA metabolites were found significantly high when compared to the control group. Although the tumors of the patients enrolled in the study were small, the metabolite levels were compared according to tumor size and tumor grade. The levels of E, L-DOPA, 5-HIAA and 5-HT metabolites were found highly significant in Grade 3 tumors. Results indicate that the metabolite level is not associated with tumor size. Similar to humans, the use of biomarkers in animals to distinguish diseases from normal individuals has a great importance. In this determination process, responses to process-related criteria such as proliferation, lifetime, chemotherapy response, etc. are sought through various invasive procedures such as various aspiration/biopsy and blood collection from the patient. The non-invasive biomarkers should have more extensive use because of the ease of application, and they should be developed by future studies.

1.2. Neutrophil gelatinase–associted lipocalin and matrix metalloproteinase 9 complex

Neutrophil gelatinase-associated lipocalin (NGAL) is expressed by neutrophils or different cells in cases such as tissue injuries (Kjeldsen et al., 2000). Besides, studies indicate that accumulation of NGAL is associated with the progression of the tumor (Fernandez et al., 2005; Provatopoulou et al., 2009). Three molecular forms of NGAL complexed with Matrix Metalloproteinase 9 (MMP9) are identified which are monomer (25-kDa), homodimer (45-kDa) and heterodimer (135-kDa) (Chen et al., 2019). MMP9 is an enzyme which participates in the initiation, growth, metastasis, and progression of the tumor (Gialieli et al., 2011). In a study conducted on the role of NGAL in human breast tumor, increased MMP9 activity and NGAL/MMP9 complex formation was observed in the urine of mice with tumor cells expressing high levels of NGAL (Fernandez et al., 2005). Apart from human studies, the literature on NGAL expression and NGAL/MMP9 complex formation in tissue and urine in canine mammary tumor cases is not sufficient. Chen et al. (2019) detected the molecular form of NGAL/MMP9 both in tissue and urine samples of dogs with benign and malign mammary tumors, and they stated that this complex was not found in healthy dogs. They also indicated that NGAL/MMP9 complex was only found in the urine of dogs with mammary tumors.

2. Saliva

Saliva is a biological secretion which is systemically and locally used as a biomarker in the diagnosis of many diseases. As the sample collection is painless and easy and does not require an invasive procedure, it provides an important advantage to reduce sampling stress of patients (Cerón et al., 2019). In humans, it is concluded that the proteomic analysis of saliva is a successful technique in identifying the biomarkers associated with the disease in many pathologies such as cancer (Sivadasan et al., 2015). Tandem Mass Tag (TMT) which is one of the gel-free proteomic approaches was successfully used in pyometra cases that are gynecologically important in addition to certain infectious diseases in dogs (Franco-Martínez et al., 2020). When the current literature is reviewed, the first comparison of serum and saliva proteomics with gel-free TMT proteomic approach between mammary tumor cases in dogs which are gynecologically important and healthy dogs was conducted by Franco-Martínez et al. (2020). In salivation of patients with mammary

tumor, S100A2, S100A4 and S100A6 from S100A protein are among the upregulated proteins. S100A proteins have a calcium binding role in many cancer types including the mammary tumors (Salama et al., 2008). In human mammary tumors, the positive correlation between S100A4 expression and cancer is proven with studies (Platt-Higgins et al., 2000; Rudland et al., 2000; Pedersen et al., 2002). S100A2 protein of the same family is considered to have a debatable role in carcinogenesis. This arises from its role both as a tumor suppressor and a promoter (Naz et al., 2014; Zha et al., 2015). However, increased expression is significantly associated with short survival time in ovarium cancers (Giri et al., 2019). S100A6 expression is associated with poor prognosis in certain cancer types, and recent studies have shown that it is associated with good prognosis in human breast tumors (Zhang et al., 2017). The use of S100A family as a biomarker to assess the prognosis in canine mammary tumors should be supported with future studies. In the same study, the existence of proteins similar to calmodulin (CALM) in salivation of dogs with mammary tumors was also presented (Franco-Martínez et al., 2020). While CALM2 and CALM3 showed up-regulation, KLK1 showed down-regulation. Both kallikrein genes and proteins have an effect of inducing and inhibiting tumor cell angiogenesis, metastasis, and the growth of these tumor cells through various mechanism (Borgoño et al., 2004). Therefore, the role of kallikrein genes and proteins in cancer is controversial. There was no significant difference between the proteomic results of serum and salivation when they were compared in the study. However, it was determined that the proteins in salivation were twice as much of those in serum, and none of the proteins was detected together both in serum and salivation. This indicates that proteins originate from different locations. Besides, it is determined that the proteins occur in the salivation are not carried in the blood; and they originate from salivary glands, nasal or bronchial secretion, and gingival crevicular fluid. Thus, salivation may be presented as a non-invasive biomarker when it is supported with further studies.

Conclusion

In human and veterinary oncology, it is very important to identify biomarkers which may distinguish healthy individuals from unhealthy ones. In veterinary medicine, there are many studies on the canine mammary tumor biomarkers. Despite this, the ideal biomarker has not yet been concluded. An ideal biomarker should bear minimum risk for the patient during application; it should have positive correlation with the malignity; and it should give a definite result about the prognosis. With the recent studies, non-invasive biomarkers which may be measured from bodily fluids such as salivation are added to biomarkers such as tissue which require an invasive procedures during sampling. Invasive biomarkers provide information on many criteria such as diagnosis, cellular differentiation, ulceration and necrosis formation, prognosis of the patient, histological grade of tumor, high mitotic index, spread to lymph nodes, and survival time. Non-invasive approaches provide benefits for the follow-up of the diseases with the imbalances in the systems. Higher expression of specific urine metabolites, which is one of these biomarkers, in patients with mammary tumors compared to healthy individuals may be considered as a beneficial biomarker for diagnosis. Salivation, which is another non-invasive biomarker, may be accepted as a potential biomarker although it is quite new. When non-invasive biomarkers are considered, they are promising but their usability should be supported by future studies.

Conflict of Interest: Authors declare that there is no conflict of interest.

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Canine transmissible venereal tumor: etiology, diagnosis and treatment

Çağla Nur Küçükbekir[1], Zeynep Günay Uçmak[2], Çağatay Tek[2*]

1. Istanbul University-Cerrahpaşa, Institute of Graduate Studies, Istanbul, Turkey. 2. Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, Department of Obstetrics and Gyneacology, Istanbul, Turkey. Küçükbekir, Ç. N. ORCID: 0000-0002-9687-1702, Günay Uçmak Z. ORCID: 0000-0003-2530-1291, Tek Ç. ORCID: 0000-0002-6429-658X.

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ABSTRACT

Transmissible venereal tumor (TVT) is a round cell type neoplasia that is transmitted by mating and physical transfer of tumor cells. It is one of the most common benign tumors in dogs that can be seen in both gender. The external genital area is the main location of the tumor. However, internal organ metastasis has been reported. The most common clinical signs are lobular masses which are seen in the caudal part of the penis, in the posterior region of the vagina and at the vestibulovaginal junction. Nodular lesions with rapid bleeding are the most pronounced clinical finding. Initially, the small tumor forms into a large ulcerated mass in the next periods. Simultaneously, the volume of the tumor increases and the lesions are seen multilobular, cauliflower-like, brittle, hyperemic, and hemorrhagic. The most practical diagnostic method of the tumor is vaginal cytology. Cytology findings are characterized by the round or oval cells which have, pale blue or colorless cytoplasm with cytoplasmic vacuoles and a prominent nucleus. Chemotheraphy is the most effective treatment method. The weekly intravenous administration of vincristine suplhate given for 3 weeks on average reveals that the treatment success rate is beyond 90%. In this review, etiology, clinical findings, diagnosis and treatment of TVT are presented .

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Introduction

Transmissible venereal tumor (TVT) is one of the most uncontrolled tumors in dogs which spread through mating (Uçar, 2016). It can also be named as sticker tumor or sarcoma, venereal granuloma, infectious granuloma, canine condyloma, and infectious lymphosarcoma (Murhia et al., 2006; Regmi et al., 2020). This tumor is usually transmitted during mating (Tella et al., 2004) and mostly occur in young, sexually active animals (Rogers, 1997). It has been described as a benign reticuloendothelial tumor that mainly affects the external genitalia and less frequently the internal genitalia (Tella et al., 2004; Abedin, 2020). Although the metastasis of TVT is rarely seen, the metastasis is more frequently observed in immunocompromised animals (Rivera et al., 2005).Unlike the canine breeds have 78 chromosomes in nature, TVT cells have abnormal number of chromosomes ranging from 57 to 64, 59 on average. Transmissible venereal tumor is the only proven example of a naturally occurring tumor that is transmitted as an allograft by cell transplantation, and the tumor becomes autonomous apart from the original host (Abedin, 2020).

*Corresponding Author: Çağatay Tek
E-mail: catek@istanbul.edu.tr

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Ethiopathology

Transmissible venereal tumor was described as a contagious neoplasm by Novinsky (Novinsky, 1876). Although the exact source of TVT cells has been unclear, which is morphologically a round cell tumor, studies have revealed its histiocytic and mesenchymal origins. Since it has been thought to originate from lymphocytes, histiocytes and reticulum cells, its etiology is not fully defined yet. The presence of inclusion bodies in the cytoplasm of the tumor cell has lead to the suspicion of a viral agent (Do Amaral et al., 2007). Predisposing factors consist of the rapid increase in the number of stray dogs, aging, and the circumstances of the immune suppression. Because rapid tumor growth and metastasis can be seen in dogs whose immune system is suppressed (Chikweto et al., 2013). It is primarily found in dogs with a weakened immune system in the southern regions, especially in the Mediterranean (Hithem et al., 2020). Compromised immune system plays a major role in acquiring and spreading of TVT (Das et al., 2020). The cases can be listed as localized necrosis in some parts of the tumor or secondary bacterial infection (Vermooten, 1987; Das and Das, 2000; Kabuusu et al., 2010; Macotpet et al., 2019).

In many countries, TVT occurs between the ages of 2 and 8 in both genders (Smith and Washbourn, 1998; Boscos and Ververidis, 2004; Strakova and Murchison, 2014; Günay Uçmak et al., 2019). However, the bitches are more likely affected than male dogs. Incidence is high in urban areas where mating of young and sexually active stray dogs is not controlled (Hayes et al., 1983; Rogers, 1997; Das and Das, 2000; Gurel et al., 2002). Stray dogs are at risk factor for contracting and transmitting this disease (Hithem et al., 2020). It occurs mostly in tropical and subtropical climates (Hayes et al., 1983; Rogers, 1997; Uçar, 2016). Besides, the researchers reported that TVT is formed in the presence of ovarian remnant syndrome and stump pyometra cases in bitches (Sontaş et al., 2010; Turna Yılmaz et al., 2013). Ayala-Diaz et al. (2017) have been detected that the red, hemorrhagic and irregularly-edged TVT mass protrudes into the vaginal mucosa during pregnancy. After the diagnosis of a TVT, chemotherapy is suitable for the expectant mother to eliminate the tumor. However, it represents risks to the developing fetus. Salpingo-oophorectomy and hysterectomy might be preferred when the bitch is in the early period of pregnancy. A recovery period of 21 days and then chemotherapeutic treatment with Vincristine has been advised subsequent with the surgery. A 50% reduction after the second treatment, no obvious tumor mass on vaginal examination and tumor cells on cytological smears have been reported (Ayala-Diaz et al., 2017).

The disease is mainly observed on the mucosal surface of the external genitalia of male dogs and bitches (Kabuusu et al., 2010; Ostrander et al., 2016; Hiblu et al., 2019). The tumor is transmitted by living tumor cells from one dog to another by mating, licking, or sniffing. This explains cases occurring in the oral mucosa and nose (Purohit, 2009; Stockmann et al., 2011; Behera et al., 2012; Lopes et al., 2015). Neoplastic cells contaminated by physical contact are thought to be transferred to the genital system, nasal and oral mucosa, rectum or skin (Albanese et al., 2002; Do Amaral et al., 2007; Rezaei et al., 2016). Therefore, in primal TVT cases, it is possible to see lesions in the skin, face, nose or oral cavity, eyes and subcutaneous tissues along with the external genital area (Rogers, 1997; Das and Das, 2000; Brandao et al., 2002; Uçar, 2016; Kumar et al., 2018). Although tumors are located in the genital tract, extragenital cases have been observed including the conjunctival mucosa (Ferreira et al., 2000; Rodrigues et al., 2001) and central nervous system (Ferreira et al., 2000; Faccini et al., 2019). The presence of tumors involving the ocular area is less common than others, and TVT metastases occur by contact in these sides (Ginel et al., 1995; Souza et al., 2020). More aggressive and metastatic cases are observed in young dogs with poor body resistance and immunosuppression (Çeşme et al., 2015). It has been reported that TVT metastasis was also detected in the caudal mammary lobes of a 4 year old bitch (Günay Uçmak et al., 2019). In addition, TVT is seen in 5% of the extragenital organs other than genital localization. This placement is either together with the genital form or only extragenitally (Çeşme et al., 2015).

Clinical findings

Transmissible venereal tumor in male dogs usually occurs in the caudal part of the penis, from the corpus to the bulbous or glans penis (Das and Das, 2000). Clinical findings appear less striking in males. In affected males, bloody discharge, redness, deformation and ulceration can be seen in the preputial opening (Das and Das, 2000; Ferreira et al., 2000). Phimosis and paraphimosis can develop (Das and Das, 2000; Birhan and Chanie, 2015). In bitches, the development of TVT is mostly seen in the posterior wall of the vagina, and the vaginal vestibule (Stockmann et al., 2011). The affected bitches may have difficulty in urination or dystocia due to mechanical obstruction. Macroscopically, solitary or multiple tumor masses with cauliflower-like ulceration, hemorrhagic, friable and irregular appearance can be seen. Lesions are fragile and mostly hemorrhagic as a result of low cohesion between neoplastic cells. Therefore, the first clinical sign is tend to be bleeding (Ferreira et al., 2000; Faccini et al., 2019). Tumor size ranges from millimeters (mm) to several centimeters (cm) with a dark red to grayish pink color (Das and Das, 2000; Purohit, 2009; Lopes et al., 2015). Lesions are small (1 - 3 mm in diameter), superficial and colored pink to red at the onset of tumor formation (Purohit, 2009). Afterwards, the shape of the tumor takes on a cauliflower-like appearance. It has a fragile, red, hemorrhagic and hard structure. The mass can reach 5 - 7 cm in diameter. If the multilobular subcutaneous lesions invade to the deeper mucosal layers, the diameter of the mass may be reached to 10 - 15 cm. The mass is observed as petiolate, nodular, papillary and multilobule structures. The tumor can cover the orificium urethra externa and may protrude through the vulva labia (Purohit, 2009). The tumor surface is often inflamed and can be infected (Brown et al., 1980). Tumors can have petechial or simple bleeding as well as infection or ulceration (Das and Das, 2000; Ferreira et al., 2000; Purohit, 2009). Weakness, loss of appetite, constipation, refusal of mating and weight loss are less common symptoms (Ganguly et al., 2016). Laboratory result of a research (Boyd, 1983; Kerr, 2002) has been showed the increasing concentrations of leukocytosis, hemoconcentration, microcytic hypochromic regenerative anemia, hypernatremia, and Aspartate transaminase (AST), Alanine Transaminase (ALT), Alkaline phosphatase (ALP), and Creatine Kinase (CK). Microcytic hypochromic regenerative anemia is associated with hemorrhagic anemia due to severe bleeding on tumor, while hemoconcentration and hypernatremia are associated with dehydration. Leukocytosis is related to infection in and around the tumor tissue. Increased serum AST, ALT and ALP activities have been reported to be associated with liver damage, and increased AST and CK concentrations are related to muscle tissue damage (Boyd, 1983; Kerr, 2002).

Serosanguineous secretion, vulvar and preputial deformities may occur due to tissue damage, intense odor, ulceration and itching, aggression, and in severe cases, urinary retention in the affected area (Costa, 1999; Martins et al., 2005; Kolawole et al., 2020). Due to the risk of bacteriuria development in TVT affected animals, urinary retention can occur (Batamuzi and Kristensen, 1996). Prolonged serosanguineous vaginal discharge is seen in infected dogs (Purohit, 2009). The general health of the dogs is not compromised unless the lesions become infected and necrotic or the mass covers the urethral opening (Ganguly et al., 2016; Uçar, 2016).

Diagnosis

Diagnosis is mostly based on anamnesis, clinical findings, cytology and histopathology. As compared with other round cell tumors, TVT has a microscopic appearance to diagnose. Immunohistochemistry can be used to differentiate this neoplasm from other round cell tumors (Ferreira et al., 2000; Faccini et al., 2019). Prooestrus bleeding, cystitis, urethritis and prostatitis should be considered in the differential diagnosis (Das and Das, 2000).

Macroscopic Findings: Clinical symptoms vary according to the location of the tumors. On physical examination, small pink to red nodules are observed at 1 mm to 3 mm in diameter which can be visible 2 or 3 weeks after the TVT cell contamination. Initial lesions are superficial or pedunculated. Later, multiple nodules combine to form larger, red, bleeding, cauliflower-like masses (Aprea et al., 1994; Martins et al., 2005). There is

hemorrhagic discharge with genital localization of TVT in bitches. Lesions in males are usually cranially on the glans penis, preputial mucosa or bulbus glandis (Higgins, 1966; Mc Evoy, 1987; Martins et al., 2005). Regional lymph node involvement is common with large tumors in males. In bitches, tumors may be localized in the vestibule and / or caudal vagina, protruding from the vulva and often causing a deformation in the perineal region. Persistent hemorrhagic discharge may cause anemia. Rarely, TVT may be localized in the uterus (Aprea et al., 1994; Martins et al., 2005). Clinical diagnosis is usually more difficult in cases of TVT with extra genital localization because TVT cause various symptoms such as sneezing, nosebleeds, epiphora, shortness of breath and tooth loss, exophthalmos, skin lumps, regional lymph node enlargement and facial or oral deformation depending on the anatomical location of the tumor (Rogers, 1997; Martins et al., 2005). Definitive diagnosis is based on tumors, fine needle aspiration or physical examination and cytological findings (Moulton, 1978; Richardson, 1981; Daleck et al., 1987; Martins et al., 2005).

Cytological diagnosis: A definite diagnosis is made according to physical examination and cytological findings (Kroger et al., 1991). Since the cytology is non-invasive and painless method, it is the best choice for diagnosis. In addition to being simple and cheap, it causes much less deterioration in cell morphology than formalin-fixed biopsy samples (Das et al., 2020; Do Amaral et al., 2007). Cytological examination is a complementary test in a simple, fast, non-invasive and cost-effective method, which is guiding the appropriate type of treatment for the animal (Lopes et al., 2015). Wet fixation smears can be stained with Harris Hematoxylin and Eosin (Bancroft and Stevens, 1996). However, for air-drying preparations, Wright-Giemsa (WG), Wright's (W), MayGrünwald-Giemsa (MGG) and Leishman-Giemsa (LG) methods are used. Fixation of smears is performed by wet fixation with absolute isopropanol or 95% ethanol (Allen et al., 1986) for 20 minutes or immediately dried in air (Das et al., 2020). Also, TVT has a typical cytological appearance. The shape of cells in cytology ranges from round to oval structures. Cells mostly have a pale blue or colorless cytoplasm with a single distinctive nucleus. They also contain small, light, clear intracytoplasmic vacuoles, and numerous mitotic figures (Rogers, 1997; Hayes et al., 1983; Purohit, 2009).

Histological diagnosis: The definitive diagnosis is made by histopathological examination of the biopsy specimen. Large cells, round or oval vague contours are observed on histological examination. Another feature of this neoplasm is that it has inflamed cells and mitotic figures. The TVT can be confused with mastocytoma, histiocytoma and lymphoma. Therefore the importance of differential diagnosis should be emphasized (Do Amaral et al., 2007; Lopes et al., 2015). In histopathology, abundant round, oval or variable-shaped tumor cells are usually found around the blood or lymphatic vessels (Purohit, 2009; Birhan and Chanie, 2015). The size of the cell nucleus is larger than the size of the cytoplasm. Cytoplasmic vacuoles are often visible. The nuclei are oval or round and centrally-located, with delicate chromatin and large nucleoli; the cytoplasm is slightly acidophilic and contains finely granular, delicate vacuoles, and cells do not display anisokaryosis, anisocytosis, hyperchromasia or nuclear macrokaryosis. The cTVT is histopathologically classified based on the predominant cell type as lymphoid, plasmacytoid or mixed. The lymphoid type of tumor predominantly includes cells with a rounded morphology, scant and finely granular cytoplasm, the presence of vacuoles, and round nuclei with coarse chromatin and the presence of one or two evident nucleoli. In plasmacytoid tumors, most cells have an ovoid morphology, a smaller relative nucleus: cytoplasm ratio and eccentrically-located nucle. Cytoplasm is slightly basophilic and usually small and slightly multiple vacuoles accompanying the cell board (Stockmann et al., 2011). Lymphocytes, plasma cells, and macrophages are frequently observed (Birhan and Chanie, 2015). Tumor growth leads to tightly integrated, irregular cell formation, with fibroblasts forming between them (Purohit, 2009). Immunohistochemistry can be used in the diagnosis of metastatic tumors following a combination of clinical findings (Birhan and Chanie, 2015; Uçar, 2016).

Molecular Diagnosis: Transmissible venereal tumor has a molecular property based on

rearrangement of a c-myc gene that is not found in normal somatic cells, gametes and other tumor cells (Katzir et al., 1985). Transmissible venereal tumor cells allow a diagnostic polymerase chain reaction (PCR) based on the diagnosis of nuclear elements (LINE) added to the stream of a myc gene. Therefore, the presence of this LINE element near c-myc (LINE-c-myc) has been used for the definitive diagnosis of TVT cases using in situ polymerase chain reaction (PCR) and conventional PCR in controversial cases (Liao et al., 2003; Park et al., 2006). Generally, the PCR test has increased the accuracy of the diagnosis. It may also facilitate the decision to terminate chemotherapy by performing the diagnosis between tumor cells and fibrotic tissue (Setthawongsin et al., 2016).

Auxiliary diagnosis: Doopler ultrasonography can be used to evaluate the blood flow in the tissue / organ, where metastasis occurs in TVT cases and it may also be useful to examine vascular perfusion in the affected tissues (Günay Uçmak et al., 2019).

Treatment

Radiotherapy, chemotherapy, immunotherapy, biotherapy and excisional surgery can be applied for the treatment of TVT (Purohit, 2009; Günay Uçmak et al., 2019). Self-healing may occur following the animal's immune system fight the tumor cells (Andrade et al., 2009; Lapa et al., 2012).

The preferred treatment for TVT is chemotherapy. Recently, many agents and chemotherapy protocols such as cyclophosphamide, vincristine sulfate, vinblastine, doxorubicin and methotrexate are widely used. These drugs are used as a single agent or in combination with each other. The most effective, safe and appropriate treatment in clinical practice is the use of vincristine sulfate as a single agent. Immunotherapy should be performed by using substances effective on the immune system in immunocompromised animals (Das and Das, 2000). However, the widespread use of vincristine in TVT treatment and the presence of malignant neoplasm features have increased the number of applications of the drug. The resistance against vincristine has been associated with overexpression of a protein molecule called P-glycoprotein of the plasma membrane (Pouliot et al., 1997). This molecule found in various tissues such as kidney, liver, colon, brain, lung, peripheral blood, and normal bone marrow. Tumors derived from tissues expressing high amounts of P-glycoprotein exhibit intrinsic resistance to chemotherapy (Gaspar et al., 2010). Because this molecular membrane acts as both a carrier and a flow pump dependent on the energy produced by ATP hydrolysis (Korystov et al., 2004; Gaspar et al., 2010). The studies (Andrade et al., 2009) have shown that vincristine sulfate in combination with ivermectin is beneficial. Because this antiparasitic drug is used as P-glycoprotein substrate, that reduces the amount of molecules in the tissue. Thus it strengthens the antitumor therapy and slows the treatment resistance (Andrade et al., 2009; Lopes et al., 2015).

Favorable results were obtained from the intravenous (iv) use of vincristine sulfate, a chemotherapeutic agent, at doses of 0.5 - 0.7 mg / m2 body surface area or 0.025 mg / kg once a week for approximately three weeks (Amber et al., 1990; De Lorimier and Fan, 2007). Before the starting chemotherapy with vincristine sulfate, the animal's general health should be evaluated. It is important to analyze the total blood count weekly during treatment (Ganguly et al., 2016). The vincristine sulfate should be diluted with a isotonic solution and this combination should be administered as a very slow iv infusion. The drug should be avoided from direct sunlight. Treatment should be continued until the symptoms disappear and the duration of administration is approximately 2 - 6 weeks. The overall success rate varies between 90 - 95% (De Lorimier and Fan, 2007). Male dogs treated with vincristine sulfate may experience a temporary deterioration in semen quality, which rapidly returns to normal within 15 days following the last administration (Saratsis et al., 2000; Gobello and Corrada, 2002). Following chemotherapy, a marked regression of neoplastic formation usually begins two weeks after the first treatment. Although no significant decrease was detected in AST and ALP enzymes, a significant decrease in total serum protein, hemoglobin, total erythrocyte and total leukocyte count can be observed. Possible side effects of the

chemotherapeutic agent include loss of appetite, vomiting, diarrhea, myelosuppression, and alopecia. When the desired result is not obtained from vincristine sulfate, doxorubicin can be used as iv at a dose of 1 mg / kg for a maximum of 3 weeks (Amber et al., 1990). One of the treatment approaches for the TVT is also the combination of vincristine sulfate (0.0125 mg / kg / week, iv), methotrexate (0.3 – 0.5 mg / kg / week, iv) and cyclophosphamide (1 mg / kg / day). It should also be used until visible symptoms disappear. The application takes about 4 - 6 weeks (Purohit, 2009). Although adriamycin (30 mg / m^2, every three weeks, iv) is also effective, it should only be used when vincristine sulfate is not effective due to the side effects of adriamycin (De Lorimier and Fan, 2007). Good prognosis can be expected in most TVT cases after the treatment with chemotherapy (Vermooten, 1987; Rogers, 1997; Uçar, 2016).

Surgery can also be performed in the treatment of TVT. Although surgery can be effective for small and localized tumors, postoperative recurrence rate can be up to 30 - 75% in metastatic cases (De Lorimier and Fan, 2007). During surgery, the surgical site may be contaminated with TVT cells and this increase the risk of recurrence (De Lorimier and Fan, 2007; Purohit, 2009). Therefore, surgery is not usually preferred treatment method for TVT. However, cauterization, electrosurgery, or cryosurgery can be utilized to prevent recurrence of TVT after surgery (Idowu, 1985; Rao et al., 1993; Hoque, 1995; Das et al., 2020).

As an alternative to chemotherapy treatment for TVT, radiotherapy can be used in treatment-resistant lesions or lesions in the brain, testicle, or eyes. However, the main disadvantages of this method are the difficulties in implementation, the lack of sufficient equipment, the economical burden of the equipment, and the need for longer application time compared to chemotherapy (Rivera et al., 2005).

Biotherapy for TVT treatment has limited effect. Three weeks of intratumoral Bacillus Calmette-Guérin (BCG) application has low success (Johnston, 1991). High recurrence rates have been reported following treatment (Richardson, 1981; Vermooten, 1987).

Conclusion

In conclusion, etiology, clinical findings, diagnotic and treatment options of TVT were reviewed. The TVT is more common in sexually active and stray male and female dogs. Preventive approaches and appropriate treatment methods are very important in terms of restrain the spread of the disease. The recovery rate is high when TVT is accurately treated. When a long treatment period is considered, the protective approaches for TVT become important.

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