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Successful management of mummified fetus in a heifer by prostaglandin therapy and episiotomy

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Abstract

Fetal mummification is one of the gestational accidents that occur due to intra-uterine death of fetus commonly at fourth, fifth and six months of gestation. This report describes the successful management of the mummified fetus in a five year old graded Holstein Friesian heifer cow using single dose of prostaglandin F2α analogue and by performing episiotomy. Antibiotic therapy was given to avoid any uterine infection.

Introduction

Bovine fetal mummification results due to death of conceptus in the uterus between third to eighth months of gestation, without accompanying lysis of corpus luteum and opening of cervix, and is characterized by failure in expulsion of dead fetus, absorption of all fetal fluids, involution of fetal cotyledons and maternal caruncles, and presence of hard, firm fetus in the uterine horn as compact mass with no clinical signs. Persistent corpus luteum helps to maintain the dead fetus within uterus by secreting progesterone.[1] Fetal mummification has been reported to occur in many domestic species but this reproductive disorder affects the economy of dairy farms by increasing inter calving period as well as fetal loss.[1] The incidence of fetal mummification in cattle is sporadic and found to be 0.13-1.8%.[2,3] The present study describes the successful management of the mummified fetus in a heifer cow using single dose of prostaglandin F2α (PGF2α) analogue and by performing episiotomy.

Case Report

A five-year-old graded Holstein Friesian heifer, weighing 450 kg, was presented with the complaint of not showing any signs of parturition even after completion of full term of pregnancy. The owner of the cow told that she had been inseminated 310 days before and pregnancy was confirmed at 60th and 90th day after insemination. Apparently the clinical parameters of heifer including heart rate, pulse rate, temperature, respiratory rate and posture were normal with no visual signs of pregnancy. On vaginal examination, the cervix showed one finger dilatation with no discharge. Per rectal examination revealed no fetal movement and a hard bony mass without the palpation of cotyledons adhering to uterine wall, no fremitus and absence of fetal fluid. Based on clinical signs and observations cow was diagnosed to be having mummified fetus and decided to treat medically.

The animal was given an intramuscular single dose of PGF2α analogue cloprostenol and dicrysticin was started (for five days) as antibiotic therapy to prevent probable uterine infection. After 72 hours of the therapy, a long thick shred of brownish mucoid discharge from vulva was reported. Per vaginally, the cervix was found fully relaxed and a huge bony mass draped within the fetal membranes was palpated. A mild traction was applied to take out the dead fetus but labial narrowness hindered the easy passage. Episiotomy was performed as it appeared that further traction will result in tearing of vulva. A 3-5 cm incision using scalpel blade was made on the dorsal commissure after locally anaesthetizing the area with 2% lignocaine hydrochloride aseptically and fully grown dead fetus covered with dark brown fetal membranes was delivered manually (Figure 1). Following parturition, the incision was cleansed of foreign materials such as fetal remnants and sutured with horizontal mattress suture pattern.

Discussion and Conclusions

Fetal mummification has been reported in several species but it is more common in cattle.[1] Several potential causes such as infectious (including bovine viral diarrhea, leptospirosis and molds)[1] and mechanical (compression or torsion of umbilical cord,[4] uterine torsion,[5] defective placentation,[6] and genetic abnormalities)[7] have been observed for causing this condition. Mummification of fetus in cattle usually occurs between 3-8 months of gestation and thereafter, the dead fetus is retained after absorption of all fetal and placental fluids into the uterus because of persistent corpus luteum. Further the fetal membranes adhere to the dead fetus and form the viscous brown material over dehydrated fetus. Apparently a hard bony mass with closed cervix but without placentomes, fremitis and fetal fluid remains in the uterus which was experienced per rectally in the present case. Similar finding on rectal examination were reported by Azizunnesa *et al.*[8]

The physical examination of the dam reveals no abnormality, except for some rare cases in which reduced milk production and gradual weight loss has been recorded.[9] The medical treatment involves the lysis of corpus luteum by PGF2α injection which results in the expulsion of mummified fetus within 2 to 4 days.[3] Arthur *et al.*[10] reported that the treat-

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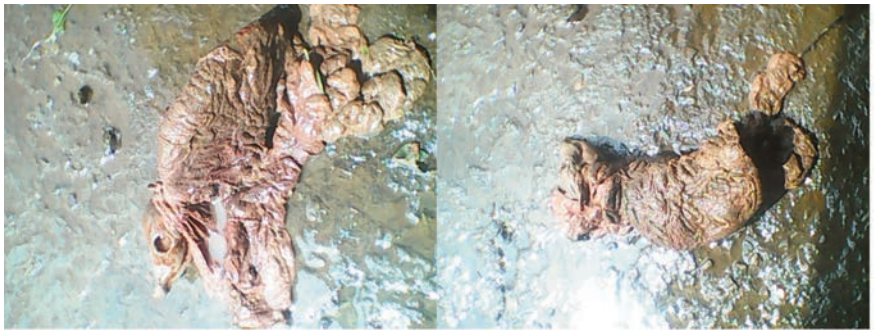

Figure 1. Mummified fetus wrapped in the dehydrated fetal membranes after expulsion.

ment of mummified fetus with PGF2α created some complexity in cattle *viz.* maceration of mummified fetus and packed in the birth canal instead of expelled out. However, no such complication was experienced in the present study. Dabas and Chaudhari also delivered mummified fetus easily by mild traction after 72 hour of the prostaglandin therapy.[11]

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Cortisol and glucose responses in juvenile striped catfish subjected to a cold shock

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Abstract

Cold-shock stress happens when a fish had been adjusted to a specific water temperature or range of temperatures and is consequently exposed to a rapid drop in temperature, resulting in a cascade of physiological and behavioral responses and, in some cases, death. In the current study, the stress response of striped Catfish (*Pangasianodon hypophthalmus*) was studied by evaluating serum cortisol and glucose level following an abrupt reduction in water temperature (from 28°C to 15°C) at different time points (prior to, and after 1h, 12h and 24h cold treatment, respectively). Regardless of some mortality occurred in cold challenged fish, none of the physiological parameters changed during evaluation period. The results, suggesting that despite of necessity of cortisol and glucose evaluation in any of stress assessment, yet, due to their high variability in different fish species, additional complementary tests such as measurement of other stress hormones *e.g.* heat shock proteins as well as blood-cell counts (preferably in chronic experiments) should also be included.

Introduction

Among the natural stressors fish can experience throughout their life cycle are thermal changes. Fluctuations in water temperature either resulting from a transient (daily change) or a seasonal change is generally associated with disease and fish mortality.[1] Cold-shock stress occurs when a fish had been acclimated to a range of water temperature and is subsequently exposed to a rapid decrease in temperature, resulting in a cascade of physiological and behavioral responses and, in some cases, death.[2] To deal with the environmental changes, fish respond by altering physiological functions including those associated with the stress response.[3] The physiological stress response in fish is mediated by the neuro-endocrine system and includes the release of hormones such as cortisol and adrenaline.[3] In response to most stressors fish will exhibit an increase in plasma cortisol levels, which is generally followed by an elevation in plasma glucose concentration. Although some e ects of temperature and (gradual) temperature changes on the stress response have been investigated in fish species,[4-6] however, little is known about the impacts of rapid temperature drops on the stress response.[7]

Temperature shock can hamper fish life by reducing metabolic rates,[8] impairing swimming performance,[9] reducing the ability to capture prey,[2] impeding predator avoidance,[10] altering rates of recovery from exercise[11,12] and disrupting physiological homeostasis.[8,12,13] Some studies have shown an endocrine stress response change in fish exposed to cold shock.[7,14-16] Cortisol and glucose are two of the most common stress indicators.[17] Increased plasma cortisol levels were observed in rainbow trout, common carp (*Cyprinus carpio*) and tilapia aurea (*Oreochromis aureus*), respectively, exposed to cold shock (in different experimental conditions).[16] Striped catfishes play an important role in Asian aquaculture and commercial fishing.[18] *Pangasianodon hypophthalmus* formerly referred to as *pangasius sutchi* is native to the Chao Phraya River in Thailand and the Mekong in Vietnam. It is abundantly available in the Amazon River, in parts of Russia and in other places of the world under different names.[19] Moreover, fingerlings of the species are often collected and transported to pet fish shops to several countries.[20] Nowadays, this species emerged as a promising species for aquaculture purposes even outside of tropical regions of Southeast Asia. However, development of culture industry for this species has faced difficulties mainly due to the limited knowledge of biology, ecology, and physiology in cultivated stocks.[21] In the current study, the stress response in a tropical fish during and after exposure to an acute cold shock (13°C decrease in water temperature) was investigated. The levels of cortisol and glucose as well as death rate prior to and during cold stress at several time intervals (over 1h, 12h and 24h cold stress) were studied. No recovery was appointed in this study.

Materials and Methods

Experimental design

Juvenile Striped catfish (average initial weight 1.27±0.24 g and initial length 5.55±0.45 cm) were purchased from a local commercial pet fish shop and held in 1000 L glass tank for three weeks to be acclimated to the experimental conditions. In the beginning of the experiment, the fish were fasted for 24h and then weighed. Two hundred and ten fish of similar sizes were divided into two treatment groups (cold shock and control group). Each group had three replicates and completely randomized design (CRD) was followed to set up the experiment.

Fish were handfed a commercial diet (Table 1) at 2-3% of body weight to apparent satiation twice daily. Water temperature (27.56±0.86°C), pH (7.82±0.08) and dissolved oxygen (5.20±0.34 mg/L) were constant throughout this period.

Stress tests and sampling

The cold shock treatment consisted of transferring directly the fish from each replicate to 150 L tanks in which the water temperature was kept at 15°C by adding ice to the tanks. During the cold shock treatment (max. 24h) the temperature in the chilling tank was monitored and held stable by adding ice if necessary. An YSI model 55 probe was used during the cold shock to monitor water temperature and dissolved oxygen concentration. To account for handling procedures, fish from all treatments (the test and control groups) were

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transferred to tanks with the same initial water temperature (27.56±0.86°C). Food was withheld 24h before the onset of the cold shock. At each sampling point (prior to and after 1, 12 and 24h cold treatment), 3 fish were sampled at random from each experimental group and anesthetized with clove oil (50 mg/L). Blood samples were collected immediately after caudal vein amputation and transferred into sterile tubes and allowed to clot at room temperature for 1 h and then kept at 4°C for 5 h. Afterwards, serum was separated by centrifugation at 3000 g for 10 minutes and stored at –20°C until required.

Assays for determination of stress

Serum cortisol levels were measured by radioimmunoassay (RIA) and expressed as ng/mL.[22] The quantitative determination of glucose was carried out using commercially available diagnostic Experimental Protocols kits (Pars Azmun, Iran, 1 500 0178),[23] at 546 nm and 37°C according to the glucose oxidase method suggested by Trinder.[24]

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the statistical software SPSS, version 11.0. All the measurements were made in triplicate. Significant differences between means were delineated by Duncan test. $P<0.05$ was considered significant.

Results

No differences in serum cortisol or glucose levels were found between fish from control and cold challenged fish at several time points of sampling (Figure 1).

No fish mortality was observed throughout 1h cold shock treatment in all experimental groups. However, the cumulative mortality reached to 50% after 12h and to 65% by the end of cold shock treatment (Figure 2). Nonetheless, the intensity of mortality was significantly reduced in second half compared to first half of 24h cold shock treatment (Figure 2).

Discussion

In the current study, none of the physiological parameters (cortisol and glucose values) measured in striped catfish changed at several time points of cold stress. Nevertheless, some of these parameters have been shown to change when fish are exposed to cold shock.[7,18,19,25,26] However, in a similar study, no significant changes either in cortisol or in glucose rate was detected immediately after 1h sudden cold exposure on the warm-water fish matrinxã (*Brycon amazonicus*).[20] Yet, after fish had been returned to the conditions prior to cold shock, a clear increase in plasma cortisol and glucose occurred in the cold-shock group. However, unlike this study, no recovery was arranged in our experiments, as fish were

Table 1. Proximate chemical composition of experimental diets.

Feed proximate composition	%
Dry mater	91.6
Protein	29.27
Fat	6.4
Ash	10.66
Carbohydrate	45.27

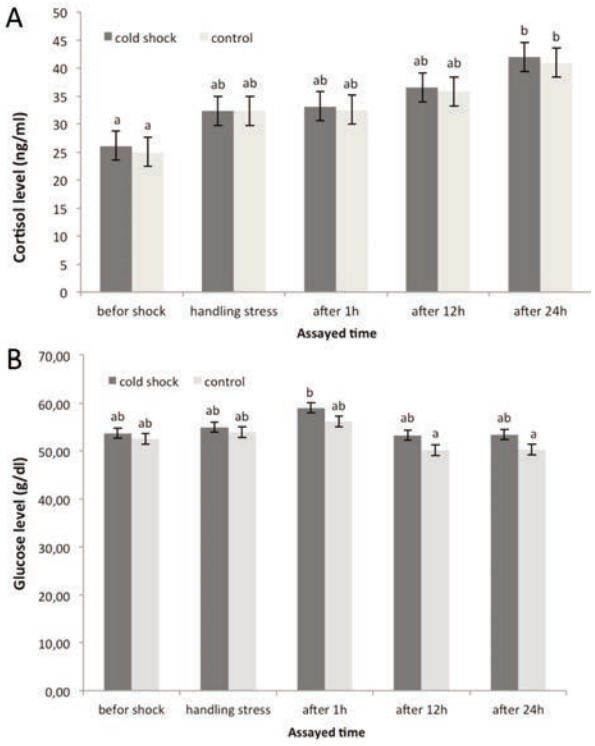

Figure 1. Values of serum cortisol level (A) and serum glucose concentration (B) of striped catfish challenged with a cold shock and sampled at time-matched sampling points (prior to, and at the end of 1, 12 and 24 h cold shock treatment). Data are expressed as mean ± standard error. Significant differences between values are indicated by different letters.

Figure 2. Cumulative mortality percentage recorded over a 12 and/or a 24h cold shock treatment in striped catfish. Data are expressed as mean ± standard error. Significant differences between values are indicated by different letters.

exposed to a constant cold stress for 24h.

In the present study, no mass mortality occurred in cold challenged fish as the highest rate of mortality reached to 65 percent detected by the end of 24h cold shock treatment. However, the intensity of mortality significantly decreased after 12h of imposing stress, likely due to a long-term acclimation to lower temperature, indicating that fish are really stressed despite no endocrine response. In fact, the lack of response would evidence the inability to adapt to cold, which could eventually lead to fish death. Indeed, in contrary to our results but in a similar condition, mass mortality of matrinxã due to sudden decrease of water temperature has been reported.[20]

It is equally difficult to explain the lack of endocrine response. One possibility is that the activity of the enzymes involved in steroid and glucose synthesis were altered (possibly downregulated) by the low temperature.[20,27] Roach *Rutilus rutilus* L., which were confined during winter (5°C) had much lower post-stress plasma cortisol levels than fish confined during the summer (16°C).[28] Other studies in striped bass (*Morone saxatilis*) and sunshine bass (*Morone chrysops* × *Morone saxatilis*) have shown that cold water temperature had no effect or lowered plasma cortisol.[29]

The rate of cortisol clearance is another step in the cortisol cycle that may be influenced by environmental factors. Liver is the key organ for cortisol disposal with the hepato-biliary system as the main biochemical pathway for cortisol clearance.[30,31] However, the efficiency of that process is reported to be altered by stress, salinity, maturity, nutritional state, etc.[32]

Conclusions

In conclusion, reasons for the apparent low responses to cold stress in striped catfish are not known but may relate to their evolutionary history, neuroendocrine mechanisms involved in their corticosteroid responses, or anatomy of their interrenal tissues structure. Similar to our work, previously many studies utilized cortisol and glucose as sole stress indicators in fish, however, regarding the several factors that can affect these responses, one should consider that cortisol and glucose are not enough as stress indicators.[21] In fact, there are some inconsistencies in the results of various experiments that in some cases would be attributed to unknown situations.[21] Iwama *et al.*[33] argued that *none of the current indicators of stress are 100% suitable in reflecting stressed states in fish and recommended to complement cortisol and glucose with other stress indicators to establish a more complete profile of the experimental organism*. For example, glutamine synthase has been observed to increase even with small response of cortisol.[34] Moreover, there are some other important stress indicators such as catecholamines,[35] melanocyte stimulating hormone (α-MSH),[36-39] lactate,[40] lysozyme,[41,42] as well as heat shock proteins that should be taken into account to study fish stress responses.[33]

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Effect of caraway on gentamicin-induced oxidative stress, inflammation and nephrotoxicity in rats

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Abstract

Different potentially therapeutic approaches to prevent or attenuate gentamicin (GEM) induced nephrotoxicity have been proposed. The aim of the present study was to investigate the possible protective effects of caraway seed oil against GEM-induced nephrotoxicity in rats. Rats (24) were randomly assigned into four equal groups: i) normal control group, ii) treated with GEM, iii) pretreated with orally caraway seed oil 10 (mg kg^{-1}) plus GEM and iv) treated with GEM and caraway seed oil 10 mg kg^{-1}. Biochemical examinations were utilized for evaluation of the oxidative stress and renal nephrotoxicity. Creatinine, blood urea nitrogen (BUN), plasma malondialdehyde (MDA) levels, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities were determined. Administration of gentamicin to rats induced a marked renal failure, characterized by a significant increase in plasma creatinine and BUN concentrations. The animals treated with gentamicin alone showed a significantly higher plasma MDA level andlower SOD, GSH-Px and CAT activities when compared with the control group. Treatment and simultaneous treatment with caraway seed oil produced amelioration in MDA and increased the activity of antioxidant enzymes SOD, GSH-Px and CAT when compared with the gentamicin treated group. In addition, GEM nephrotoxicity increased renal inflammatory cytokines (TNF-α, IL-6 and IFN-γ). Pro-inflammatory cytokines were significantly decreased ($P<0.05$) in the test groups administered caraway seed oil. These findings suggest that caraway seed oil treatment attenuates renal dysfunction and structural damage through the reduction of oxidative stress and inflammation in rats.

Introduction

Aminoglycoside antibiotics are widely used for treatment of severe gram negative infections. One of the most aminoglycoside antibiotics which is applied in human clinical practice and veterinary medicine is gentamicin (GEM). Aminoglycosides induce dose-dependent nephrotoxicity and ototoxicity which limits their clinical use.[1,2] GEM nephrotoxicity is described as direct tubular necrosis, without morphological changes in glomerular structures.[3] The mechanisms involved in GEM-induced nephrotoxicity are not completely understood; however, reactive oxygen species (ROS) are involved in this progress and are one of the most important mediators.[4] ROS may damage some macromolecules which induces cellular injury and necrosis. Peroxidation of membrane lipids, protein denaturation and DNA damage are pathways which increase cellular injuries caused by ROS. Experimental evidence suggests the role of ROS is associated with increased lipid peroxide formation.[5,6] In this regard, malondialdehyde (MAD) which is formed during oxidative degeneration is accepted as an indicator of lipid peroxidation.[7] Furthermore, activity of antioxidant enzymes is altered during GEM-induced nephrotoxicity. The most important antioxidant enzymes are catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). Accordingly, these enzymes protect cells against ROS damage.[8] An increased or unbalanced ROS production and oxidative stress mediate the inflammatory response unleashed by GEM. Hydrogen peroxides and superoxide anions activate NF-κB.[9,10] NF-κB is a key mediator for several inflammatory pathways and induces the expression of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and interferon gamma (IFN-γ).[11-13]

Many researchers reported that renal damage induced by GEM, ameliorates by dietary supplement of antioxidants and nowadays attention has been focused on herbal medicines which have antioxidant activities.[2,14] *Carumcarvi* (caraway), belonging to the family Apaiaceae, have been used in herbal medicine since prehistoric times for various indications in traditional healing systems in wide geographical areas. Caraway is a highly efficient antioxidant with a singlet-oxygen and free radical scavenging capacity.[15] The intrinsic antioxidant activity of caraway is due to the presence of monoterpene alcohols, linalool, carvacrol, anethole and estragol, flavonoids, and other polyphenolic compounds.[16-20] The antioxidant activity of caraway is responsible for its various pharmacological properties such as antimicrobial, antidiabetic, anticarcinogenic/antimutagenic, antistress, and antiulcerogenic.[21] The present study was therefore designed to evaluate the possible protective or ameliorate effects of caraway seed oil on oxidative stress, lipid peroxidation and inflammation in GEM-induced nephrotoxicity in rats. For this purpose, antioxidant enzymes SOD, GSH-Px, CAT, the level of plasma MDA, TNF-α, IL-6 and IFN-γ were measured.

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Materials and Methods

Animals

In this investigation, 24 healthy adult male albino Wistar rats weighing 200-250 g were obtained from Razi Institute (Shiraz, Iran). The animals were housed under standard laboratory conditions (12 h light/12 h dark) in a room with controlled temperature (23±1°C) during the experimental period. All experimental procedures were conducted in accordance with the guide to the care and use of laboratory animals. The rats were provided tap water *ad libitum* and standard diet.

Animal ethics

This experiment was accomplished under the approval of the State Committee on Animal Ethics, Shiraz University, Shiraz, Iran. The recommendations of European Council Directive (86/609/EC) of November 24, 1986 regarding the standards in the protection of animals used for experimental purposes were also followed.

Preparation of caraway seed oil

Essential oil of caraway for this experiment was prepared by grinding the seeds, and the resulting powder was hydrodistillated for 3 h.[22] In aqueous and solvent derived seed extracts, different kind of flavonoids, isoflavonoids, flavonoid glycosides, monoterpenoid glucosides, lignins and alkaloids and other phenolic compounds have been found.[23] Caraway seed oil also includes several nutrients (vitamins, amino acids, protein, and minerals), starch, sugars and other carbohydrates and dietary fiber components (Table 1).[24]

Experimental design

The animals were randomly divided into four groups containing six rats each (Table 2). Nephrotoxicity was induced by GEM (Sigma, Aldrich, USA) in rats with an intraperitoneal dose of 100 mg k^{-1}, for six consecutive days.[1,25,26] Dose of caraway seed oil used in this study was selected on the basis of the previous studies.[27]

Experimental groups

Group I (Control)*:* controls received a daily intraperitoneal injection of normal saline (0.5 mL).

Group II (GEM): received GEM alone for six successive days plus the administration of 0.5 mL normal saline for 10 days.

Group III (GEM-S10): received caraway seed oil (10 mg kg^{-1}) during 10 consecutive days, after 10 days injection of GEM was initiated and lasted in daily manner for a 6 consecutive days.

Group IV (GEM-T10): received GEM for six successive days plus 10 days of caraway seed oil (10 mg kg^{-1}) treatment.

The animals in all groups were decapitated 24 h after the last application. Blood samples were collected into EDTA tubes. The samples were centrifuged at 750*g* for 20 min, and then, the plasma was pipetted into different aliquots.

Biochemical analysis

Blood urea nitrogen and serum creatinine

Urea nitrogen and creatinine were measured by commercial kits (Pars Azmoon Co., Tehran, Iran). Biochemical analyses were measured using a standard autoanalyser with veterinary software (Cobas-Mira, ABX-Diagnostics, Japan).

Antioxidant enzymes activities

SOD activity was measured with a commercial kit (RANSOD kit, Randox Com, UK). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) toform a red formazan dye. The enzyme activity was then determined by the degree of reaction inhibition, as one unit of SOD corresponded to 50% inhibition of INT reduction under assay condition. GPX activity was measured by a commercial kit (RANSEL kit, Randox Com, UK) based on the method of Paglia and Valentine.[28] GPX catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance was measured at 340 nm. The values of both enzymes were expressed as units/gr of hemoglobin. The activity of catalase was determined with the commercial catalase assay kit (OxfordBiomedical Research, Inc., USA) based on the colorimetric method described by Slaughter and O'Brien[29] and the activities of the enzymes were expressed as U/g of hemoglobin. Hemoglobin concentration was measured by Cyanmethaemoglobin method.

Lipid peroxidation of red blood cells

Malondiadehyde (MDA), an end product of polyunsaturated fatty acid oxygenation, is a reliable and commonly used biomarker for assessing lipid peroxidation.[30] The lipid peroxidation level of the RBC membrane was evaluated by means of a modified HPLC method with UV-Visible spectrophotometry according to Lykkesfeldt.[31] The measurement was based on MDA reactions with thiobarbituric acid (TBA) to form a colored MDA-TBA adduct, and the values were expressed as U/gHb of MDA.

Assay of pro-inflammatory cytokines

IL-6 was assayed in serum using a rat Interleukin-6 ELISA kit (CUSABIO®, Wuhan, China) which employs the quantitative sandwich enzyme immunoassay technique, and expressed as pg/mL. The concentrations of IFN-γ and TNF-α were measured by a solid phase sandwich ELISA (AbC 606 and AbC 607, respectively; Votrefournisseur AbCysS.A. Paris, France) and expressed as pg/dL and ng/mL, respectively.

Statistical analysis

The data were expressed as means±SEM differences between groups. Means were esti-

Table 1. Essential oil composition of *Carum carvi* L.

Compound	%
a-Pinene	0.57
b-Pinene	4.68
Myrcene	0.4
p-Cymene	7.99
Limonene	1.48
c-Terpinene	17.86
p-Cymen-8-ol	0.94
Cuminaldehyde	22.08
a-Terpinene-7-al	2.88
Bornyl acetate	1.12
c-Terpinene-7-al	15.41
Cuminyl acetate	3.78
Myristicin	1.87
Elemicine	3.54
Germacrene B	1.58
Dillapiol	1.39
Total	87.57

Table 2. Treatment schedule of male Wistar rats exposed to gentamicin (GEM) and caraway seed oil (n=24).

Group/days	1 to 6		7 to 10		11 to 16	
	GEM, mg/kg	Caraway seed oil, mg/kg	GEM, mg/kg	Caraway seed oil, mg/kg	GEM, mg/kg	Caraway seed oil, mg/kg
Control	0	0	0	0	0	0
GEM	100	0	0	0	0	0
GEM- S 10	0	10	0	10	100	0
GEM- T 10	100	0	10	0	10	0

Control group: healthy rats, received the normal saline as placebo, (0.5 mL/kg) daily; GEM group: Received GEM alone for six successive days plus the administration of 0.5 mL normal saline for 10 days; GEM-S10 group: Received caraway seed oil (10 mg kg^{-1}) during 10 consecutive days, after 10 days injection of GEM was initiated and lasted in daily manner for a 6 consecutive days; GEM-T10 group: Received GEM for six successive days plus 10 days of caraway seed oil (10 mg kg^{-1}) treatment.

mated using one-way analysis of variance followed by Duncan's multiple range test using SPSS the software package, version 18. Results were considered statistically significant at P value ≤0.05.

Results

The effect of treatments on plasma creatinine and BUN levels is shown in Table 3. Plasma creatinine and BUN levels (P<0.05) were significantly higher at the end of administration of GEM for six successive days when compared to the control group. However, creatinine and BUN levels (P<0.05) in caraway seed oil treated groups (GEM-S10 and GEM-T10) were lower than the group treated with GEM alone.

The data in Table 4 indicate the effect of treatments on plasma levels of MDA and activities of SOD,GSH-Px and CAT. GEM treated group had significantly higher levels of plasma MDA (P<0.05), while having significantly lower GSH-Px (P<0.05), SOD (P<0.05) and CAT (P<0.05) activities when compared with the control group. Treatment with caraway seed oil (GEM-S10 and GEM-T10) caused significant reduction in MDA levels (P<0.05) when compared with the GEM treated group. However, treatment with caraway seed oil provided a significant increase in CAT levels (P<0.05), SOD levels (P<0.05) and GSH-Px activities (P<0.05). Activity of antioxidant enzymes SOD, GSH-Px and CAT was higher in GEM-S10 group (P<0.05) than GEM-T10 (P<0.05) and GEM (P<0.05) treated group but was lower than the control group.

Pro-inflammatory cytokines IL-6, TNF-α and IFN-γ levels are shown in Table 5. GEM administration produced a significant (P<0.05) elevation of IL-6, TNF-α and IFN-γ levels when compared to control rats. Caraway treatment in GEM-S10 group significantly (P<0.05) decreased the IL-6, TNF-α and IFN-γ levels when compared with GEM group. IL-6, TNF-α and IFN-γ levels were significantly (P<0.05) decreased in GEM-T10 group compared to GEM alone treated rats. In GEM-S10 group caraway treatment caused more reduction in mean levels of pro-inflammatory cytokines in comparison with GEM-T10 group.

Discussion and Conclusions

GEM is a typical aminoglycoside and it is widely used as a bacterial agent for treatment of severe gram-negative bacterial infections.[2] Nephrotoxicity is a major complication of GEM administration and is the limiting factor for GEM clinical usage.[32] A slight increase in GEM blood concentration is accompanied by a significant, incommensurate increase in the amount of the drug in renal cortex.[1,32,33] Several investigators reported that treatments with GEM produce nephrotoxicity and cause reduction in renal functions, which is characterized by an increase in serum creatinine and serum urea level accompanied by impairment in glomerular functions.[34,35] Serum creatinine level is more significant than the urea levels in the earlier phase of the renal damage. In this study, intraperitoneal injection of GEM caused nephrotoxicity in rats, which was correlated with increased creatinine and BUN levels (Table 2). These observations indicated that GEM-induced nephrotoxicity and the results are in accordance with otherresearchers.[8,36,37] Increase in BUN and creatinine levels, induced by GEM was ameliorated by oral treatment of caraway seed oil. In this regard, various antioxidant agents have been shown to reduce GEM-induced renal injury, including vitamin C, *Ginkgo biloba* extract, Grape Seed Extract, green tea extract and *Bauhinia purpurea*.[38-42]

The exact mechanism by which GEM induces nephrotoxicity is unknown; however, several investigators reported that ROS are the causative factors for the renal side effects of this drug.[43,44] Under normal conditions, ROS, which are generated during cellular functions, are eliminated by intrinsic antioxidant enzyme systems like SOD, CAT and GSH-Px.[45] On the other hand, lipid peroxidation, mediated by oxygen-free radicals, is believed to be an important cause of destruction and damage to cell membrane and MDA which is formed during oxidative degeneration is accepted as an indicator of lipid peroxidation.[7,46] Several agents that scavenge or interfere with the production of ROS have been used successfully to ameliorate GEM nephropathy.[32] In this study, the role of ROS in GEM-induced nephrotoxicity was assessed by the usage of antioxidantagent, caraway seed oil, and further evaluation of alterations in the biochemical indicators of oxidative stress mainly SOD,CAT and GSH-Px activities and MDA plasma levels.

In the present study, GEM caused depletion inactivities of antioxidant enzyme SOD, CAT and GSH-Px in blood and elevated MDA plasma

Table 3. Effect of caraway seed oil on blood urea nitrogen (BUN) and creatinine in rats.

Groups (n=6)	Treatment	Parameters	
		BUN, mg/dL	Creatinine, mg/dL
I	Control	11.6±0.3	1.1±0.4
II	GEM	52.0±2.3*	3.7±0.1*
II	GEM-S10	15.9±0.4**	1.5±0.0**
IV	GEM-T10	39.4±1.4**	3.1±0.2**

Values are mean ± standard mean error. *P<0.05 when compared to the normal control. **P<0.05 when compared to gentamicin induced non treated disease control.

Table 4. Effect of caraway seed oil on catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) in rats.

Groups (n=6)	Treatment	Parameters			
		SOD, U/mL	GSH-Px, U/mL	CAT, U/L	MDA, U/gHb
I	Control	448.4±42.2	527.5±4.0	0.5±0.0	5.1±0.0
II	GEM	237.0±7.4*	276.6±5.7*	0.2±0.0*	5.8±0.0*
II	GEM-S10	319.5±1.6**	322.6±5.1**	0.4±0.0**	5.0±0.0**
IV	GEM-T10	279.2±4.7**	434.8±5.2**	0.3±0.0**	4.7±0.0**

Values are mean ± standard mean error. *P<0.05 when compared to the normal control. **P<0.05 when compared to gentamicin induced non treated disease control.

Table 5. Effect of caraway seed oil on TNF-α, INF-γ and IL-6 in rats.

Groups (n=6)	Treatment	Parameters		
		TNF-α, pg/mL	INF-γ, pg/L	IL-6, pg/mL
I	Control	380.8±17.2	11.9±0.3	18900.1±592.4
II	GEM	941.0±22.1*	45.4±3.1*	48223.5±1267.4*
II	GEMS-10	439.0±14.4**	14.6±0.9**	21942.8±1324.1**
IV	GEMT-10	772.5±26.4**	34.4±2.4**	41143.1±784.5**

Values are mean ± standard mean error. *P<0.05 when compared to the normal control. **P<0.05 when compared to gentamicin induced non treated disease control.

levels. Other investigators also reported that GEM induced nephrotoxicity is associated with low activity of GSH-Px, CAT and SOD in the renal cortex and high levels of MDA.[42,47] These decreases in renal antioxidant enzymatic protection could aggravate the oxidative damage. Furthermore, the increased production of ROS in GEM-induced nephrotoxicity may cause inactivation of antioxidant enzymes such as GSH-Px, CAT and SOD. In this study, SOD, GSH-Px and CAT were increased in blood of rats inGEM-S10 and GEM-P10groups compared to GEM group. On the other hand, treatment with caraway seed oil decreased the elevated MDA. These results could be in accord with several other researches, which reported that, compounds with antioxidant properties like garlic, lycopene and diallyl sulfide inhibited the reduced antioxidant enzymes in GEM-induced rats.[1,4,48] The antioxidant activity of caraway may be due to the presence of monoterpene alcohols, linalool, carvacrol, anethole and estragol, flavonoids, and other polyphenoliccompounds.[21] Many researchers have studied flavonoids and their major role in reducing ROS.[49] Abdel-Raheem *et al.*'s[45] investigation showed that quercetin, a flavonoid, increases antioxidant enzymes (SOD, GSH-Px, and catalase) activity and reduces tissue thiobarbituric acid reactive substance (TBARS) as an index of lipid peroxidation in GEM-induced rats. It has been reported that, quercetin exerts its antioxidant effects by scavenging free superoxide and hydroxyl radicals on one hand and by inhibiting xanthine oxidase activity and lipid peroxidation on the other. Harlalka *et al.*[50] suggested that aqueous extract of *Kalanchoe pinnata*, which has a notable amount of flavonol glycosides, has protective effect on GEM-induced nephrotoxicity. Sahu *et al*.[47] have reported that treatment with naringin, a major and active flavanone glycoside, leads to increase antioxidant enzymes GSH-Pxand CAT in GEM-induced nephrotoxicity. The renoprotective effect of *Cuminum cyminum,* which has related compounds similar to caraway, was studied by Burkenet *et al*.;[51] they showed that treatment of cisplatin-induced rats with different doses of aqueous extract of *Cuminum cyminum* significantly alters serum urea, creatinine, lipid peroxidation and antioxidant enzyme levels near to normal rats. As a result, the significant antioxidant and renoprotective activity of *C. carvi* is probably related to the presence of flavonoids.

In response to various inflammatory conditions, white blood cells release pro-inflammatory cytokines. In this regard, Balakumar *et al*.[5] reported that GEM-induced nephrotoxicity stimulates inflammatory events at the site of injury and enhances the migration of monocytes and macrophages to the site of tissue damage. The key factors in renal inflammatory process is activation and nuclear translocation of NF-κB, in response to oxidative stress which is regulated by gene expression of cytokines, chemokines and adhesionmolecules.[9,10]In the present study, addition of caraway seed oil (10 mg kg^{-1}) treatment along with gentamicin (GEM-T10 group) significantly decreased pro-inflammatory cytokines(IL-6, TNF-α and IFN-γ) compared to GEM alone treated rats. Earlier studies also demonstrated that an increase in NF-κB activation by GEM nephrotoxicity is followed by an increase in the concentration of inflammatory cytokines like TNF-α and IL-6.[36,47,52] Numerous studies have shown that GEM induces renal damage by free radical generation. Hence antioxidants and free radical scavengers of natural and synthetic origin have been studied by many researchers to provide nephro-protection in GEM-induced renal injury.[53] Among many antioxidants, consumption of flavonoid containing foods and beverages has been proposed as a useful practice to limit oxidative damage in the body.[54]

In conclusion, the present study indicated that caraway seed oil can provide protective effect against GEM induced oxidative stress and nephrotoxicity. However, further investigations are essential to elucidate the exact mechanism of protection and potential usefulness of caraway seed oil as a protective agent against drugs or xenobiotic toxicity in clinical trials.

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Clinico-pathological characteristics of canine gingival squamous cell carcinoma

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Abstract

In the present study, a case of gingival squamous cell carcinoma is described in a 9-year-old sheepdog with a swelling of the left mandible. Plain radiographs of the head revealed a soft tissue mass behind the ventral border of the left mandible. At necropsy, the tumor presented as reddish-brown ulcerated and irregular tumoral masses of the gingiva. In the cytology smear, there were oval to angular-shaped squamous epithelial cells with varying immaturity and variable staining and nuclear to cytoplasmic ratios (N:C). Some of the cells showed dyskeratosis. Histopathologically, the tissue sections were composed of the cords and islands of squamous epithelial cells with an abundant eosinophilic cytoplasm, large and ovoid nuclei with a prominent nucleolus. The mitotic figures were moderate. Based on the histopathological findings, the tumor was diagnosed as a moderately differentiated gingival squamous cell carcinoma.

Introduction

Squamous cell carcinoma is, by far, the most important skin tumor affecting most external sites, but is less reported affecting the internal organs. Oral cancers constitute approximately 2-4% of all malignant tumors in humans.[1] Malignant tumors of the oral cavity, approximately 6% of all malignant neoplasms, are one of the most common cancer types in dogs.[2] Approximately 85 to 90% of all oral cancers are squamous cell carcinomas in humans,[3] whereas they account for approximately 20% of oral tumors in dogs.[4] The prevalence of tumor increases with advancing age, and there is no gender and breed predilection.[5]

Squamous cell carcinomas have localized neoplastic invasion into the adjacent stroma or subepithelium including local bone invasion and only 10% of tumors spread to regional lymph nodes and 3% metastasize to the lungs.[6]

Squamous cell carcinoma arising from internal sites such as tonsils, gastric epithelium, and urinary bladder does not share the relatively innocuous behavior of those initiated by sunlight, which are slow to metastasize, usually only to local lymph nodes.[7]

The clinical forms of gingival squamous cell carcinomas are quite variable, exhibiting an ulcerated area or an exophytic, granular or verruciform growth, easily leading to misdiagnosis with benign tumors or other inflammatory responses.[8]

This report describes the clinical signs and histopathological findings of moderately differentiated gingival squamous cell carcinoma in 9-year-old sheepdog.

Case Report

A 9-year-old male sheepdog was referred for clinical evaluation of an asymmetrical swelling of the mandibular region. The dog had a clinical history of lethargy, poor appetite and weight loss. At clinical examination, there was a firm mass behind the ventral border of the left mandible. The dog had a rectal temperature of 38.2°C and was depressed. Complete blood count (CBC), biochemical analysis and radiography from head and thorax were performed. CBC and serum biochemical results and thoracic radiographs were normal. Plain radiographs of the head revealed a soft tissue mass behind the ventral border of the left mandible.

Because of very poor clinical condition, the possibility of severe bleeding during the operation and possible post operative complications, including injury to salivary duct, lingual dysfunction and probability of the lifelong necessity of tube feeding, the owner elected to euthanize the dog.

Grossly, a reddish-brown ulcerated and irregular mass of approximately 5×5×5 cm in diameter was observed within the gingiva and demonstrated a firm consistency. Some enlarged lymph nodes were observed and removed.

The tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 µm and stained with Hematoxylin & Eosin for light microscopic examination.

In the cytology smear, oval to angular-shaped squamous epithelial cells with varying immaturity and variable staining and nuclear to cytoplasmic ratios (N:C) were seen. Some of the cells showed dyskeratosis. Histopathological features revealed the cords and islands of squamous epithelial cells, which extended into the submucosal layer. The tumor cells were large and had an abundant eosinophilic cytoplasm, large and ovoid nuclei with a prominent nucleolus. Keratin tonofibers were seen to some degree. The mitotic figures were moderate (Figure 1). Similar neoplastic cell islands were also detected in the regional lymph nodes. Hence, the mass was found to be a moderately differentiated squamous cell carcinoma.

Discussion and Conclusions

The tissue sections from this case revealed a malignant tumor of epidermal cells in which the cells showed differentiation of keratinocytes with an abundant eosinophilic cytoplasm and large nuclei. With the exception of the tonsillar tissue, the gingiva are more often affected than the other soft tissues and most frequently affected at the maxilla. In the present case, the neoplastic mass was found to originate from the gingiva of the ventral border of the left mandible.

Squamous cell carcinoma is, by far, the most important skin tumor, but less reported affecting the internal organs. Squamous cell carcinoma in dogs infrequently involves the eye.[9] Occurrence on multiple digits simultaneously or consecutively is seen in dogs in a low percentage of cases.[10] A single squamous cell carcinoma is reported arising from the pyloric gland mucosa in a dog.[11] Squamous cell carcinoma occurs most often in the urethra of bitches.[12] Squamous cell carcinoma of the thyroid is an infrequent tumor in animals, but only occasionally encountered.[7]

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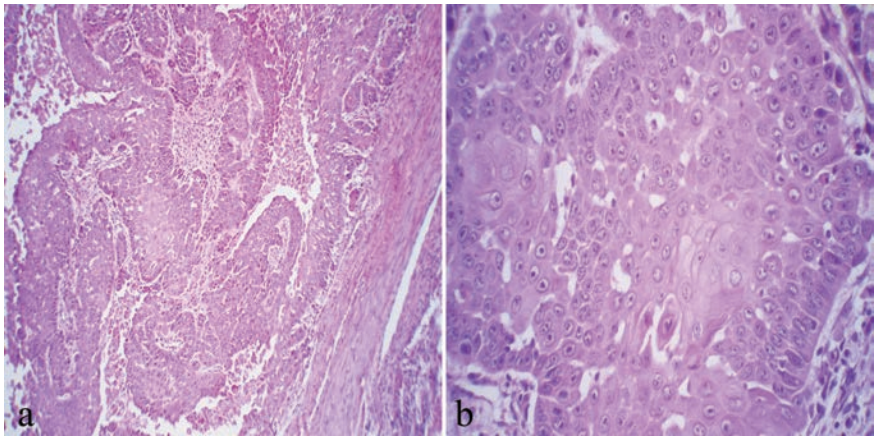

Figure 1. a) The cords and islands of squamous epithelial cells, H&E (×180). b) The neoplastic cells consisted of large cells with abundant eosinophilic cytoplasm, ovoid nuclei and a prominent nucleolus, H&E (×720).

Gingival squamous cell carcinoma is the second most common malignant neoplasm of the canine oral cavity. Only 10% of tumors spread to regional lymph nodes and 3% metastasize to the lungs.[13,14] Despite the low percentage of metastasis of squamous cell carcinomas, the present neoplastic cells were also seen in the regional lymph nodes.

There are several factors associated with the development of a squamous cell carcinoma, including prolonged exposure to ultraviolet light, lack of pigment within the epidermis at the sites of tumor development.[15] The etiology of oral squamous cell carcinoma is unclear in dogs.[16] In humans, induction of cyclo-oxygenase-2 has been implicated in the oncogenesis of various cancers, including squamous cell carcinomas. Oral squamous cell carcinoma in dogs can also be associated with overexpression of cyclo-oxygenase-2.[17] In addition, poor oral hygiene associated with chronic inflammation may promote the development of oral cancer.[8]

Based on histopathological findings, a gingival squamous cell carcinoma was diagnosed.

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The effects of prebiotic, probiotic and synbiotic diets containing *Bacillus coagulans* and inulin on serum lipid profile in the rat

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Abstract

An *in vivo* trial was conducted to evaluate the effects of *Bacillus coagulans*, and inulin, either separately or in combination, on lipid profile using a rat model. Thirty-two male Wistar rats were randomly divided into four groups (n=8) and fed as follows: standard diet (control), standard diet with 5% w/w long chain inulin (prebiotic), standard diet with 10^9 spores/day spores of *B. coagulans* by orogastric gavage (probiotic), and standard diet with 5% w/w long chain inulin and 10^9 spores/day of *B. coagulans* (synbiotic). Rats were fed for 30 days. Serum samples were collected 10, 20 and 30 days following onset of treatment. Total, HDL and LDL cholesterol and triglycerides concentrations were analyzed. Results of this study showed that inulin potentially affected the lipid profile. An obvious decrease in serum total cholesterol and LDL-cholesterol of rats fed with inulin in symbiotic and prebiotic groups was seen in all sampling days. Inulin fed rats also demonstrated higher levels of HDL-cholesterol concentration; however this value in probiotic and control fed rats remains without significant change. According to the results of this study, *B. coagulans* did not contribute to any lipid profile changes after 30 days. Thus, further *in vitro* investigations on the characteristic of these bacteria could be useful to gain insights into understanding the treatment of probiotics in order to achieve the maximum beneficial effect.

Introduction

WHO has predicted that by 2030, cardiovascular diseases will be the most important cause of death, affecting approximately 23.6 million people around the world.[1] People affected with hypercholesterolemia are at a three times higher risk of heart attack compared to those who have normal blood lipid profiles.[2] Pharmacological agents are able to reduce cholesterol levels effectively; however, they are expensive and the undesirable side effects have caused concerns about their therapeutic use. Therefore many investigations have been done to evaluate new approaches toward the identification of other dietary means of reducing blood cholesterol levels. These include dietary supplementation of probiotics and/or prebiotics. Probiotics are defined as *living microbial supplements that beneficially affect the host animals by improving its intestinal microbial balances*.[3] Prebiotics are *indigestible fermented food substrates that selectively stimulate the growth, composition and activity of microflora in gastrointestinal tract and thus improve hosts' health and well-being*.[4] When probiotics and prebiotics are used in combination they are known as *synbiotics*.

Micro-organisms used as a probiotic for human mainly belong to the *Lactobacillus* and *Bacillus spp*. Bacillus probiotics differ in many characteristics from those based on *Lactobacillus spp*. While *Lactobacillus* represents a normal resident gastrointestinal tract (GI) microflora of humans, Bacillus belongs only to the transitory GI bacteria.[5] Most *Lactobacillus* probiotics are inactivated by bile and low gastric pH, whereas members of genus *Bacillus* are endospore forming bacteria that make it extremely heat-stable and resistant to adverse GI tract conditions and when germinate in GI tract, cause positive effects for the host. *B. coagulans* (reported incorrectly as *Lactobacillus sporogenes*)[6] is a shelf stable bacteria that secretes L (+) lactic acid, short-chain fatty acids such as butyric acid and a bacteriocin called Coagulin, which has activity against a broad spectrum of enteric microbes.

Although many controversial studies have demonstrated the cholesterol-lowering effects of probiotics, prebiotics and synbiotics in animals and humans, there is also limited information on cholesterol-lowering effects of *B. coagulans* spores. Studies on the effects of *B. coagulans* on lipid profile have been limited to those who investigated the influence of administration of *B. coagulans* capsules (each containing 360 million spores) per day in hyperlipidemic patients for three months, they reported total serum cholesterol, LDL cholesterol and total cholesterol to HDL cholesterol, and LDL-cholesterol to HDL-cholesterol ratios were reduced significantly. They also found HDL-cholesterol was marginally increased.[7] Panda *et al*.[8] also reported this probiotic is able to reduce total cholesterol, VLDL and triglycerides in broiler chickens.

Considering our previous findings indicated significant changes in rat GI tract microbiota following administration of *B. coagulans* and inulin,[9] this study was conducted to evaluate the *in vivo* effects of *B. coagulans* and inulin, separately and in combination on lipid profile using a rat model.

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Key words: *Bacillus coagulans*; inulin; lipid profile; synbiotic diet; rat.

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Materials and Methods

Preparation of spore suspension of probiotic bacteria

Lyophilized probiotic *B. coagulans* were donated by the Pardis Roshd Mehregan Company, Iran. It was grown aerobically in Nutrient Yeast extract Salt Medium (NYSM) agar[10] at 37°C for 24 h. A single colony from the NYSM plate was inoculated into 500 mL of NYSM broth and incubated at 37°C with shaking at 250 rpm for 48 h. The bacterial suspension was pelleted three times by centrifugation at 3000×g for 20 min, and washed with sterile normal saline. Final pellet was re-suspended in 100 mL sterile normal saline. To determine the spore per ml of suspension, the solution was heated at 80°C for 15 min to kill the vegetative cells before appropriate serial dilution and plating in NYSM agar. Finally, the spore suspension was prepared at a concentration of 1×10^9 spore/mL in sterile saline and kept in the refrigerator until use.

Animals and diets

Thirty-two male Wistar rats (200±8.4 g) were provided by the Animal Centre of Razi Research Institute, Shiraz, Iran. Animals were randomly assigned to four dietary groups (n=8/group) and housed in groups of six rats per cage in a temperature controlled environment (22±2°C) with 55±10% relative humidity and controlled lighting (12 h light/dark cycle).

Rats were randomly divided into 4 groups and fed as follows: i) standard diet (control), ii) standard diet supplemented with 5% w/w long chain inulin (Sensus, Netherlands) (prebiotic), iii) standard diet with 10^9 spores/day *B. coagulans* (gavage 1ml of prepared spore suspension using a blunt ended needle) (probiotic), and iv) standard diet supplied with 5% w/w long chain inulin and 10^9 spores/day *B. coagulans* (synbiotic). The standard pellet feedstuff contained 14.5% protein, 4.7% ash, 51.2% starch, 4.3% sugar and 4% fat (3.2 kcal/g). Regarding micronutrients, the feedstuff contained 0.72% calcium, 0.6% phosphorus, 0.23% magnesium and 0.25% chloride among others. The inulin content in the rat diet was calculated based on food intake. The food intake of each rat with mean of 200 g body weight is 10 g/day, it means each rat received 0.5 g inulin/day. Food and distilled water were provided *ad libitum*.

To assimilate the experimental conditions, the control and prebiotic group were gavaged with 1 mL of sterile normal saline once a day.

Animal ethics

This experiment was accomplished under the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran. Also, the recommendations of European Council Directive (86/609/EC) of November 24, 1986 were followed, regarding the standards in the protection of animals used for experimental purposes.

Experimental design and sampling

All animals were acclimatized for 2 weeks before the experimental session. Rats were fed the diets for 30 days. On day 10, 20 and 30 of trial, animals were anesthetized with Diethyl ether and 3 mL blood samples were collected from each animal through heart puncture and left to stand for 30 min at room temperature (20°C) for coagulation before being centrifuged at 750 *g* for 15 minutes. Serum samples were stored at -20°C until analysis.

Lipid components analysis

The analysis of the serum for total cholesterol was done using a commercial kit (Ziest Chem Diagnostics, Tehran, Iran) by a modified

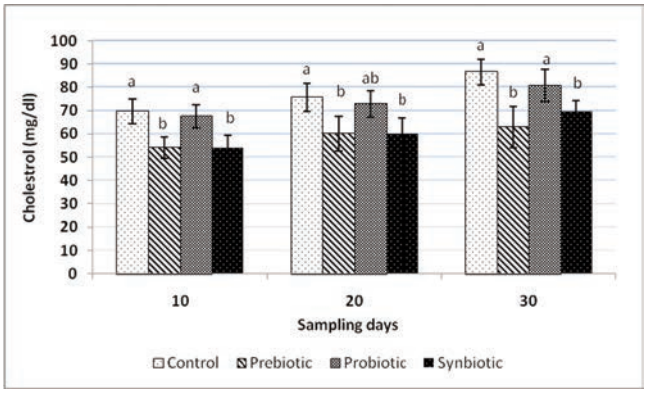

Figure 1. Effect of oral administration of *Bacillus coagulans* (probiotic diet) and inulin (prebiotic diet) separately and in combination (synbiotic diet) on serum total cholesterol levels in male Wistar rats. Values are expressed as mean for 8 animals. Bars represent standard deviation values. The different letters in the same sampling day indicate significant differences (P<0.05)

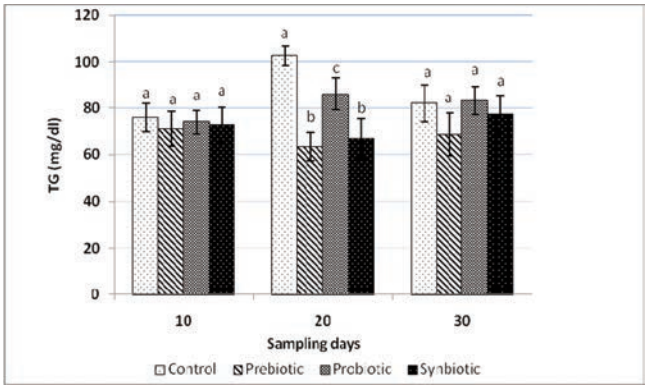

Figure 3. Effect of oral administration of *Bacillus coagulans* (probiotic diet) and inulin (prebiotic diet) separately and in combination (synbiotic diet) on serum HDL-cholesterol levels in male Wistar rats. Values are expressed as mean for 8 animals. Bars represent standard deviation values. The different letters in the same sampling day indicate significant differences (P<0.05).

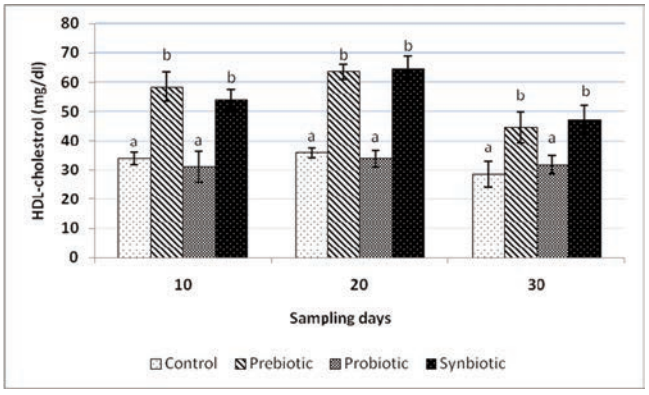

Figure 2. Effect of oral administration of *Bacillus coagulans* (probiotic diet) and inulin (prebiotic diet) separately and in combination (synbiotic diet) on serum triglycerides levels in male Wistar rats. Values are expressed as mean for 8 animals. Bars represent standard deviation values. The different letters in the same sampling day indicate significant differences (P<0.05).

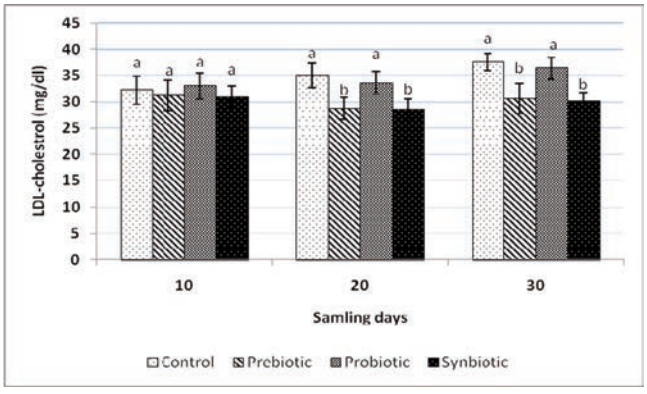

Figure 4. Effect of oral administration of *Bacillus coagulans* (probiotic diet) and inulin (prebiotic diet) separately and in combination (synbiotic diet) on serum LDL-cholesterol levels in male Wistar rats. Values are expressed as mean for 8 animals. Bars represent standard deviation values. The different letters in the same sampling day indicate significant differences (P<0.05).

Abell-Kendall/Levey-Brodie (A-K) method and the measurement of serum triglyceride was accomplished based on the enzymatic procedure by a commercial kit (Ziest Chem Diagnostics, Tehran, Iran).

Lipoproteins including HDL-cholesterol (mg/dL), and LDL-cholesterol (mg/dL) were analyzed by quantitative enzymatic colorimetric method using test kits supplied by STAN-BIO Laboratories, Boerne, TX, USA. All reactions were measured using Digital UV/VIS Spectrophotometer (CE 292, series 2, Cecil Instruments, Cambridge, England).

Statistical analysis

In order to determine the difference among treatments, an Analysis of Variance (ANOVA) was used and when differences were detected, a Duncan's multiple comparison test was used to differentiate the treatment means. The analysis was carried out using SPSS (version 19, SPSS Inc) at a significance level of 0.05.

Results

The effect of different diets supplementation on serum total cholesterol is shown in Figure 1. Supplementation with inulin caused significant decrease in serum total cholesterol of rats, on prebiotic and synbiotic diets after 10, 20 and 30 days of treatment initiation.

On day 10, there were no significant differences in triglycerides values between treatments and control (Figure 2). By day 20, serum triglycerides concentrations in control, probiotic, prebiotic and symbiotic groups were 102.6, 86.1, 63.2 and 66.9 mg/dL, respectively, which shows inulin has a potential role in triglycerides decrease (P<0.001). On day 30, lower serum triglycerides (P=0.23) was seen in prebiotic and synbiotic groups compared to probiotic and control but it was not significant.

On day 10, 20 and 30 of trial HDL-cholesterol level increased significantly (P<0.001) in serum of rats fed with inulin, in prebiotic and symbiotic groups, while rats fed by only *B. coagulans* remained without significant change (Figure 3).

Changes in LDL-cholesterol level during 30 days treatment with experimental diets are shown in Figure 4. Treatment with different diets appeared to have no effect on the LDL cholesterol concentration in rats by day 10. However, supplementation with prebiotic and synbiotic diets significantly lowered LDL-cholesterol concentration over 20 and 30 days; rats on the probiotic and control diets showed increased in LDL-cholesterol values.

Discussion

This feeding trial was conducted to investigate the effect of *B. coagulans* and inulin, separately and in combination on serum lipid profile using rats as a model. Administration of inulin in rats on prebiotic and synbiotic diets showed significantly (P<0.05) lower total cholesterol level compared to probiotic and control rats in day 10, 20 and 30. Significant changes in triglyceride concentration are limited to day 20, when rats on prebiotic and symbiotic diets showed lower levels of triglyceride concentrations, while on day 10 and 30 no significant differences between groups were observed.

According to the results of this study, inulin played an important role in changing lipid profile effectively. An obvious decrease in the serum total cholesterol and LDL-cholesterol of rats fed with inulin in synbiotic and prebiotic groups was seen in all sampling days. Inulin fed rats also demonstrated higher levels of HDL-cholesterol concentration; however this value in probiotic and control fed rats showed no significant change.

There are two suggested mechanisms that have been attributed to hypocholesrolemic effect of prebiotics such as inulin; decreased cholesterol absorption by enhancing cholesterol excretion via feces and the selective fermentation by intestinal bacterial microflora causing production of short-chain fatty acids (SCFAs).[11]

Results of this study concerning hypocholesterolemic effect of inulin are in agreement with several studies conducted *in vivo* trials. Kim and Shin[12] reported administration of inulin in hypercholesterolemic rats for 4-weeks decreased serum LDL-cholesterol with increased serum HDL-cholesterol levels (P<0.05) compared to the control.[12] Causey *et al*.[13] showed consuming 20 g/day inulin for 3 weeks caused significant decrease in serum triglycerides in twelve subjects.[13] Similarly, in another study involving eight healthy volunteers with a daily consumption of 10 g of inulin for 3 weeks, significant decrease in plasma triacylglycerides concentrations compared to the placebo was observed.[14] In another study by Brighenti *et al.*,[15] significant (P<0.05) reduction in plasma total cholesterol and triacylglycerols of twelve healthy rats was seen following 12-week consumption of 50 g of a rice-based ready-to-eat cereal containing 18% inulin.[15] According to the results of this study, *B. coagulans* did not contribute to any lipid profile changes after 30 days. Although many studies have demonstrated cholesterol-lowering effects of probiotics in both animals and humans, debatable results have also been reported concerning inability of a particular strain of probiotic bacteria to improve lipid profile. A study by Hatakka *et al.*[16] reported that administration of *L. rhamnosus* LC705 (10^{10} CFU/g per capsule; two capsules daily) did not influence blood lipid profiles in thirty-eight men after a 4-week treatment.[16] Simons *et al*.[17] and Lewis and Burmeister[18] also refuted effect of *L. fermentum,* (2×10^9 CFU per capsule; four capsules daily) and *L. acidophilus* on human lipid profiles, respectively.

Several possible mechanisms for hypocholesterolemic effects of probiotics are: incorporation of cholesterol into the cellular membranes by growing cells and deconjugation of bile via bile salt hydrolase. Once deconjugated, bile acids are less soluble and absorbed by the intestines, leading to increasing their rates of excretion in the feces. Cholesterol is used to synthesize new bile acids in a homeostatic response, resulting in lowering of serum cholesterol.[19-21]

Ooi and Liong[20] in a review article attributed these controversial findings to various factors such as different strains of probiotics, administration dosage, analytical accuracy of lipid analyses, duration of treatment period, clinical characteristic of subjects, inadequate sample sizes, and lack of suitable controls or placebo groups.[2]

Considering the mentioned reasons hypothesized by Ooi and Liong,[20] normolipidemic condition of used rat model in the current study may be a reason for failure of these probiotic bacteria in changing lipid profile.[20] In addition to all of the above, the feeding period of 30 days may not be sufficient to observe a significant change in lipid profile.

However, the strains of bacteria used as a cholesterol lowering agent must be bile tolerant, have the ability to deconjugate bile acids, and bind cholesterol. In addition, the ability of particular strain of probiotic bacteria to attach permanently to the gut wall and hence continuous supply might be necessary to exert the effects.[21,22] The results of our previous study indicated that these bacteria are not able to colonize the intestine and are quickly eliminated in feces.[9] As such, daily consumption of probiotic products is necessary for any long-term effect on metabolism.

Conclusions

Prebiotic inulin significantly reduced the serum total cholesterol, LDL-cholesterol and increased the HDL cholesterol of rats during the treatment period. But no influence of probiotic *B. coagulans* on lipid profile was observed. In order to justify the cholesterol-lowering effect of *B. coagulans*, *in vitro* studies proposed mechanisms of action are necessary. Thus, further investigations on the characteristic of these bacteria could be useful to gain insights into understanding the treatment of

probiotics in order to achieve the maximum beneficial effect.

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Canine osteoarthritis and treatments: a review

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Abstract

Arthritis is a commonly occurring chronic illness in human and animals alike. Among all domestic and pet animal species, dogs suffer from arthritis more often because of excessive running or exercise, injury, and/or genetic predisposition. Presently, one in four of 77.2 million pet dogs in the United States are diagnosed with some form of arthritis. In dogs, osteoarthritis is more common than rheumatoid arthritis and pain is the number one observation. Osteoarthritis, also known as degenerative joint disease, is a slowly progressive inflammatory disease, which is characterized by degeneration of the cartilage, hypertrophy of bone at the margins, and changes in the synovial membrane, and that eventually results in pain and stiffness of joints. Alterations in joint structures, decreased flexibility, and severe pain ensues, due to lack of hydration and inflammation. Cells within the damaged joints release pro-inflammatory cytokines, which further the inflammatory process. This causes more breakdown of the cartilage collagen type II and proteoglycans, which results in a perpetual destructive cycle. This perpetuating cycle ultimately results in cartilage destruction, subchondral bone thickening, and synovial membrane inflammation. This review focuses on osteoarthritis, the disease, causes, treatments, and presents a glimpse of some new therapies under study.

Introduction

Osteoarthritis, also known as degenerative joint disease (DJD), is a chronic inflammatory joint disease, which causes pain/soreness, stiffness, swelling, and lameness due to the diminished cushion and changes in the synovial fluid.[1-3] Osteoarthritis affects the entire synovial joint including the cartilage, synovial fluid, and bone. This disease is characterized by degeneration of the cartilage and soft tissues, hypertrophy of bone at the margins, and changes in the synovial membrane.[1-3] Mechanical stress is thought to induce changes in biochemical factors within affected joints, leading to articular cartilage degradation.[4] The disease process limits the amount of protein, released from the cartilage's cells, to repair cartilage in the joints; this is referred to as pitting and fraying of cartilage.[5] This pitting and fraying results in the cartilage losing its elasticity and protective surface due to enzymatic cleavage of proteoglycans.[6] As the cartilage continues to break down and deteriorate completely, it causes friction between the bones, which leads to inflammation, thickening of soft tissues, and loss of mobility of the joint.[7] Trying to maintain its normal balance of injury and repair, as the cartilage wears away the joints begin to lose its normal shape and the space between the joints narrow. Osteophytes (spurs) formation begins where the ligaments and joint capsule attach to the bone. In addition, fluid filled cysts form and fragments of bone and cartilage can be found floating in the joint space.[5] All of the changes in the joints and bones can cause pain, swelling, and the joint may even appear enlarged.

Disease overview

Osteoarthritis is a disease that has been described for over a hundred years.[8] Currently there are about 27 million Americans diagnosed, but is expected to reach 67 million by 2030.[9] Osteoarthritis is the most common form of arthritis in humans and in dogs. In almost every form of arthritis there is a loss of bone or cartilage that results in changes in the shape of joints.[10] There are three different types of joints; fibrous, cartilaginous, and synovial. Fibrous and cartilaginous joints consist of fibrous tissues or hyaline cartilages, which allow little or no movement. Synovial joints are made up of synovial fluid and dense irregular connective tissue, which creates a synovial joint capsule allowing the joints to freely move.[3] The main focus will be on the synovial joint, especially the ball and socket (hip and shoulder) and hinge joints (elbow), because these joints are most commonly affiliated with osteoarthritis, especially in canines.[3] The synovial fluid in the synovial joint capsule provides nutrients, lubrication, and a cushion for articular cartilage.[1-3] Articular cartilage, which is composed of hyaline cartilage, is avascular tissue consisting of chondrocytes embedded within an extracellular matrix of collagens, proteoglycans, and non-collagenous proteins. Articular cartilage reduces friction and makes movement of the synovial joints painless.[11] The hyaline cartilage, which has a high content of collagen type II, serves as a shock absorber by distributing pressure from the load over the subchondral bone. In healthy joints, there is a fine balance between injury and repair amongst chondroblasts and chondroclasts.[5] However, in osteoarthritis this balance is disrupted by an overproduction of osteoblasts that can cause pain and swelling. Osteoarthritis has multiple causes and risk factors; however, once the cartilage is lost, the joint fails.[12]

Osteoarthritis is a progressive disease that consists of four stages. In stage one of osteoarthritis, minor bone spurs begin to develop. The cartilage matrix begins to break down due to chondrocyte's metabolism being affected and increasing the production of matrix destroying enzymes, metalloproteinases (MMPs). The severity of cartilage lesions can be correlated with the levels of collagenase present (MMP-1).[13] Cartilage lesions disrupt the function of cartilage, increasing friction and inflammation in the joints, resulting in pain. Stage two of osteoarthritis is considered the *mild* stage. This stage involves erosion of the bone due to the cartilage lesions. This can cause new bone growth, osteophyte, also called bone spurs, which affect normal joint movement. In this stage, proteoglycan and collagen fragments are released into the synovial fluid.[10] In the adult dog, protecoglycan turnover is quicker (300 days) than estimated collagen turnover (120 years). Marked protecoglycan loss of articular cartilage is irreversible and results in joint degeneration.[4] Stage three is considered *moderate* osteoarthritis. The cartilage, in-between the bones, thins out and loses cushion. The space between the bones is also narrowing, causing grinding between the adjacent subchondral bones.[10] During stage three, symptoms are more severe and inflammation begins to occur. Production of synovial

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macrophages occurs, including MMP, cytokines (interleukin 1), and tumor necrosis factor-alpha.[4,6] Once the synovial macrophages are produced they can destroy tissues by diffusing back into the cartilage and can also stimulate chondrocytes. The fourth and final stage of osteoarthritis is considered *severe* osteoarthritis. In this stage the joint space is dramatically reduced, the cartilage is almost gone, and joint mobility is reduced greatly.[4,10] Early diagnosis of osteoarthritis is key to prevent further damage to the joint and alleviate symptoms.

Diagnosis of canine osteoarthritis

Osteoarthritis is the most common type of arthritis in dogs and is the most common source of chronic pain in older dogs.[7] This is due to the constant wearing away of the cartilage from dogs running, jumping, and other strenuous exercise. Arthritis commonly affects large breed dogs, *i.e.* German Shepherds, Labradors Retrievers, Siberian Huskies, and Rottweilers, more than small breed dogs. Prevalence of osteoarthritis can be as high as 20% in dogs more than a year old, with middle-aged and older dogs being at higher risk. Dogs that are diagnosed with arthritis tend to be lethargic, have difficulty moving from a sitting or lying position, cracking joints, stiffness, muscle wastage, and visible pain.[7] Diagnosing osteoarthritis in dogs begin with owners observing the pain and stiffness while the animal is running, walking, jumping, or rising from a lying or sitting position. Radiographic evidence, patient symptoms, and osteoarthritis risk factors such as age, gender, and body mass index, can all aid in predicting the risk of rapid, highly predictable joint degradation. During physical exams, the patient may show signs of pain, including whining, biting, or trying to move away. Radiographic evidence can show the breaking down of cartilage between bones and inflammation in the joints. By properly diagnosing patients with osteoarthritis, this will help establish a future plan to help ease pain, prevent further damage, and overall increase the quality of life.

Canine hip dysplasia

Along with osteoarthritis, dogs may also suffer from hip dysplasia, a form of osteoarthritis present in the ball and socket joints. Hip dysplasia can be a genetically inherited condition from improperly formed hip joints typically seen in large breed dogs.[14] Dogs that suffer from inherited hip dysplasia, show signs within the first year and should be spayed or neutered to avoid passing this genetic tendency to malformation to offspring. Bulldogs, St. Bernard's, Blood Hounds, and Boykin Spaniels are a few examples of breeds that are at a higher risk factor for developing hip dysplasia. Dogs can also be at risk for hip dysplasia if there is excessive weight gain during the early stages of growth, typically 3-8 months of age, and from putting excessive pressure on the hip joint from strenuous exercise. Hip dysplasia is caused from an abnormal development of the hip joint, leading to excess laxity in the hip joint. Laxity in the hip joint can cause stretching of the supporting ligaments, joint capsules, and surrounding muscles, leading to permanent damage to the anatomy of the hip joint.[15] The permanent damage to the anatomy causes the poorly developed head of the femur to loosely fit into a shallow acetabulum.[16] Orthopedic Foundation for Animals (OFA) radiographs can also be done to diagnose hip dysplasia. According to the Orthopedic Foundation for Animals, OFA radiographs must be performed with the animal in dorsal recumbancy with rear limbs extended parallel. The stifles are rotated inward and the pelvis is symmetric. This type of radiograph allows veterinarians to assess how the femoral head fits into the acetabulum, which aids in the diagnosis of hip dysplasia.[16]

Measuring joint mobility

Osteoarthritis patients struggle with limited range of motion (ROM), a reduction in the ability to move one's joints. Pain, stiffness, and swelling, all symptoms of osteoarthritis, can hinder mobility. Measuring the range of motion can help identify what condition the articular surface, joint capsule, ligaments, and muscles, are in.[17] Assessing the range of motion is widely used in human medicine and is becoming more popular in canine veterinary medicine, as more patients are being diagnosed with arthritis (Table 1). Universal Goniometry is a commonly preferred way to measure range of motion in humans and other species.[18] A goniometer is an affordable, reliable, commonly used, non-invasive tool used to measure flexion and extension degrees of joint mobility in the forelimbs and hind limbs in canine, as well as humans during physical therapy sessions. When using a goniometer, place the tool over the fulcrum of the joint, aligning the stationary arm with the stationary line of the body. Move the desired joint, either flexed or extend, and follow the moving line of the body with the moving arm of the goniometer; look at the readings on the goniometer for the degree of range of motion.

Erythrocyte sedimentation rate

In addition, a multitude of blood tests can be used to determine the degree of inflammation in the joints from arthritis, aiding in the diagnosis. One test used to assess inflammation is the erythrocyte sedimentation rate test along with complete blood counts and chemistry panels. The erythrocyte sedimentation rate (ESR) test, also known as the sed rate, sedimentation rate, and Westergren sedimentation rate, is a quick and simple test that has been used for many years to detect inflammation associated with infections, autoimmune diseases, and arthritis. A Polish pathologist, Edmund Biernacki, invented the ESR test in 1897. In 1918, two Swedish pathologists, Robert Sanno Fahraeus and Alf Vilhelm Albertsson Westergren used sodium citrate-anticoagulant specimens. This method of the test is widely used today and known at the Westergren method.[19]

Due to the ESR test not being specific, it is used in addition to other blood tests including c-reactive protein, antinuclear antibody (ANA), and rheumatoid factor. Typically, ESR tests are ordered when a condition or disease is suspected to cause some form of inflammation in the body. For example, people who suffer from arthritis may have an ESR test run to detect the amount of inflammation in the joints. ESR is the rate at which red blood cells sediment in a period of one hour. The test is performed by anticoagulated blood, typically in an ethylenediaminetetraacetic acid (EDTA) tube that is placed in an upright 150 mm tube, also known as a Westergren tube. After an hour, the rate at which the red blood cells have fallen is reported in millimeters of plasma per hour (mm/hr).[20] Normal ranges for canine and

Table 1. Maximum joint range of motion in canine.

Joint	Extension	Flexion
Shoulder	142 degrees-ground	125 degrees-ground
Elbow	124 degrees-ground	98 degrees-ground
Carpus	124 degrees-ground	97 degrees-ground
Hip	141 degrees-ground	115 degrees-ground
Stifle	141 degrees-ground	109 degrees-ground
Hock	135 degrees-ground	115 degrees-ground

feline ESR are listed in Table 2. The ESR test works by a precise balance of pro-sedimentation factors, specifically fibrinogen, and resisting sedimentation factors, such as the negative charge of erythrocytes. During a state of inflammation, the fibrinogen increased causing the red blood cells to stick together in a stacked pattern known as rouleaux. The stacked erythrocytes are denser and cause the cells to settle faster than normal.[21]

Table 2. Erythrocyte sedimentation rate normal ranges.

Species	Normal range (mm/hr)
Feline	0-12
Canine	0-5

Drugs and disease management

Animals with osteoarthritis are treated with various approaches, involving invasive and non-invasive measures. The objectives in managing osteoarthritis include minimizing joint pain by reducing the inflammation and slowing the progression of the cartilage damage, resulting in increased joint flexibility and ultimately improving quality of life. To achieve these goals, a variety of conventional pharmaceuticals, experimental treatments, nutraceuticals and supplements, and life change, such as stem cell therapy, physical therapy with acupuncture, and weight loss and exercise programs.

Conventional treatments

Non-steroidal anti-inflammatory drugs

Pharmacological management of osteoarthritis includes steroidal or non-steroidal anti-inflammatory drugs (NSAID). These drugs do not address the underlying issue; they just control pain and inflammation. NSAIDs work against prostaglandins, which are a family of chemicals that are produced by cells and promote inflammation. Their inflammation properties also result in pain, fever, and increased platelet clumping.[1,7] The cells that produce prostaglandins are called cyclooxygenase (COX). There are two forms of COX enzymes, COX-I produces prostaglandins that support platelet clumping and protect the stomach, and COX-II enzymes produce prostaglandins that are responsible for pain and inflammation. Since NSAIDs inhibit both forms of COX enzymes, NSAIDs can result in gastrointestinal side effects, including ulceration, vomiting, anorexia, melena, and abdominal pain.[1,17]

Acetylsalicylic acid was the first NSAID to be used in modern medicine and still is widely used. Acetylsalicylic acid, despite its side effects, is commonly recommended in veterinary medicine for dogs that suffer from osteoarthritis due to it being relatively inexpensive. However, studies have shown that it can decrease chondrocyte production of collagen and proteoglycans and can enhance cartilage degradation over time.[1] Acetylsalicylic acid is also a unique NSAID in the fact that it prolongs blood clotting for 4-7 days. This makes in an ideal drug for preventing blood clots that cause heart attacks and strokes, rather than an osteoarthritis event.[1,7] Since there are many problems associated with acetylsalicylic acid for osteoarthritis treatment, other NSAIDs are becoming more popular. The six types of NSAIDs that are commonly prescribed by veterinarians, other than acetylsalicylic acid, for osteoarthritis patients include: carprofen, deracoxib, etodolac, meloxicam, tepoxalin, and firocoxib.[7]

Corticosteroids

Corticosteroids and gluccorticosteroids, often referred to as steroids, may be lifesaving and certainly increase the quality of life of dogs and humans.[22] Cortisone is a hormone that naturally occurs in the cortex of the adrenal gland. This is where the *cortico* prefix comes from. Corticosteroids are produced from the same chemical base that produces the sex hormones.[23,24] Cortisol is naturally produced when an animal gets stressed; however, man-made cortisol is 5-6 times stronger than naturally produced cortisol. Any production, natural or drug induced, of cortisol has a negative feedback and slows or stops natural production. Suppression of naturally produced cortisol typically occurs within 12-48 hours and takes a few days to start the process back up.[25] Stopping the use of steroids quickly can result in a withdrawal syndrome, which includes fatigue, joint pain, stiffness, tenderness, and fever.[26]

Corticosteroids are the most used, and misused, pharmaceuticals in veterinary medicine.[22] Steroids, generally in an oral tablet, are used for stress response, immune system issues, inflammation, nutrient metabolism, and maintaining electrolyte levels in the blood.[22] Corticosteroids are a popular treatment plan for patients suffering from arthritis because they are extremely effective in relieving pain and inflammation.[23] Steroids inhibit the production of arachidonic acid, which can stop the inflammation and stop the production of prostaglandins, similar to NSAIDs.[26] However, when using steroids the body cannot separate the anti-inflammatory properties from the immunosuppressant properties.[26] Therefore, low doses of steroids are used to suppress inflammation and high doses of steroids are used as immunosuppressants.[22] Since steroids affect nearly all cells of the body, their benefits are widespread, however, their side effects can be long lasting and devastating.[26] The side effects, which vary depending on the dose and duration of steroid use, include sore mouth, weight gain, osteoporosis, high blood sugar levels (diabetes), cataracts, insomnia, gastrointestinal bleeding and ulcers, suppressed immune systems, fluid retention, atherosclerosis resulting in increased risk of heart disease, and aseptic necrosis. To reduce the probability of side effects from steroid use, one must avoid using steroids on a daily basis and no longer than 3-4 months without re-evaluating organ functions. Due to the devastating side effects of steroid use, alternative medicine such as acupuncture, nutraceuticals, and therapy can be used to treat osteoarthritis patients.

Experimental treatments

Insulin growth factor-I

Insulin growth factor-I (IGF-I) is peptide produced by the liver that promotes growth by reaching the articular cartilage through the synovial fluid. IGF-I can also be synthesized by chondrocytes.[27] This peptide is vital for childhood growth, but continues to have anabolic affects in adults. IGF-I is stimulated by growth hormones and helps cartilage maintain structural and functional integrity by inhibiting interleukin-1's ability to stimulate proteoglycan degredation.[27] However, under malnutrition conditions, hormone and receptor insensitivity, and failure to downstream signaling, can affect growth and stimulate damage to cartilage health. Past studies have shown that IGF-I can be important to the development of osteoarthritis and osteoporosis due to IGF-I being abundant in the cartilage and bone; therefore, it is suggested that it can prevent cartilage damage and the progression of osteoarthritis.[27] This is because IGF-I plays a role in the regulation and homeostasis of normal cell growth and cartilage. In adults, if damage occurs, *i.e.* tumors, this can lead to overgrowth of bony tissue, which can cause osteoarthritis.[28] However, due to many inconclusive studies, more work needs to be done to understand the mechanism and to what extent IGF-I has on osteoarthritis.

Oral doxycycline

Doxycycline, broad spectrum, bacteriostatic antibiotic, is commonly used for bacterial infections and to treat malaria. However, preliminary studies on animals have been conducted to study the effects of doxycycline on osteoarthritis. It is speculated that low dosages of tetracycline analogue, specifically doxycycline, can inhibit the MMPs, which play an essential role in cartilage degradation. Due to doxycycline being more lipid soluble it is able to penetrate areas such as the synovial joint, which in the main site of damage in dogs with osteoarthritis. In one study conducted by the Johns Hopkins' Arthritis Center (1995), oral administration of doxycycline showed to prevent narrowing of the knee joint in subjects. Studies have also shown that oral administration of doxycycline can reduce the severity of articular cartilage, which plays a vital role in the process of osteoarthritis.[29] In Nganvonpanit *et al*., the therapeutic effect of oral doxycycline on canine hip osteoarthritis, by reducing the rate of joint pathology in osteoarthritis, was studied. Overall, after a six-month period, dogs showed significant signs of improvement in joint mobility, pain upon limb manipulation, lameness, and were able to bear more weight on their hips. Signs of improvement were shown as early as two months.[30] However, the results were not consistent and further studies should be conducted at the therapeutic effects of oral doxycycline and canine osteoarthritis and lameness. In addition, the overuse of doxycycline can result in microbial resistance; therefore, long-term side effects and dosage recommendations need to be further studied.

Sodium pentosan polysulfate

Originally made for humans who suffer from interstitial cystitis, sodium pentosan polysulfate is a semi-synthetic, poysulphated polysaccharide that has anti-inflammatory and anti-arthritis properties and is classified as a disease modifying osteoarthritis drug (DMAOD). Sodium pentosan polysulfate is structurally similar to glycosaminoglycans. Sodium pentosan polysulfate is also similarly structured to the anti-coagulant, heparin; however, does not have the same strength. Sodium pentosan polysulfate has been studied in Europe for over 40 years, but only recently has been combined with calcium to increase its anti-arthritic properties.[31] These properties are due to it being able to stimulate chondrocytes to synthesis cartilage, stimulate synoviocyte biosynthesis, and inhibit degradation of cartilage matrix and acrachidonic acid, which promotes an inflammatory cascade.[32] Sodium pentosan polysulfate is recommended to be injected into the joint in 5-7 day intervals with provided three months of relief.[32] Currently, it is leading experimental treatment for osteoarthritis in canines and other animals by inhibiting MMPs and maintaining the cartilage structure and biochemistry.[32] Other than mild gastrointestinal upset, not other side effects have been noted. Therefore being relatively safe, even at three times the recommended dose and having minimal side effects, sodium pentosan polysulfate is becoming a more popular alternative treatment for canine osteoarthritis.[31] However, more *in vivo* studies need to be performed to evaluate the bioavailability of oral route versus intramuscular.

Glycoaminoglycans: glucosamine and chondroitin sulfate

As the body ages the production of glucosamine slows down; therefore, it is important to supplement glucosamine to avoid joint issues.[37] Glucosamine (2-amino-2-deoxy-D glucose), the most abundant monosaccharide, is a naturally occurring compound composed of sugar and amino acids. Glucosamine has been used for nearly 40 years in human medicine.[25] It is strictly used as a dietary supplement in the United States, but is a regulated pharmaceutical throughout Europe.[16] There are three different types of glucosamine; glucosamine sulfate, glucosamine hydrochloride, and N-acetyl-glucosamine. However, glucosamine sulfate may be more effective for arthritis treatment because sulfate is needed to produce cartilage and the other two forms of glucosamine do not contain sulfates.[25] Glucosamine supplements are extracted from crustacean exoskeletons or from fermentation of grains such as corn or wheat.[21] Glucosamine is one of the most commonly used nutraceuticals, especially for arthritic patients, due to it being involved in the body's production of joint lubrication and shock absorption and maintaining healthy cartilage and joint function.[25] Glucosamine is the precursor in the biochemical synthesis of glycosylated proteins and lipids, glycosaminoglycans. Glycosaminoglycans are a major component of joint cartilage and the extracellular matrix of articular cartilage.[25] Glucosamine also aids in the rebuilding of damaged cartilage and is a building block for articular cartilage.[25] Glucosamine has anti-inflammatory properties by inhibiting synthesis of degradation enzymes, increasing synthesis of extracellular matrix, and reduces apoptosis of articular chondrocytes.[25] Glucosamine is also good for nail growth, tendons, skin, eyes, synovial fluid, ligaments, heart valves, and mucous secretions of the digestive, respiratory, and urinary tract.[21] Glucosamine supplements have little to no side effects when used at the recommended dose; however, if taken above the recommended dose, it can cause damage to pancreatic cells and increase the risk of diabetes. Short-term side effects include stomach upset, constipation, diarrhea, headaches, and rashes.[16] In recent years, in a series of preliminary experiments, researchers have evaluated several nutraceuticals individually and in combination with several other supplements, and found that they are significantly effective in ameliorating arthritic pain.

Glucosamine supplements are often combined with chondroitin sulfate. Chondroitin sulfate, a type of gylcoaminogycan, addresses the disease process of arthritis by aiding in the repair of damaged connective tissue. Chondroitin sulfate is one of the most abundant glycoaminoglycans in joint cartilage, bones, tendons, cornea, and heart valves.[21] It is also beneficial to stress injuries, by keeping joints hydrating and protecting existing cartilage breakdown.[21] Studies has theorized that supplementation of chondroitin sulfate will maximize blood circulation to subchondral bone and synovial joints. Chondroitin sulfate is vital for articular cartilage and joint structure because it can bind collagen fibrils and is used as a chondroprotective agent by inhibiting the degradations of cartilage matrix and synovial fluid.[21] Supplementation of chondroitin sulfate is important because as the body ages, less chondroitin sulfate is produced and other glycoaminoglycans, such as keratin sulfate, are produced which predisposes the joint to osteoarthritis. In addition to the joint benefits, chondroitin sulfate supplements are noted to have up to 70% bioavailability when taken orally, this is significantly more than the bioavailability of other supplements and nutraceuticals. Overall, glucosamine chondroitin sulfate and other joint related glycosaminoglycans, seem to be relatively safe and do not display any long term side effects. Therefore, making glycoaminoglycans a popular alternative treatment for osteoarthritis in canines.[21,25]

Stem cell therapy

Stem cell therapy, acupuncture, and massage therapy are all becoming popular aliments used to treat dogs that suffer from osteoarthritis symptoms. While most osteoarthritic treatments are the same for humans and dogs, stem cell therapy is only available for dogs. When anti-inflammatory agents are no longer improving the quality of life for arthritic dogs, stem cell therapy can be the next option. First discovered in 2005, by Dr. Brian Voynick of the American Animal Hospital, this therapy is an option for dogs with osteoarthritis or hip dysplasia. Stem cells are platelet rich plasma that can help inhibit the inflammatory process and repair damaged tissue.[33] Currently, several therapeutic regenerative strategies have investigated whether autologous mesenchymal stem cells (MSCs) have significant effects on regeneration and maintenance of articular cartilage.[33] Stem cell

therapy is based on the isolation of these cells from fat or bone marrow tissues and then after culture expansion, they are injected back to the patient's damaged joints.[33] Veterinarians claim that harvesting stem cells from fat is less invasive than a spay. A positive aspect to this therapy is that it is the dog's cells and therefore the risk of rejection is lower. Mesenchymal stem cells are responsible for releasing anti-inflammatory chemicals, which are speculated to repair damage in the joint.[33] However, little is known about the mode of action when injected into the damaged joint. While stem cell therapy is still relatively new and seems to have promising effects for treating and preventing osteoarthritis in canines, it is a relatively expensive treatment, averaging around $3,000, and results may vary from individual and severity of osteoarthritis.

Nutraceuticals and natural products

Pharmaceuticals have a high risk of toxicity and adverse side effects, because of this; there is push for alternative treatments in the form of food supplements. A nutraceutical is defined as a food, typically plant based, which provides medical or health benefits including the prevention and treatment of a disease.[13] Stephen DeFelice, MD, the founder and chairman of the Foundation for Innovation in Medicine, coined the word *nutraceutical* in 1989 from the words *nutrition* and *pharmaceutical*.[34] However, the use of food supplements to treat diseases dates back to Hippocrates, the father of medicine, (460-377 BC) when he predicted the health benefits of foods.[35] Since certain foods play an important role in maintaining normal functions in the human body, nutraceuticals are gaining popularity with health professionals and the public. Currently, there are over 470 nutraceuticals with documented health benefits.[13,15] Nutraceuticals are classified into two types; traditional foods and non-traditional foods. Traditional food is defined as natural, whole food with new information about potential health qualities. For example, omega-3 fatty acids in salmon and other seafood help reduce undesirable cholesterols. Non-traditional foods result from agriculture, crop and animal breeding or adding nutrients and ingredients to boost traditional food's nutritional value. Examples include orange juice that is fortified with calcium; milk fortified with vitamins; crops fortified with vitamins, minerals, and omega 3s. However, to date few focus directly on osteoarthritis.

Unlike pharmaceuticals, there are no FDA regulations for the health claims of nutraceuticals or non-traditional foods.[13] Even though there are few regulations on the health claims of nutraceuticals, safety must be assured in advance. Therefore, extensive, independent, testing must be reported on a nutraceutical before health professionals recommend it to their patients. During the research process, nutraceuticals can be classified as potential or established nutraceuticals. Nutraceuticals provide a promising approach towards a particular health or medical benefits, while established nutraceuticals have multiple, independent, peer-reviewed, research reports backing up their claimed benefits.[31,36]

Herbal medicine is increasing its popularity in veterinary medicine.[7] Popularity may be due to low cost and a belief it has minimal to no side effects. Herbal medicine is becoming a common treatment for mastitis occurrences, foot-and-mouth disease outbreaks, skin allergies, food poisonings, tympany, and expulsion of placentae. In the past, nutraceuticals were a common therapy for livestock in treating a variety of diseases including hepatitis, chronic heart disease, skin disorders, wounds, and arthritis.[24] Some nutraceuticals affect the progression of arthritis by preventing degradation and enhancing the repair of joint cartilage.[37]

Weight control, exercise, and physical rehabilitation

When treating osteoarthritis the main goals are to reduce pain and inflammation, improve joint function, eliminate or control the cause of arthritis, and even halt the process. Treatment can either occur through therapy or through medication. Osteoarthritis is more common in overweight dogs, so by putting the dogs on a strict diet to promote weight loss it can decrease mechanical stress that is placed on the joints. Obesity, and inactivity, which leads to obesity, can cause the joint to wear away faster due to extra pressure that is exerted on a joint.[1,38] According to the Arthritis Foundation, for every pound gained, three pounds of pressure are added to the knees and six pounds of pressure are added to the hips.[1,38] By incorporating a weight loss program into the treatment plan this can lower the amount of medication the dog will need to take. Females, older dogs, and specific breeds, such as Beagles, Dachshunds, Collies, and Labrador Retrievers, are more prone to obesity. Therefore, a diet plan should be enforced to prevent and control body weight. Diets should be high protein and low fat, with a negative energy balance. Along with strict dieting, a modified exercise plan should also be established for the dog. An exercise program can help in reducing weight while maintaining range of motion and muscle mass. Modified exercises, low-impact like walking or swimming, can also strengthen joint supporting structures, muscles, ligaments, tendons, and joint capsules.[1,7] Animal hospitals and rehabilitation facilities are starting to promote underwater treadmill therapy. This provides exercise with the lowest possibly impact due to the dog being in water. Overall, to create a successful treatment program the pet owner must be committed and willing to learn, the diet and exercise must be monitored, and post-diet weight monitoring by a veterinary should be done on a monthly basis.

Future perspectives

Currently, osteoarthritis is treated or managed by invasive as well as noninvasive means.[24] In the recent past, the treatment options for arthritis were typically NSAIDs given alone or in combination with other disease-modifying agents. NSAIDs (COX enzymes inhibitors) eliminate pain, but do not eliminate the signs and symptoms of active disease nor do they repair cartilage. In recent years, chronic use of NSAIDs has been linked to numerous side effects, including gastrointestinal (GI) bleeding, and renal and hepatic dysfunction. Anti-inflammatory drugs such as aspirin and ibuprofen are non-specific inhibitors of COX enzymes (COX-I and COX-II).[37] They inhibit the production of inflammatory prostaglandins, resulting in their therapeutic effect, but also inhibit the production of constitutive prostaglandins, resulting in side effects, such as GI bleeding.[37] Therefore, under these circumstances, a safe therapy is warranted for arthritic dogs. Nutraceuticals are also gaining popularity due to being readily available, inexpensive, and having minimal to no side effects.[34,37-41] Nutraceuticals, such as type-II cartilage, shilajit, 5-loxin, avocado/soybean unsaponifiables, and curcumin have gained immense popularity for their anti-arthritic and anti-inflammatory uses in humans and animals. However, with alternative medicine, there is no guarantee that the condition will improve; therefore, further safety and efficiency tests need to be performed to ensure the quality of these new treatments.

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The effect of short term rest after handling stress on electrocardiogram indices in goat

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Abstract

This study examined the effects of short term rest after handling stress on electrocardiographic parameters, and evaluated the probable effects of age and sex on them. It was performed on 40 clinically healthy pure Raini goats. The animals were divided into four groups consisting of 10 male kids (up to one year old, 15-20 kg body weight), 10 female kids (up to one year old, 15-20 kg body weight), 10 female adult goats (1-5 years old, 25-35 kg body weight) and 10 male adult goats (1-3 years old, 20-40 kg body weight). Five minutes after transporting animals to a standing stock, the electrocardiograms were obtained from each goat. Our results showed that the proportion of sinus tachycardia in stress was significantly ($P<0.05$) higher than other cardiac arrhythmia in this study. It seems that the insignificant change in heart rate as a result of short term rest was due to insufficient time to reduce the effects of handling stress, and it was concluded that cardiac arrhythmias observed in the clinically healthy Cashmere goats in stress periods could be accepted as the physiological arrhythmias, so no treatment is necessary.

Introduction

Skilled diagnosis of heart disease in livestock has improved in recent decades.[1,2] Electrocardiography (ECG) is a non-invasive diagnostic method for diagnosis of disturbances in the genesis and spread of the cardiac impulses.[3] ECG records and heart auscultation have been introduced as accurate and very useful tools for evaluation and comparison of cardiac function, diagnosis of cardiac arrhythmias and cardiac murmur in small ruminants.[3,4]

Goats, economically important producers of meat, hair and milk, have a high economic value in many countries.[5] Although the importance of obtaining normal values of ECG for specific breeds of animals besides the high variability in the ECG parameters in goats has been emphasized,[3,6] little studies have been done the effects of short term rest after handling stress on ECG parameters in healthy goats. Additionally, there is no previous study exploring the effects of age and sex on changes of ECG parameters during short term rest after handling stress in ruminants.

The Raini goat, one of the most famous breeds in Iran, is raised in large numbers in the Kerman province of Iran where goat production contributes significantly to the agricultural economy.[7] There is little information regarding Raini goat and to the best of our knowledge, there is no previous study regarding the effects of handling stress on ECG parameter in this valuable breed. This study was undertaken to evaluate the effects of short term rest after handling stress on ECG parameters, and to evaluate the probable effects of age and sex on them in this breed.

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Materials and Methods

This study was performed in July 2013 on 40 clinically healthy pure Raini goats, selected randomly from research farm of the Agriculture School of Shahid Bahonar University of Kerman, central Iran. The animals were divided into four groups consisting of 10 male kids (up to one year old, 15-20 kg body weight), 10 female kids (up to one year old, 15-20 kg body weight), 10 female adult goats (1- 5 years old, 25-35 kg body weight) and 10 male adult goats (1-3 years old, 20-40 kg body weight).

The animals were reared under the same husbandry conditions in the same group pen. Five minutes after transportation of animals to a standing stock (rest time), the electrocardiograms were obtained from each goat on a bipolar base apex lead using a single channel ECG machine (Cardiomax FX-2111, Fukuda, Japan) with a paper speed of 25 mm/s and calibration of 10 mm equal to 1 mV. The ECG was recorded when the animals were thought to be in a quiet standing position using an alligator-type electrode attached to the skin. The positive electrode of lead I (left arm) was attached to the skin of the fifth intercostal space just caudal to the olecranon and the negative electrode (right arm) on the jugular furrow about the lower 1/3 of the left side of the neck and the earth was attached away from these two electrodes.[1,2] Alligator clips were fixed to the skin after application of methyl alcohol.

A magnifying glass was used for analyzing and measuring ECG parameters. Using this method of measurement, the precision of duration and amplitude was 0.02 s. and 0.05 mV, respectively. The heart rate was calculated by measuring the average six R-R intervals of each trace. To describe the QRS complex, the first negative deflection was designated as Q, the first positive wave was named R and the negative deflection after R was designated S. If the QRS complex was a single negative deflection, it was described as the QS pattern.[1,2,8] In the case of biphasic P or T waves (-/+ or +/-), the amplitudes of two phases were summed.

Statistical analysis

Statistical analysis was performed using SPSS12 (Chicago, IL, USA). Comparisons of heart rate, waves' amplitude and duration, and P-R, Q-T and R-R intervals were performed using paired-samples t tests, while Chi-square test was used for comparison of QRS wave conformation, and Fisher exact tests were used for comparison of P and T waves' conformation. Differences were considered significant at $P<0.05$.

Results

The results of the measurement of the heart rate, besides amplitude, duration and configuration of ECG waves before and after short term rest in different genders and age groups of Raini goats are shown in Figure 1 and in Supplementary Tables S1 and S2.

The heart rates before and after rest was 108±4.7 and 108.3±4.6 beats/min, respective-

ly. There was no significant difference between the two periods in heart rates, and amplitude, duration and configuration of ECG waves, except for Q-T interval, which showed a marginally significant difference (P=0.09).

The average age of female goats was significantly higher than male goats (mean ± mean standard error: 2.39±0.38 and 1.44±0.15 years, respectively). Each sex was also evaluated separately. In male goats, the duration of P wave increased significantly (P=0.041) and so did the duration of R-R interval (P=0.036). In female goats, R-R interval significantly increased (P=0.046) and the heart rate showed a marginally significant decrease in second evaluation (P=0.08).

The goats were divided into two groups according to their maturity: kids (animals up to 1 year old) and adults (animals higher than 1 year old). Before rest, the heart rates of kids were significantly higher than adults (P<0.01), while the difference was not statistically significant after rest. Each group was evaluated separately. In kids group, Q-T interval had a marginally significant difference between the two periods (P=0.065), while in the other group, none of the measured parameters showed significant difference between the two periods.

The goats were divided into three groups according to their age, as: G_1<1 year, 1 year's ≤G_2<3 years and, G_3≥3 years. Before rest, the heart rate of G_1 was significantly higher than the other two groups (P<0.01) and heart rate showed significant difference between the two periods in the three groups (P<0.01).

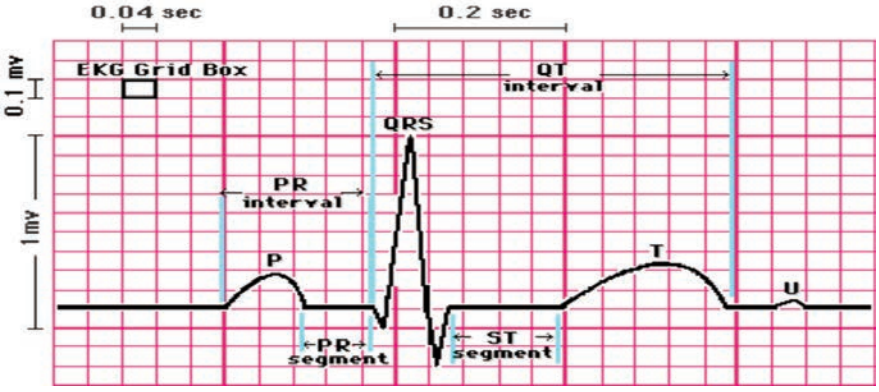

Figure 1. Electrocardiogram profile (comprising: waves amplitude and duration, and duration of P-R, Q-T and R-R intervals) in base apex lead system (paper speed 25 mm/s, sensitivity 10 mm/mv).

Discussion and Conclusions

Electrocardiography and auscultation, as non-invasive diagnostic methods, have been introduced as the preferred method for evaluation of electrical and mechanical activity of heart and diagnosis of cardiac dysrhythmia and cardiac murmur.[1,2] Base apex lead has been proposed as the best and standard lead for monitoring cardiac arrhythmias in large animal medicine and is used routinely.[1,2] There are a few previous studies regarding the normal ECG parameters in healthy goats and to the best of our knowledge, there is no previous study exploring the effect of handling stress on ECG parameters in small ruminant.[5]

According to our results, the mean heart rate in Raini goats was 108 beats/min which was within the reported normal range of goats by Smith (70-110 beats/min for adult goats and 120-160 beats/min for kids)[9] and was higher than the reported normal range by Radostits *et al*. (70-90 beats/min for goat).[10] Sinus tachycardia denotes an increase in heart rate that is initiated by the sinoatrial node. The term sinus tachycardia is caused by detectable influences such as pain, excitement, stress, pregnancy, exercise, hyperthermia, decrease in arterial blood pressure and administration of adrenergic drugs.[10] The heart rate returns to normal when the influence is removed or relieved. Stress has been proposed as an important reason for tachycardia and our results showed that the proportion of the sinus tachycardia in stress was significantly (P<0.05) higher in this study.[11,12] It is well known that stress increases in plasma corticoid concentrations,[13,14] which are regulated by the corticotrophin-releasing factor (CRF) that is secreted by the hypothalamus in response to stress.[15] Corticotrophin-releasing factor acts at additional sites in the central nervous system to stimulate sympathetic noradrenergic outflow to the heart rate and inhibit cardiac parasympathetic nervous activity, resulting in increased heart rate.[16-18] It seems that the insignificant change in heart rate due to short term rest was due to insufficient time to reduce the effects of handling stress. Because there were no clinical signs of cardiac problems (edema, jugular distension or pulsation) in evaluated animals, this cardiac rhythm irregularity could be categorized as physiologic arrhythmias.[19]

A heart rate higher than 120 and 90 in goats up to 1 year old and higher than 1 year old, respectively, has been considered as sinus tachycardia.[10] In the current study, 31.6% of Raini goats in kid animals (up to one year old) and 64% of adult animals (over 1 year old) had a heart rate higher than 120 and 90, respectively, and could be considered as sinus tachycardia (47.2% of all examined animals). It may be suggested that the higher heart rate of kids might be due to stress and excitation caused by isolation of kids from their dams. Sinus tachycardia has been reported as the most common cardiac arrhythmia in newborn Iranian fat-tailed lambs.[20] We found no clinical sign of heart problem or cardiac insufficiency in any of the animals with cardiac arrhythmia. Pourjafar *et al*. suggested occurrence of sinus tachycardia, sinus arrhythmia and sinoatrial block without appearance of clinical signs of heart problem in goats as the physiologic cardiac arrhythmias.[20]

No significant change in ECG parameters (configuration, amplitude and duration of ECG waves) was observed, except Q-T interval, in the current study. It seems that short term rest in our study was not sufficient to remove the effect of handling stress on ECG parameters. Although we found no specific cause for Q-T interval change, it seems that the short term rest was sufficient to affect ventricular depolarization-repolarization interval.

Sinus arrhythmia was the other common arrhythmia seen in this study. The observation of ECGs revealed that the sinus arrhythmia was associated with stress which is similar to the result of Pourjafar *et al.*,[19] Rezakhani and Khajedehi studies.[21] The animals in stress period showed this arrhythmia more frequently.[22] Sinus arrhythmia is a normal physiologic arrhythmia that occurs at slow resting heart rates and is associated with variation in the heart rate. This arrhythmia has been detected in anorectic cattle,[23,24] clinically healthy Najdi goats and late pregnant sheep,[19,20] but has not been reported in piglets during handling stress.[12] None of the animals with sinus arrhythmia in this study had obvious clinically systemic disease, pregnancy or anorexia. Low vagal tone (high sympathetic tone) could be suggested as the cause of this arrhythmia in these animals.[1,2] Furthermore, stress is a time of hemodynamic, hormonal, and catecholamine fluctuations, which may provoke cardiac arrhythmias.[25]

Sheep and goat ECG parameters in stress are of interest to researchers and there are a

few previous studies exploring this issue.[21] Although hearing cardiac dysrythmia is possible in horses and cattle with low heart beat, in small ruminant with high heartbeat, due to small size of heart, the heart sounds have little value in diagnosis of heart arrhythmia.[26]

It was concluded that the cardiac arrhythmias observed in the clinically healthy Raini goats in stress periods could be accepted as the physiological arrhythmias, so no treatment is necessary. Furthermore, the results of the present study showed that the physiological cardiac arrhythmias in Raini goats might be increased coincidentally with stress and showed little effect of sex and age on ECG parameters in base-apex lead in Raini goats during stress period. Additionally, sufficient time to rest should be considered before taking ECG to remove the effects of handling stress.

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Impact of vitamin C on concentrations of thyroid stimulating hormone and thyroid hormones in lambs under short-term acute heat stress

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Abstract

The present study evaluated the effect of vitamin C on alteration in thyroid hormones induced by short-term acute heat stress. Eight male lambs were divided into two groups of 4 animals each. Both groups were placed in an environment with hyper-acute heat stress based on the temperature – humidity index (THI). Groups I and II were injected intramuscularly normal saline and vitamin C (20 mg/kg), respectively, for the first five consecutive days of the experiment. All lambs were fed *ad libitum*. Blood samples were collected from both groups on days one, two, four, six and eight. Thyroxine and free thyroxine numerically increased (91.03 *vs*. 70.78 nmol L-1, P=0.080 and 29.8 *vs*. 24.8 pmol L-1, P=0.080; respectively) in heat stressed lambs supplemented with vitamin C compared to control group. Respiration rates and heart rates were elevated until day five of the experiment and then decreased. Mechanism for increasing the levels of thyroxine and free thyroxine by vitamin C is not well known. However, it may occur in part because of vitamin C antioxidant properties. The present study revealed that vitamin C might ameliorate the adverse effect of heat stress in lambs.

Introduction

Heat stress is a main issue in the welfare of livestock that can induce profound effects on metabolism of energy in sheep. Heat-stressed animals are presumed to increase maintenance requirements because of enhanced energy consumption for heat loss via panting and sweating.[1] The thyroid hormones regulate basal metabolism in various tissues by affecting the metabolism of lipids and carbohydrates.[2] Thyroid gland is regulated by the release of thyroid stimulating hormone (TSH) from the anterior pituitary gland. TSH production and release is under the influence of thyrotropin releasing hormone (TRH) from the hypothalamus.[3] The activity of TRH cells can be influenced by the temperature regulation center that integrates the environmental temperature.[4] It has been shown that heat stress in sheep alters TSH secretion and triiodothyronine (T3) and thyroxin (T4) concentration in serum.[5] Heat stress has been implicated in promoting oxidative stress either through excessive production of reactive oxygen species (ROS) or decreased antioxidant defenses, including vitamin C.[6] Moreover, a drop in vitamin C concentration has been reported in heat-stressed lactating cows,[7] pigs[8] and chickens.[9] On the other hand, vitamin C supplementation improves feed intake and growth rates in heat-stressed birds.[10] Domestic animals like sheep are believed to meet their vitamin C requirements in normal conditions from synthesizing it in liver.[11] It has been demonstrated that heat stress induces oxidative stress and vitamin C as an important water-soluble antioxidant might reduce the adverse effects of heat stress.[12] Based on our knowledge, the vitamin C and heat stress impact on thyroid hormone levels has not been clarified. This investigation was designed to examine the possible effect of vitamin C on the level of plasma thyroid hormones and TSH in clinically healthy male Iranian lambs under short-term acute heat stress.

Materials and Methods

This study was performed in summer 2010 in the Animal Husbandry Unit of The Faculty Of Agriculture, Birjand University, Iran. Birjand as a semitropical area is located in the east of Iran, (32°52'26" north latitude, 59°12'52" east longitude, and average elevation of about 1445 meters above sea level). Birjand has a dry climate with significant difference between day and night temperatures. On average, the warmest month is July and June is the driest month.[13] The animals were placed under controlled temperature condition. This experiment was accomplished under the approval of the state committee on animal ethics, Birjand University, Birjand, Iran (10/90-2-R-A/BU). In addition, we used the recommendations of European Council Directive (86/609/EC) of November 24, 1986, regarding the standards in the protection of animals used for experimental purposes. The present experiment was carried out on eight healthy Iranian male lambs about 4-5 months of age. Lambs were fed hay, mainly alfalfa and concentrate according to NRC (1985).[14] All animals were treated against internal and external parasites by Albendazole (Damloran Company, Borujerd, Iran) 10 mg/kg, and Ivermectin (Razak Company, Tehran, Iran) 0.22 mg/kg, 30 days prior to the study. After two weeks of acclimatization to the indoor facility, the lambs were allocated randomly to heat stress conditions induced by diesel heater. The temperature was monitored with a thermocouple thermometer. Animals were divided into two equal groups: i) control group (normal saline); ii) treatment group that received vitamin C (20 mg/kg) intramuscularly in five consecutive days. Animals were subjected to heat stress (40±2°C) between 7.00 and 15.00 for 8 days and for the remaining time kept at 28°C. Relative humidity of the room was measured three times daily and the mean of the measurements was used for the calculation of the temperature-humidity index (THI). THI was calculated according to the formula reported by some researchers.[15,16] Physiological parameters [rectal temperature (RT), heart rates (HR) and respiration rates (RR)] were recorded daily and then blood samples were collected

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from the jugular vein between 10.00 and 11.00 on days 1, 2, 4, 6, and 8 and serum or plasma was separated by centrifugation at 750 *g* for 15 min and stored at –20ºC. Determination of serum T3 was carried out by the microplate enzyme immunoassay method (Monobind Inc, Lake Forest, USA). Serum T4 concentration was measured using a competitive enzyme immunoassay kit (Monobind Inc, Lake Forest, USA). Serum free triiodothyronine (fT3) and free thyroxine (fT4) concentrations were determined by the fT3 and the fT4 ELISA kits (Monobind Inc, Lake Forest, USA). TSH was measured using a sheep TSH ELISA Kit (My Bio Source Company, San Diego, CA, USA). The intra- and inter-assay coefficient of variations of the assays for the mentioned parameters were (12.6% and 13.2%; T3), (3.0% and 3.7%; T4), (4.1% and 5.2%; fT3) and (4.5% and 3.7%; fT4) respectively. The sensitivity of the tests was (0.2 ng/mL; T3), (0.4 mg/dL; T4), (0.05 pg/mL; fT3) and (0.05 ng/dL; fT4) respectively. The data were expressed in SI units and analyzed by repeated measurements ANOVA and t-test using SPSS/PC software (version 18). $P<0.05$ was considered significant and all values were expressed as mean and standard error (SE).

Results

The severity of heat stress was estimated using THI formula.[15,16] Under heat stress condition the obtained value of THI was in the range of 23-26, indicating severe heat stress. RT, HR and RR are presented in Table 1. Mean (±SE) of RT was 39.6 (±0.5) and 39.6 (±0.03) °C in treatment and control lambs respectively. The HR was numerically lower in the treatment than control lambs (96.5±3.2 *vs* 101±3.3 beats/minutes respectively). RR under heat stress did not vary significantly between treat-

Table 1. Recorded vital signs during the experiment in control and treatment groups of healthy male lambs under experimental induced heat stress (mean ± standard error).

Variable	Rectal temperature, °C	Heart rate, beats/min	Respiratory rate, breaths/min
Control (n=4)			
Day 1	39.6±0.11	69±1.9	55±6.4
Day 2	39.8±0.02	94±5	61±3
Day 3	39.6±0.09	97±10.8	61±4.4
Day 4	39.5±0.2	108±3.6	65±10.8
Day 5	39.6±0.1	120±1.6	68±12.5
Day 6	39.6±0.02	114±8.4	52±4.3
Day 7	39.7±0.04	108±7.3	54±1.1
Day 8	39.6±0.02	91±5.7	51±3
Mean±SE	39.6±0.03	101±3.3	58.37±2.3
Treatment (n=4)			
Day 1	39.4±0.17	65±5.2	45±5.2
Day 2	39.6±0.19	80±7.6	59±1.9
Day 3	39.6±0.02	92±1.6	61±5.7
Day 4	39.7±0.08	109±5.7	65±2.5
Day 5	39.8±0.12	115±1.9	67±7.8
Day 6	39.7±0.13	104±4.6	60±3.2
Day 7	39.7±0.22	109±7.1	61±3.4
Day 8	39.6±0.22	98±1.1	52±2.8
Mean±SE	39.6±0.5	96.5±3.2	58.43±1.81
P-value	0.91	0.21	0.98

SE, standard error.

Table 2. Thyroid hormones concentrations and thyroid stimulating hormone in control and treatment group of lambs under experimental induced heat stress (mean ± standard error).

Variable	T4, nmol/L	T3, nmol/L	fT4, pmol/L	fT3, pmol/L	TSH, mIU/L
Control (n=4)					
Day 1	74.65±7.8	2.81±0.81	24.77±1.92	0.03±0.0	0.22±0.05
Day 2	75.08±5.9	2.60±0.54	24.93±0.96	0.03±0.0	0.17±0.08
Day 4	62.21±7.5	2.17±0.59	23.48±1.6	0.03±0.0	0.12±0.0
Day 6	68.21±11.6	2.95±0.78	25.8±2.78	0.03±0.0	0.17±0.06
Day 8	73.79±10.6	2.94±0.80	25.09±2	0.03±0.0	0.16±0.11
Mean±SE	70.78±3.53	2.69±0.28	24.81±0.79	0.03±0.001	0.17±0.02
Treatment (n=4)					
Day 1	88.15±5. 8	2.94±0.47	30.88±1.89	0.06±0.02	0.27±0.13
Day 2	91.37±11.9	2.77±1.01	29.17±2.38	0.04±0.01	0.19±0.09
Day 4	91.37±7.3	2.72±0.38	29.6±2.03	0.03±0.01	0.12±0.05
Day 6	92.02±3.5	3.12±0.26	28.95±0.64	0.04±0.01	0.19±0.07
Day 8	92.34±4.1	3.55±0.35	30.24±0.83	0.03±0.0	0.25±0.14
Mean±SE	91.03±2.6	2.99± 0.21	29.8±0.66	0.04±0.005	0.2±0.04
P-value	0.08	0.75	0.08	0.56	0.98

SE, standard error; T4, thyroxin; T3, triiodothyronine; fT4, free thyroxin; fT3, free triiodothyronine; TSH,-thyroid stimulation hormone. Treatment group received vitamin C in 20 mg/kg; control group received equal amount of normal saline.

ment and control lambs, and averaged 58.43 (±1.81) (S.E.) and 58.37 (±2.3) breaths/minute respectively. After the induction of heat stress, HR and RR started to increase to a peak (day 5 of the experiment) and then decreased, while alterations of RT were in a narrow range (Figure 1). Results of thyroid hormones and TSH concentration tests are summarized in Table 2. Thyroid hormones and TSH concentrations were numerically higher in treatment than control lambs. The higher levels of T4 and fT4 tended to be significant (P=0.08 for both of them). Average level of T4 was 91.03 (±2.6) (S.E.) and 70.78 (±3.53) (nmol/L) respectively, and average level of fT4 was 29.8 (±0.66) (S.E.) and 24.81 (±0.79) (nmol/L) in treatment and control lambs respectively.

Discussion

In the present study, average RT was the same (39.6 C) in both groups of control and treatment lambs. Sheep were able to maintain their body temperature within a narrow range, even when exposed to elevated temperatures. The increase in RR or panting is the most obvious reaction to heat stress.[17,18] During heat stress, the HR was significantly increased to a peak until day 5 of the experiment and then decreased. To combat heat stress, cardiac output and as a result blood flow to the skin increases as well as visceral organs. The same trend was observed in respiratory frequency as an indicator of heat stress by changing the rapid shallow breathing to slower deeper panting.[17] Our finding was in agreement with the findings of Hales and Webster (1976).[19] In the present study, coping with heat stress and vitamin C injection did not result in significant changes in the thyroid hormones and TSH concentrations, although T4 and fT4 tended to significantly increase with treatment by vitamin C injection (P=0.08 and 0.08 respectively). Thyroid hormone levels in serum tend to be lower during the summer months.[20] Moreover, many investigators reported a significant depression in thyroid gland activity under heat stress conditions.[5,20,21] Oxidative stress has an impact on thyroid physiologies[22] and vitamin C has an important function as an antioxidant due largely to its redox properties.[23] In addition, its role in cell respiration, synthesis of adrenocorticotropic hormone (ACTH) and metabolism of some other vitamins and amino acids has been revealed. Vitamin C is an essential vitamin for many normal physiological functions in humans and animals including immune functions, collagen formation, and sparing action of other vitamins (vitamin A, E and some B-complex) from oxidation.[24] Many peptide hormones require vitamin C in their synthesis. Vitamin C is also required for the hydroxylation reactions in the synthesis of corticosteroid hormones.[25] Thyroid hormones are of importance in the heat adaptation process.[1] Sivakumar *et al.*[26] state that the thyroid hormone (free T3 and free T4) levels were decreased in heat-stressed goats in an attempt to reduce metabolic rate and heat production.

Mechanism for increasing the levels of T4 and fT4 by vitamin C is not well known. However, it may occur in part because of vitamin C antioxidant properties. Endogenous vitamin C production may be insufficient under some conditions such as heat stress. The present study indicates the likely beneficial effect of injection of vitamin C on adaptation mechanisms against heat-stressed lambs. A limitation of this study was the small num-

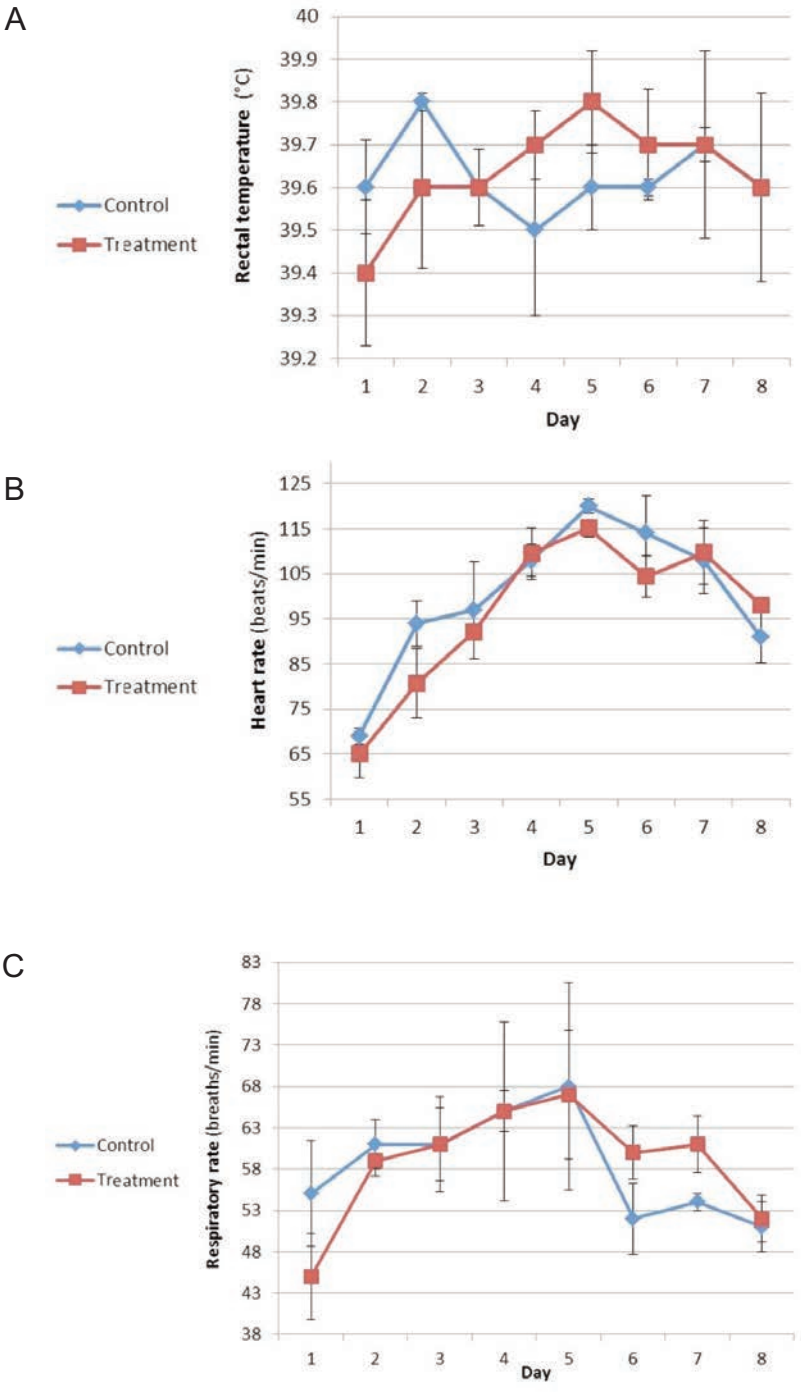

Figure 1. Vital signs of healthy male lambs under heat stress are shown (mean ± standard error). A) rectal temperature (RT); B) heart rate (HR); C) respiration rate (RR).

bers of lambs used as control and treatment group. However, more research is needed to investigate the efficacy of vitamin C in cases of heat stress.

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Prevalence of opportunistic fungi and their possible role in postpartum endometritis in dairy cows

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Abstract

The aim of this study was to identify fungal infections by culture from uterine lavages of 172 Holstein dairy cows between 25 and 35 days postpartum and two weeks later. In the first examination, 61/172 (35.5%) cows were suffering from clinical endometritis. The positive rate of fungal growth was fifteen (8.7 %) swabs and the remaining 157 (91.3%) showed no fungal growth. The most frequently isolated fungi were *Aspergillus* spp. (60%) followed by *Penicillium* spp. (26%) and Yeast (13%). In the second examination, 20/128 (16%) cows showed endometritis. Nine (5.5%) swabs were fungal positive. No significant differences between cows with positive and negative fungal cultures in the percentage of polymorphonuclear leukocytes of cytological samples were seen. In conclusion, treatment of cows affected with postpartum endometritis with intrauterine infusion of oxytetracycline, hygiene of bed, number of cows in one yard, age and parity of cows may cause increase in incidence of mycotic endometritis.

Introduction

Endometritis is an inflammation limited to the endometrium without clinical signs.[1] Physical damages to birth canal during parturition could result in an upsurge of microbial infections in the cow.[2] During the first weeks after parturition, immune responses of cows eliminate the microbes. But up to 40% of animals still have a bacterial infection three weeks after calving.[2,3] Also, the fungi are capable of infection in the uterus in cows.[4,5] Increased time of pregnancy and lower conception rates occur after uterine infections.[1,6] There have been many studies regarding pathogenic bacteria that showed their important role in occurrence of endometritis. But fewer surveys have been done on fungal infections of the postpartum uterus in dairy cows. Fungal infections of genital tracts are becoming more common because of indiscriminate use of antibiotics and hormonal therapy.[4] The aim of this study was to identify fungal infections by culture of uterine lavages in Holstein dairy cows between 25 and 35 days postpartum and two weeks later.

Materials and Methods

Animals

The study was carried out in a large commercial dairy farm near Shiraz, Fars province, in the south of Iran (29°58 34 N, 52°40 45 E). One hundred and seventy two postpartum dairy cows (1st and 2nd calving) were examined twice, between 25 and 35 days postpartum and two weeks later. The farm milked 1900 Holstein cows three times daily. Cows were housed in freestall barns with mat bedding for primiparous and sand bedding for multiparous cows. Cows calved throughout the last year and the herd had annual average milk yields of 8800 liters per cow. The cows received corn silage, alfalfa hay and concentrates (containing corn meal, soybean meal, vitamins and minerals). The cows were maintained in close-up dry group for three weeks before calving. The cows calved in an open shed barn. Fresh cows were kept in a transition group for one month. None of the cows received any intrauterine or reproductive hormonal therapy for at least 14 days before sampling. All cows were examined once between 25 and 35 days postpartum and reexamined in the next 14 days. In the first examination, samples were obtained from 172 cows and in the second examination samples were obtained from 128 cows in 2011 and 2012 during the winter season. After examinations, Prostaglandin F2 and Oxytetracycline (OTC) were administered to all cows with clinical endometritis.

Clinical examination

During examination, the cow's vulva was thoroughly cleaned and disinfected using a Savlon (chlorhexidine and cetrimide) solution, also lubricated, gloved hand was inserted through the vulva and the mucus contents of the cranial vagina were withdrawn manually for examination. The vaginal mucus was assessed regarding its color and proportion of pus. Endometritis was classified into three categories: clear mucus with flakes of pus (E1), mucopurulent discharge or fluctuating contents in the uterus (E2), and purulent discharge with or without palpable contents in the uterus (E3) (7). Then, cows were classified into 2 groups: healthy (vaginal discharges score ≤E1) and endometritis affected (vaginal discharges score ≥E2).[8]

Uterine samples collection

Uterine secretion samples were collected as follows: cows were restrained and the perineum area was cleansed and disinfected using a Savlon (chlorhexidine and cetrimide) solution. Sterile covered plastic infusion pipettes (pipettes were first autoclaved and then put on inside plastic sheaths, UV light was used to sterilize covers) were inserted into the cranial vagina and passed through the caudal cervix. The sheath was subsequently ruptured and the sterile pipette tip was manipulated through the cranial cervix into the uterus. A total of 60 mL of sterile saline solution was injected into the uterus, agitated gently, and a sample of the fluid was aspirated. The volume of recovered fluid ranged from 2 to 5 mL. Samples were maintained in ice prior to laboratory processing.[9]

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Cervical sample cytology

Cytological samples were obtained from the discharge of cervical mucus. Cervical aspirated samples were collected by gentle suction from the cervical external os with a plastic uterine pipette and aspirated by suction with a 50 mL syringe. Once the samples had been taken, the swabs were rolled on glass slides. Thin smears were prepared for cytological examination by smearing a drop of cervical mucus on a clean slide. The smears were then allowed to dry at room temperature for 30-35 minutes. Slides were transported to the laboratory and examined within two hours of collection. A differential cell count of each smear was done on Giemsa-stained slides. One hundred to 200 cells were counted in each of 20 microscopic fields (×900). Recorded cell types were epithelial, large vacuolated epithelial, neutrophils, lymphocytes and macrophages.[10]

Fungal culture

Fungal isolation Sabouraud's Dextrose agar (SDA) spot inoculation technique was employed. The samples were inoculated against SDA and incubated at 25°C for 2 weeks. Chloramphenicol was used in the agar media for initial fungal isolation. Duplicate culture was used for each sample. The cultures were examined daily for any mycobiotic growth during the incubation period. Visual examinations of the fungal colonies were made, and their colonial morphology or characteristics, such as texture, pigment and rate of growth on media, were recorded. For microscopic examination, the fungal culture was stained as wet monut with lactophenol cotton blue stain. Identification of fungal agents was made on the basis of colony characteristics and staining reaction was observed under the microscope.

Statistical analysis

In the first and second examinations, percentage of cows with and without endometritis for fungal culture results was statistically analyzed with the Chi-square test using SPSS (SPSS for Windows, version 11.5, SPSS Inc, Chicago, IL, USA). Comparison of the neutrophil percentages between different groups of studied cows for fungal culture was done by Independent-Samples T-test at the first and second examinations. Data presented as the number (percentage) and probability values of $P \leq 0.05$ were considered statistically significant.

Results

In total, 172 Holstein cows were selected and sampled at 25-35 days postpartum. Of these, 128 cows were sampled again, two weeks later. In the first examination (25 to 35 days postpartum), assessment of vaginal mucus showed 61 out of 172 (35.5%) cows were suffering from clinical endometritis (vaginal score ≥E2). Fifteen (8.7%) swabs were found fungal positive and the remaining 157 (91.3%) showed no fungal growth (Table 1). In the first examination, the most frequently isolated fungi were *Aspergillus* spp. 9 (60%) *Penicillium* spp. 4 (26%) and Yeast 2 (13%) respectively (Table 2).

In the second examination (39 to 49 days postpartum) 20 out of 128 (16%) cows showed endometritis. Among them, 9 (5.5%) swabs were fungal positive (Table 1). All 9 swabs were collected from clear mucus in the second examination.

In the first examination, 10 cases were affected by endometritis and treated by intrauterine Oxytetracycline. But 5 cases were clean and did not receive any intrauterine infusion. In the first examination, 10 out of 15 positive fungal were in the first parity cows and just one case was positive, yet in the second examination all fungal agents were isolated from healthy cows (Table 1).

In the first examination, corpus luteum (CL) was presented in five cases of positive fungal culture. In the second examination, four positive fungal culture cases had corpus luteum.

During 100 days after calving, 45.2% (71/157) of negative fungal culture cows became pregnant. Among positive fungal culture cows, 40% (6/15) of cases became pregnant during 100 days postpartum ($P>0.05$) and the other 9 cases were open in this period.

In the second examination, five out of nine positive fungal culture cows received intrauterine OTC in the first examination.

The number (percentage) of the negative and positive fungal cultures at the first and second examinations of healthy and endometritic cows is shown in Table 1. In the first examination, endometritic cows had a significantly higher rate of infection to fungi compared to healthy cows ($P<0.05$).

The results showed that 39.1% of cows with positive fungal cultures needed ≥3 inseminations and 25.4% of cows with negative fungal

Table 1. Number (%) of the fungal negative and positive culture at the first and second examination for healthy and endometritic cows.

	Healthy cows (%)	Endometritic cows (%)
First exam		
Negative	105 (94.6)	52 (85.2)
Positive	6 (5.4)[a]	9 (14.8)[b]
Total	111 (100)	61 (100)
Second exam		
Negative	99 (91.7)	20 (100)
Positive	9 (8.3)	0 (0)
Total	108 (100)	20 (100)

[a,b]Values within row having different superscripts differ significantly ($P<0.05$).

Table 2. Number of isolated fungi in the uterine lavage at the first and second examination.

	First examination		Second examination		Total
	Affected cows	Healthy cows	Affected cows	Healthy cows	
Aspergillus spp.	2	7	0	1	10
Penicillium spp.	3	1	0	0	4
Cladosporium spp.	0	0	0	3	0
Scopulariopsis spp.	0	0	0	2	2
Rhizopus spp.	0	0	0	1	1
Yeast	1	1	0	2	4
Total	6	9	0	9	24

cultures were conceived after three or more inseminations (Table 3; P=0.17), however, this difference was not significant.

The results of cytological change percentages of PMNs (mean ± SD) of the negative and positive fungal cultures at the first and second examination are shown in Table 4. There was no significant difference between cows with positive and negative fungal cultures in the first and second examination (P>0.05).

Discussion

The uterine lumen was sterile before parturition. After parturition, the microorganisms inflow from the animal's environment, skin, and feces to the uterine lumen.[11] The fungi can invade tissues and cause clinical infections.[4] About 100 species of fungi are generally identified as pathogens of humans and animals.[4] In the present study, six different genera, *Aspergillus*, *Penicillium*, *Cladosporium*, *Scopulariopsis*, *Rhizopus* and Yeast were isolated from healthy and endometritic cows (Table 2). The isolation of fungi in the first examination was higher than the second (8.7% *vs* 7%); however, this difference was not significant (P>0.05). In the first examination the growth of fungi in endometritis affected cows was higher than healthy cows. But in the second examination there was no isolation of fungi in the endometritic cows and there were nine isolations in the healthy cows. Verma *et al*. reported mycobiotic agents from 27.8% and 33.3% of endometritic buffaloes and cows, respectively.[4] Contamination of fungi in endometritic and healthy cows was 39.34% and 28.57% respectively.[12] Sharma and Singh found 15.5% mycotic isolation in repeated breeder cows.[13] Ramsingh *et al*. have reported that out of 168 uterine discharge samples from repeated breeder endometrtic cows, a total of 168 (10.5%) mycotic isolates were recorded and identified.[14] In our study, there was a lower prevalence of mycobiotic agents. This may be because of the hot and dry climate of Shiraz. The cows in our farm were housed in mat or sand bed. However, the examined cows in the present study were young and have greater ability to clean their uteri from contaminations. Other effective factors are the density of animals and topographic variations.[4]

Aspergillus spp. were the most important fungi isolated from all samples (10 from 24) which means from samples in first examination (9 from 15) and from endometritis affected cows (7 from 9). According to the survey of Verma *et al., Aspergillus* spp. was reported as the most important mycobiotic agent, 43.7% (7/16), among endometritic cases in cows and buffaloes.[4] Also, *Aspergillus* spp., *Penicillium* spp. and *Cladosporium* spp. were the highest isolated fungi from vaginal mucus of cows with complicated puerperium.[15] In another survey, *Penicillium* spp. and *Candida albicans* were the most common isolated fungi. *Penicillium* spp. was more commonly isolated from uterus of cows with reproductive diseases and *Candida albicans* was more frequently isolated from healthy uterus.[12]

Cows with lower rate of infected uteri to fungi get pregnant during a 100 day period and a higher percentage of them require three or more inseminations to become pregnant, although it was not significant. Vlcek *et al*. found increase of the insemination interval, service-period and insemination index in cows that yielded pathogenic and potentially pathogenic micromycetes.[15]

No significant difference between cows with positive and negative fungal cultures in the first and second examinations (P>0.05) confirmed that the immune system did not respond to the exposure of uterus to fungi. This issue is reported for the first time and to the best of the researchers' knowledge there have been no reports regarding this subject. No significant difference between conception in positive and negative fungal cultures may indicate that the immune system in reproductive tract of cow with positive fungal culture was unaffected. In the second examination, there were five cows with contaminated uteri to fungi that received intrauterine OTC in the first examination. It is suggested that use of intrauterine antibiotics can cause fungal infections.[4,14] Antibiotic therapy, especially from intrauterine route, or corticosteroid therapy, might eliminate bacterial agents from the reproductive tract but produce immunosuppression and trigger fungal infections.[4]

Table 3. Number (%) of cows that had fungal negative and positive culture in both exams based on the service per conception.

Service per conception	Fungal culture	
	Negative (%)	Positive (%)
≤2	103 (74.6)	14 (60.9)
≥3	35 (25.4)	9 (39.1)
Total	138 (100)	23 (100)

Table 4. Comparison of cytological change of neutrophils percentages (mean ± SD) of the fungal negative and positive culture at the first and second exam.

Fungal culture	Examination	
	First exam	Second exam
Negative	11.3±14.3 (142)	8.2±13.6 (135)
Positive	11.6±9.3 (13)	4.1±3.2 (9)

Conclusions

In conclusion, treatment of cows affected by postpartum endometritis with intrauterine infusion of antibiotic such as OTC, hygiene of bed, number of cows in one yard, age and parity of cows may increase incidence of mycotic endometritis. The clinical signs of endometritis may be not found in cows that were affected by mycotic endometritis.

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Electrocardiographic indices of clinically healthy Chios sheep

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Abstract

Information regarding normal electrocardiographic features of different breeds of animals can help veterinarians to detect any abnormalities in cardiac electrical activities. The current research was conducted to present the normal electrocardiographic indices of clinically healthy Chios ewes and lambs. The electrocardiograms were recorded from clinically healthy Chios ewes (n=27; 2-3 years old) and lambs (n=20; 4-6 months old) by using base apex lead system. T and QRS-duration in lambs were significantly lower than adult Chios ewes. The electrocardiographic amplitudes in lambs were lower than ewes, non-significantly. P-R, R-R, Q-T and S-T intervals in Chios lambs were significantly lower than ewes. No normal sinus rhythm was detected in Chios lambs. The proportion of sinus arrhythmia and sinus tachycardia in lambs was significantly more than ewes. Sino-atrial block was also detected in lambs. Based on the presented data it could be stated that aging can affect electrocardiographic findings of Chios sheep. Finally, our results will provide a good basis for judging the electrocardiograms in base apex lead system of Chios lambs and ewes.

Introduction

The heart electrical activities can be recorded over a period of time by electrocardiography, as a non-invasive procedure. The heart beats caused from depolarization and repolarization of the myocardium, which are detectable by electrocardiography and finally record and produce electrocardiograms (ECGs). Recording ECG in large animals has several indications such as evaluating the heart rate and rhythm, size of cardiac chambers, myocardial damages, electrolyte imbalances and drugs side effects.[1]

There are several electrocardiographic lead systems in large animals to record ECGs contain bipolar (I, II, III, base-apex, X, Y and Z of the orthogonal lead system) and unipolar leads (aVF, aVR, aVL, thoracic); but the recorded waves by these leads in each animal's breed, size, body type and sex are different from others and these factors should not affect the ECGs.[2] Base apex lead in large animals has not the complications in other leads and can record the clear and large waves and animal movements have minimum effects on the recordings.[1,3]

The Chios is known as a breed of semi-fat tailed domestic sheep which originated from the Greek island of Chios and they are commonly bred for their milk production.[4] Several researchers studied the electrocardiographic parameters in clinically healthy sheep and goats by base apex lead and leads other than the base apex;[5-9] but according to the author's knowledge, the study on normal electrocardiographic indices of clinically healthy Chios sheep is lacking. Hence, the current study was performed to present and clarify the normal electrocardiographic characteristics of Chios lambs and ewes tracing by base apex lead system. The data presented here may be used as guideline to evaluate the cardiac electrical activities of this breed.

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Materials and Methods

The present study was performed in March 2015 on clinically healthy Chios ewes (n=27; 2-3 years old) and lambs (n=20; 4-6 months old), around Shiraz, Fars province, southwest of Iran. All animals were grazing in a green pasture with free access to water and shade. The animals were examined prior to ECG recordings and proved to be clinically healthy. None of the ewes and lambs used in this study had any clinical signs of heart diseases (edema, jugular distension or pulsation and cardiac murmurs), coughing and exercise intolerance.

The ECGs were recorded on a bipolar base apex lead, using limb lead I. Animals were kept standing without any sedation and minimum restraint, also. When animals got calm (decreased panting behavior and muscle tremors), the ECGs were recorded, using alligator-type electrodes which were attached to skin after cleaning it with ethanol and applying electrocardiographic jelly to improve skin contact. The positive electrode (left arm) was placed over cardiac apex in the fifth left intercostal space at the level of the elbow, the negative electrode (right arm) was placed in the left jugular furrow at the top of heart base, the neutral electrode (right foot) was used on the skin of thoracic inlet and the ground was placed on the dorsal spine or another site away from the heart.[1] All ECGs were obtained in a single channel electrocardiographic machine (Kenz-line EKG 110, Suzuken Co., Ltd., Japan) with paper speed of 25 mm/sec and calibration of 10 mm equal to 1 mV. The precision of duration was 0.02 second and amplitude was 0.05 mV. The ECGs were examined to evaluate normal electrocardiographic indices and cardiac arrhythmias, subsequently.

Mean and standard deviation (SD) were calculated for all studied electrocardiographic indices. Two independent samples t-test was used to detect statistical differences between ewes and lambs about similar electrocardiographic parameter. Fisher's exact test was used to detect any proportional distribution of arrhythmias between ewes and lambs. All data were analyzed by using SPSS software (SPSS for Windows, version 20, SPSS Inc, Chicago, Illinois). $P<0.05$ was considered statistically significant.

Results

The normal electrocardiographic indices of clinically healthy Chios sheep are presented in Tables 1 to 3. T and QRS-duration in lambs were significantly lower than adult Chios ewes ($P<0.05$; Table 1). The electrocardiographic amplitudes in lambs were lower than ewes, non-significantly ($P>0.05$; Table 2). P-R, R-R, Q-T and S-T intervals in Chios lambs were significantly lower than ewes ($P<0.05$; Table 3). No normal sinus rhythm was detected in Chios lambs. The proportion of sinus arrhythmia and sinus tachycardia in lambs was significantly more than ewes. Sino-atrial block was also detected in lambs (Figures 1 and 2).

Discussion

Electrocardiography can be used as a clinical method of choice to assess the cardiac problems regarding heart electrical activities and the initiation and conduction of waves of depolarization and repolarization.[3] There are several literature about electrocardiographic studies on different breeds of the small ruminants,[8,9] but information regarding the base apex electrocardiography of clinically healthy Chios sheep was lacking. The data in basic electrocardiographic indices of this breed could be used as reference values to assess heart electrical activities.

The base apex lead appears to be most useful in measuring conduction times (*i.e.,* durations of component deflections, intervals, and segments) because the origins and terminations of deflections could be identified easily.[3] It has been reported that the base apex lead gave the least variable ECG tracings in all the animals; furthermore, the P waves, QRS complexes, and T waves in the base apex lead had the highest mean amplitude of all the leads recorded.[3] It seems that our study on Chios ECG parameters in base apex lead system may be helpful in standardizing the base apex lead of this breed.

The conductive properties of the body mass of ruminants, attributable to the volume of the gastrointestinal tract, influence the distribution of body surface potentials comprising the ECG.[3] This may explain the differences among different ECG parameters between the different ages in our study (Tables 1 to 3). The gradual development of body mass may cause difficulty in reaching the waves to the body surface due to relative electrical insulation by increasing body mass and decrease of amplitude in adults.[10]

In the present study the electrocardiographic durations and intervals were increased significantly during aging. The transition time of heart electrical impulses produces durations and intervals. It may be stated that the smallest cardiac size and more superficial purkinje fibers in lambs in comparison with ewes can create lowest durations and intervals in younger ones. It could be suggested that as the mass of heart in larger animals became larger in the process of growth, the duration of transfer of cardiac electrical activity also increases.[11] The amplitude of P, R, S and T waves recorded in the studied sheep were not followed a specific pattern during aging. The changing of amplitudes presumably could be due to a high degree of synchronized ventricular polarization passing in any given direction. Furthermore, alterations between heart situation and attached positive electrode can change amplitudes. This may be also due to the presence of high degree of synchronization of depolarization of individual myocardial fibers. At the birth, significant hypoxemia and acidaemia may develop.[12] Moreover, after the onset of breathing, the change in pulmonary vascular resistance with expansion of the lungs results in a great increase in pulmonary blood flow, accompanied by a rise in left atrial pressure.[13,14] This generates distension of the atrial walls and stretching of the atrial muscles and these might be related to the occurrence of cardiac arrhythmias.[15] It was suggested that the development of hypoxia was relative to the arrhythmias. It was considered that a hypoxic condition during delivery may contribute to the occurrence of neonatal arrhythmias in horses.[16] Belenky *et al.*[17] demonstrated that the hypoxic carotid chemoreflex, in lamb, is present at birth, but has a significantly longer response time than later in the newborn period. Moreover, the CNS-mediated ventilatory response to hypoxia was also noted to be present in the newborn animal through at least 12 days or longer times of postnatal age.[17]

Cardiac arrhythmias also occur commonly in association with gastrointestinal disorders in the dairy cow and less commonly in the horse and resolve without specific antiarrhythmic treatment when the primary gastrointestinal disorder is corrected.[1] At birth the rumen is a rudimentary nonfunctional sac. Normal development of the rumen requires the establishment of a viable microbial population and the formation of volatile fatty acids.[18] Establishment of ruminal microbial fermentation begins between two to four weeks of age

Table 1. The durations (sec) of standard electrocardiographic indices (mean ± standard deviation) of base apex lead of clinically healthy Chios ewes and lambs.

Age groups	P-duration	T-duration	QRS-duration	S-duration
Lambs (n=30)	0.020±0.005	0.029±0.007	0.095±0.005	0.010±0.001
Ewes (n=30)	0.021±0.007	0.039±0.006	0.125±0.017	0.011±0.002
P-value	0.091	0.026*	0.019*	0.089

*Significant differences between groups at similar indices (P<0.05).

Table 2. The amplitudes (mV) of standard electrocardiographic indices (mean ± standard deviation) of base apex lead of clinically healthy Chios ewes and lambs.

Age groups	P-amplitude	T-amplitude	R-amplitude	S-amplitude
Lambs (n=30)	0.052±0.016	0.110±0.057	0.054±0.012	0.261±0.089
Ewes (n=30)	0.055±0.010	0.122±0.050	0.059±0.018	0.272±0.065
P-value	0.162	0.132	0.092	0.076

Table 3. The intervals (sec) of standard electrocardiographic indices (mean ± standard deviation) of base apex lead of clinically healthy Chios ewes and lambs.

Age groups	PR-interval	RR-intervalQT-interval	ST-interval	
Lambs (n=30)	0.045±0.008	0.203±0.032	0.071±0.014	0.060±0.014
Ewes (n=30)	0.056±0.008	0.263±0.040	0.112±0.019	0.090±0.049
P-value	0.035*	0.002*	0.001*	0.022*

*Significant differences between groups at similar indices (P<0.05).

as a result of the initiation of solid feed intake.[19,20] Changes in physiological states of gastrointestinal tract, feed intake and alterations in energy metabolism, may affect the cardiac performance and changes in ECG parameters. Furthermore, changes in food regimen and electrolyte profile may affect cardiac musculature and its activation.

The results of the present study showed that sinus tachycardia and sinus arrhythmia were the most frequent cardiac arrhythmias in the studied animals (Figure 2). Sinus tachycardia means an increase in heart rate that is initiated by the sino-atrial node. The term sinus tachycardia is used to describe an increase in heart rate caused by detectable influences such as pain, excitement, exercise, hyperthermia, a fall in arterial blood pressure or the administration of adrenergic drugs.[1] The heart rate returns to normal when the influence is removed or relieved. It may be suggested that the higher heart rates might be due to stress and excitation resulting from isolation of lambs from their dams as well, but since the animals were placed in a quiet state, it is unlikely to be the origin for this higher heart rate in studied animals. Because there were no clinical signs of cardiac problems (edema, jugular distension or pulsation) in all studied animals, this cardiac rhythm irregularity could be categorized as physiologic arrhythmias. Matsui *et al*.[21] reported an elevation of the heart rate in newborn pony foals with administration of a combined blockade with atropine and propranolol. These observations indicate the possibility of high vagal activity in the newborn Thoroughbred foal at birth.[15] Sustained tachycardia is an important clinical problem in the fetus and newborn. The physiological properties of the fetal and neonatal myocardium make it intrinsically more vulnerable to high heart rates.[22] Fetal tachycardia is an important cause of fetal morbidity and mortality.[23] The rate of fetal tachycardia is of no real help in defining the mechanism, as most tachycardia occurs at about higher than 120 beats/min and average of this parameter was 170 beats/min at the time of ECG recordings.

Sinus arrhythmia was also seen frequently in Chios ewes and lambs (Figure 2). This arrhythmia has been reported in cattle which have been deprived of food or had anorexia due to some gastrointestinal problems.[24,25] None of the animals with sinus arrhythmia in this study had any clinically obvious systemic problems or were suffering from anorexia. High vagal tone could be suggested as the cause of this arrhythmia in any ages of these animals.[26] Sinus arrhythmia is a normal physiological arrhythmia that occurs at slow resting heart rates and is associated with variation in the rate of discharge from the sino-atrial node associated with variation in the intensity of vagal stimulations. It is commonly correlated with respiration so that the discharge rate and heart rate increase during inspiration and decrease during expiration. Sinus arrhythmia is more clinically obvious in tame sheep and goats.[1] It may be possible to link the genesis of the sinus tachycardia and sinus arrhythmia in apparently healthy lambs to the increased load imposed on the heart or the fluctuation of the sympathetic or parasympathetic tone associated with excessive exertion during the stage of the birth.[27]

Sino-atrial block was also detected in Chios lambs (Figures 1 and 2). In sino-atrial block the sinus node fails to discharge or its impulse is not transmitted over the atrial myocardium. It is associated with the complete absence of heart sounds, of jugular atrial wave and of an arterial pulse for one beat period. The underlying rhythm is regular unless sinus arrhythmia is present. In the ECG there is complete absence of the P, QRS and T complex for one beat. The distance between the preblock and postblock P waves is twice the normal P-P

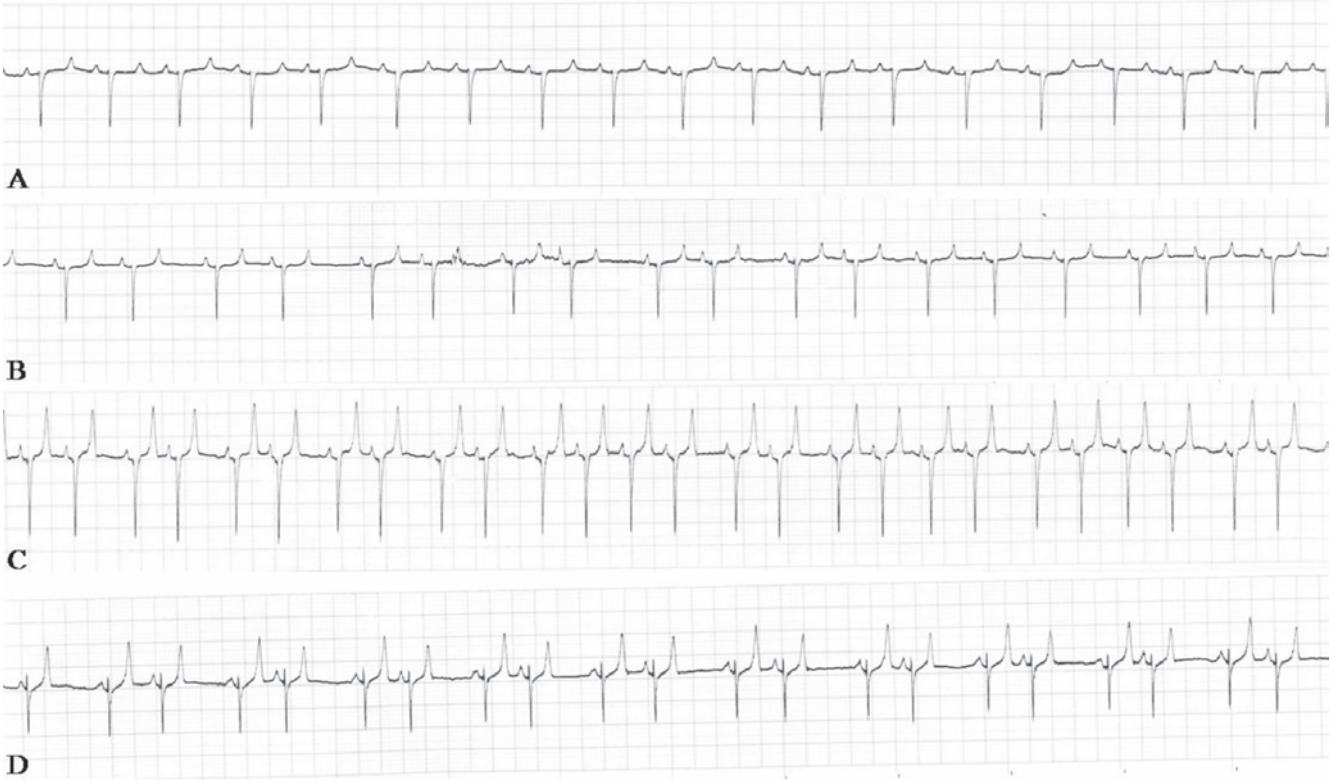

Figure 1. The electrocardiograms tracing from clinically healthy Chios sheep by base apex lead system (paper speed 25 mm/sec, sensitivity 10 mm/mV). A: Normal sinus rhythm in an ewe; B: Sinus arrhythmia in a lamb; C: Sinus tachycardia and sinus arrhythmia in a lamb; D: Sino-atrial block in a lamb.

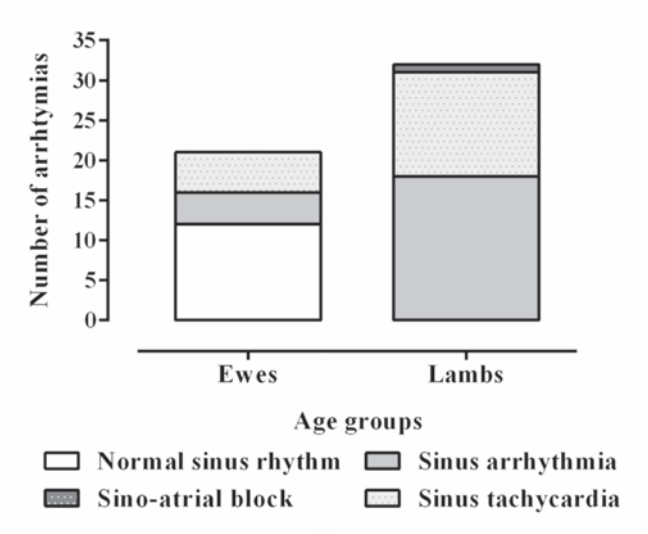

Figure 2. Distribution and proportion of cardiac arrhythmias in clinically healthy Chios ewes (n=27) and lambs (n=20) tracing by standard base apex lead system electrocardiography.

interval or sometimes slightly shorter. This arrhythmia is not uncommon in fit racing horses at rest and can be induced in horses and cattle by procedures that increase vagal tone.[1] Since, there were no clinical abnormalities in lambs which had sino-atrial block, it can be stated that this arrhythmia is categorized as physiological cardiac rhythm irregularities in Chios lambs.

Conclusions

In conclusion, it is obvious that these data will provide a good basis for judging the ECGs in base apex lead system of Chios lambs and ewes. It could be stated that aging can affect electrocardiographic findings. Furthermore, it may be suggested that the cardiac arrhythmias observed in the clinically healthy Chios ewes and lambs in this study could be accepted as the physiological arrhythmias and so no treatment is necessary. Finally, the results of the present research can help veterinarians to detect any abnormalities in heart electrical performance of Chios sheep and may be used as the guideline for the assessment of any cardiac rhythm irregularities in Chios sheep suffering from cardiac problems.

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Innocuousness of conjunctival vaccination with *Brucella melitensis* strain Rev.1 in pregnant Iranian fat-tailed ewes

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Abstract

Brucella melitensis strain Rev.1 is the most effective vaccine against brucellosis in sheep and goats. In Iran, mass vaccination is carried out all over the country in which adult animals are immunized by subcutaneous injection of reduced doses of the vaccine. However, due to antibody responses elicited by vaccination, concomitant implementation of test-and-slaughter is impossible. To overcome the problem, vaccination through conjunctival route is recommended. In this study, serological responses of six pregnant Iranian fat-tailed ewes to conjunctival vaccination with standard doses of the vaccine were evaluated using modified Rose Bengal test, serum agglutination test and indirect ELISA. Besides, vaccine strain excretion in milk and vaginal discharges was also examined by microbiological culture of milk and vaginal swab samples taken one day post-parturition. Animals were vaccinated during the second half of gestation. As the results, antibody titers of five (83.3%) ewes decreased to the levels not detectable by the tests within three months after vaccination. No vaccine-induced abortions occurred and vaccinated ewes delivered healthy lambs 50.33±15.56 (mean ± standard deviation) days post-vaccination. Vaccine strain was not isolated from milk and vaginal swab samples. Generally, our study shows full doses of *B. melitensis* strain Rev.1 can be used conjunctively to vaccinate pregnant Iranian sheep during late pregnancy without abortifacient effects, prolonged antibody responses and vaccine strain excretion in milk and vaginal discharges. Nevertheless, further studies are required to determine safety and immunogenicity of the vaccine in field conditions.

Introduction

Brucellosis in sheep and goats is an important zoonotic disease caused mainly by *Brucella melitensis*.[1,2] Vaccination of the host animals with *B. melitensis* strain Rev.1 is used worldwide for disease control which has been proved to be the most effective vaccine.[3,4] It is recommended to immunize replacement animals from 3 to 6 months of age with standard doses of vaccine containing at least 10^9 live cells.[5] However, there is evidence that effective control of the disease in countries with high prevalence requires immunization of all susceptible young and adult animals in a mass vaccination campaign which is considered as the most practical measure.[6-8]

One problem with vaccination of adult animals is antibody responses induced by the vaccine which may last for a long time and cause sero-positivity of vaccinated animals in routine serological tests interfering with detection of infected ones.[4,9,10] This makes simultaneous implementation of vaccination and test-and-slaughter impossible since vaccinated animals are falsely diagnosed as infected.[4] Moreover, vaccine-induced abortion and vaccine strain excretion in milk and vaginal discharges may occur.[5,10] Vaccination of flocks through conjunctival route is known as one way to solve these problems.[6,10,11]

Small ruminant brucellosis is an enzootic disease in Iran causing abortion in different parts of the country.[12] Mass vaccination has been the main control measure since 2003 in which adult animals are vaccinated subcutaneously using reduced doses of the vaccine.[12] Nevertheless, there are field reports showing long-lasting sero-positivity of vaccinated adult sheep and goats and abortions in pregnant animals attributed to the vaccination. Therefore, this study was done to evaluate serological responses of pregnant fat-tailed ewes to ocular vaccination with standard doses of *B. melitensis* strain Rev.1 as well as its safety in terms of abortion induction and vaccine strain excretion in milk and vaginal secretions.

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Key words: Brucellosis; Vaccination; Sheep; *Brucella melitensis* strain Rev.1; Conjunctival vaccine.

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Materials and Methods

Animals and vaccination

Eleven pregnant Iranian fat-tailed ewes were randomly selected from a known brucellosis-free flock. Selected animals were negative in modified Rose Bengal test (mRBT), serum agglutination test (SAT) and indirect ELISA (iELISA) carried out twice with a month's interval. Six ewes were vaccinated during third to fifth month of pregnancy with conjunctival *B. melitensis* strain Rev.1 vaccine containing 10^9 colony forming units (CFU) per dose. Other ewes were used as controls in which normal saline was used instead of vaccine at the same time. The vaccine used in the study was produced in Razi Vaccine and Serum Research Institute according to standard procedures.[5,13] The original seed for vaccine production was obtained from Animal Health and Veterinary Laboratories Agency (AHVLA), Weybridge, UK. Vaccinated and control animals were kept separately in the same conditions.

Serological evaluation

Animals were bled every 2 weeks for three months after immunization to evaluate serological responses to vaccination. Serum samples were examined using mRBT, SAT and iELISA. RBT and SAT antigens were produced in Razi Vaccine and Serum Research Institute based on standard methods,[5,13] as described previously.[14] For mRBT, one drop of the antigen was mixed with three drops of the serum sample.[15] Indirect ELISA was performed using PrioCHECK® Brucella Ab (Prionics AG, Schlieren, Switzerland) according to the manufacturer's instructions.

Bacteriological examination

To determine vaccine strain excretion in

milk and vaginal discharges, milk and vaginal swab samples were taken within 24 hours after abortion or parturition. Milk samples were first centrifuged at 6000-7000 rpm for 15 minutes and then supernatant cream and precipitated pellet were cultured.[13] Swab samples were cultured directly on solid media. *Brucella* agar medium (BD, USA) was used for vaccine strain isolation which was supplemented with *Brucella* selective antibiotics (Oxoid, Basingstoke, UK) and 5% (v/v) horse serum following manufacturer's instructions. For each sample, at least 3 plates were inoculated.

Results

Serological responses after vaccination

While control ewes remained negative during the study, vaccinated animals showed antibody responses from the second week after immunization. Percents of positive vaccinated ewes in mRBT and iELISA at two-week intervals over the study period are presented in Figure 1. Two weeks post-vaccination, all animals were positive in mRBT but none of them in iELISA. Five ewes (83.3%) showed positive results in iELISA after four weeks. The mRBT and iELISA results of each ewe were similar from fourth week on. After 12 weeks, only one vaccinated ewe (16.7%) remained positive detected by the two tests.

Evaluation of antibody titers using SAT demonstrated a falling trend over time (Figure 2). All animals showed increased antibody titers after two weeks which declined gradually afterwards in a way that 5 ewes (83.3%) had no SAT titers twelve weeks after vaccine inoculation. The only ewe, which had antibody titers detectable by SAT, was also reactive in mRBT and iELISA.

Vaccine safety

No abortion occurred following vaccination and all vaccinated animals had normal delivery with healthy lambs 50.33±15.56 (mean±SD) days after vaccine inoculation. *B. melitensis* strain Rev.1 was not isolated by microbiological methods from milk and vaginal discharges of vaccinated sheep within 24 hours postpartum.

Discussion and Conclusions

In Iran, brucellosis in small ruminants is an important enzootic disease which is a public health burden. Nomadic raising of sheep and goats, traditional production practices, illegal animal imports and uncontrolled movements of flocks within the country contribute to the difficulties in disease control. In these conditions, control of the disease has been mainly based on mass vaccination of young and adult animals using subcutaneous administration of full and reduced doses of *B. melitensis* strain Rev.1, respectively.[12] However, the disease still remains prevalent in different parts of the country diagnosed as a significant cause of abortion in sheep and goats.[12]

Although we previously demonstrated that reduced doses containing less than or equal to 10^6 bacteria can be safely used to immunize pregnant ewes with short-lasting serological responses,[14] field reports show persistence antibody responses and abortions caused by vaccination. It is known that the innocuousness of the vaccine in adult and pregnant animals depends on vaccine dose, time of vaccination during gestation and administration route.[8,16] Hence, these observations may be partly due to the fact that according to a standard approved by National Brucellosis Expert Committee, the reduced dose of vaccine used for adult animals immunization can contain up to 4×10^6 colony forming units (CFU) per dose. In addition, subcutaneous use of vaccine and extended lambing season in Iran, which results in presence of pregnant animals throughout the year in flocks, could be influential. Therefore, ocular inoculation of the vaccine is considered as an alternative proved to be safer.[6,8,10,16]

In our experiment, all animals were detected as positive using mRBT two weeks after immunization. Stournara *et al.*[9] also reported a hundred percent positive results in mRBT of non-pregnant ewes 21 days post-vaccination. In another study by Zundel *et al.*,[16] all ewes vaccinated at mid-pregnancy using the same dose as ours were positive in RBT 2 weeks following vaccination.

The proportion of positive vaccinated ewes in iELISA reached its maximum after 4 weeks. A similar result has been observed by Stournara *et al.*[9] which was attributed to the higher affinity of the conjugate used in the assay to immunoglobulin G (IgG). It has also been reported that more than 70 percent of non-vaccinated ewes were detected as negative by iELISA 14 days after challenge with the virulent strain during pregnancy.[17] Because the ewes used in our study were from a brucellosis-free flock without previous exposure to the pathogen, and according to the explanation provided by Stournara *et al.*,[9] negative iELISA results of these naive ewes two weeks after vaccine inoculation suggest antibody responses may be mainly of IgM class at this time. The percentage of positive animals detected by iELISA and mRBT decreased rapidly from 6 weeks post-vaccination to the end of study. Similar performance of iELISA and mRBT in our study is in agreement with results of the study carried out by Stournara *et al.*[9] However, in the latter study 72.6% and 84% of animals remained positive in iELISA and mRBT, respectively 91 days after immunization, but in our study only 16.7% were positive in both tests 12 weeks post-vaccination. This differ-

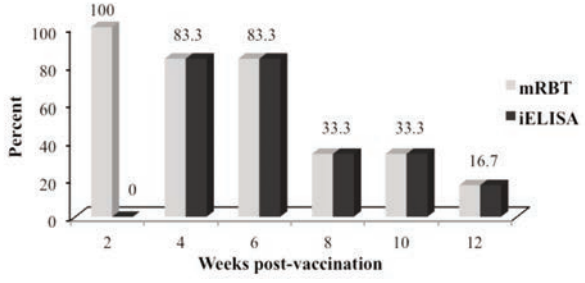

Figure 1. Percent of positive vaccinated ewes in modified Rose Bengal test and indirect ELISA.

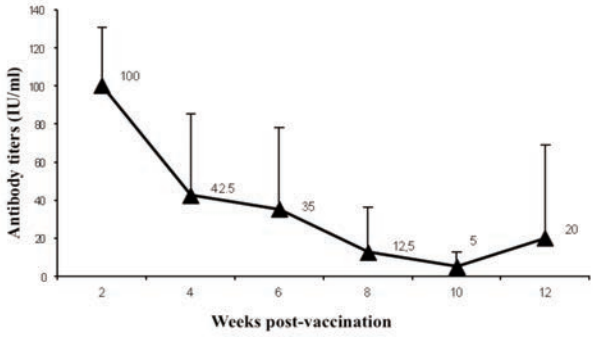

Figure 2. Mean ± standard deviation of serum agglutination titers in vaccinated animals.

ence may be due to age and physiologic status of animals when vaccinated or breed variation in antibody responses.[7,18]

Evaluation of serological responses during the study period using SAT revealed antibody titers fell from the peak reached at week two toward the end of study. SAT results are compatible with the other two tests and the only ewe, which had antibody titer of diagnostic value 12 week after immunization, was also detected as positive in iELISA and mRBT at this interval. While serum antibody level of this ewe was decreasing from 120 IU/mL six weeks following vaccination to 15 IU/mL at week 10, there was a further surge in its antibody titer (120 IU/mL) two weeks later at 12th week. For this animal, parturition occurred 66 days post-immunization 4 days prior to blood collection for the 10th week. This suggests that parturition might have effects on antibody responses to vaccination.

Use of vaccine through conjunctival route during second half of pregnancy was safe in terms of abortion induction, and no vaccine excretion in milk and fetal materials was detected soon after delivery. Rev.1 strain delivered conjunctivally is known to have a spread confined mainly to head lymph nodes.[8] Considering normal delivery of all animals one to two months following vaccine inoculation and as vaccine strain was not isolated immediately after parturition, it seems Rev.1 strain was not generalized to the uterus and mammary gland. Although there is no a completely safe way to use Rev.1 vaccine in pregnant small ruminants,[4] it is known that conjunctival administration of the vaccine during late pregnancy or before breeding can reduce risks of vaccine-induced abortions and vaccine strain excretion in milk and vaginal discharges,[5] which was also proved in Iranian fat-tailed ewes in this study.

In general, the present experiment showed serological responses to ocular vaccination of pregnant Iranian fat-tailed sheep with standard doses of *B. melitensis* strain Rev.1 disappeared in a considerable proportion of animals within 12 weeks. Moreover, use of vaccine during late pregnancy did not cause vaccination-induced abortion and Rev.1 strain excretion in milk and vaginal discharges during immediate postpartum period. Nevertheless, further investigations are required to assess vaccine performance in field conditions.

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Normal laparoscopic anatomy of the caprine pelvic cavity

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Abstract

Due to the several advantages over conventional procedures, the laparoscopic disease diagnosis and surgery has now started receiving attention in small ruminants. The normal laparoscopic anatomy needs to be described for comparison with the findings in animals with various diseases. The objective of the present study was therefore to describe the laparoscopic anatomy of the caprine pelvic cavity. Adult Bakerwal and Pashmina goats (n=25) of both the sexes were included in this laparoscopy study. All the animals were restrained in dorsal recumbency and Trendelenburg position under lumbosacral epidural anesthesia and sedation. After creating the pneumoperitoneum, the primary port for 5 mm laparoscope was placed at linea alba (3.0 cm cranial to mammary glands in does), and at right paramedian (3.0 cm cranial to the rudimentary teat in the bucks) site. Secondary port was placed under direct laparoscopic observation 5-6 cm away from the primary port in horizontal plane, to allow insertion of the grasping forceps. Scan was performed first at the primary port and subsequently through the secondary port for orientation and exploration of the pelvic cavity. The ventral laparoscopic approach provided satisfactory exposure of the pelvic cavity in goats. Comprehensive description of the pelvic organs could be obtained. However, dorsal aspect of the urinary bladder neck and accessory genital organs of male animals could not be visualized. Major complications were not encountered during or after laparoscopy. Laparoscopy a minimally invasive procedure has several advantages over alternate methods of understanding anatomy, physiology and pathology of most of the intraperitoneal pelvic structures in goats. The technique has high pedagogic value. The procedure is safe in experienced hands.

Introduction

Laparoscopy (keyhole or minimally invasive surgery) is a type of surgical procedure that allows a surgeon with the use of an instrument (laparoscope) inserted transabdominally to access the inside of the abdomen and pelvis without having to make large incisions in the skin.[1] This modality has diagnostic, therapeutic and prognostic applications. Laparoscopy has many advantages over laparotomy. They include reduced tissue trauma, postoperative adhesions and infections, fast recovery, stimulation of the immunity, better cardiovascular stability and lower pain scores.[1-5]

In recent years, laparoscopy has gained acceptance in veterinary medicine.[4] In small ruminants, it has been recognized as one of the most promising tools for improvement of the reproductive efficiency,[6-8] disease diagnosis and treatment.[9-11] In goats particularly those maintained for dairy purpose or as pets, this state of the art technology is expected to be more popular in the near future.

Laparoscopic anatomy of the abdominal cavity in goats has recently been described.[12] That study included female goats only. Additionally, organs located in the abdominal cavity received major consideration. With this background in mind, the present study was aimed to provide description of the laparoscopic anatomy of the pelvic cavity, in goats of both the sexes. The description will be useful for those interested in augmenting animal production as well as for those involved in disease diagnosis, treatment and prognosis of the pelvic disorders in goats. A significant pedagogic value would be an additional attribute.

Materials and Methods

The prospective study was carried out on twenty apparently healthy, adult, nonpregnant female and five male Pashmina and Bakerwal goats (weighing 25-31 kg and aged 1.5-2.5 years) maintained by the Division of Animal Biotechnology, Faculty of Veterinary Sciences and Animal Husbandry, India. The study was undertaken after receiving due approved from the institutional animal ethics committee. Two days prior to laparoscopy, wide ventral abdominal area from the mammary glands/rudimentary teats up to the umbilicus was clipped and shaved in all the animals. The food and water was withheld for 36 and 24 hours respectively. Regional lumbosacral epidural anesthesia was achieved using 2% lignocaine hydrochloride (4.0 mg/kg) plus xylazine hydrochloride (0.05 mg/kg). Immediately after satisfactory induction, every goat was shifted to a cradle, placed in dorsal recumbency with all the legs tied apart. The abdominal area was subjected to aseptic surgical preparation and properly draped. The cradle was then tilted to approximately 45° angle with head down (Trendelenburg) position of the animal. Laparoscopic equipment and instruments of Karl Storz, DmbH, Germany were used. Hopkins II straight forward (0° viewing angle of vision) telescope having 5.0 mm diameter and 29.0 cm length, was used. One cm longitudinal skin incision at linea alba, 3.0 cm cranial to the mammary glands in does and 3.0 cm cranial to the rudimentary teat in the left paramedian area in the bucks was followed by development of pneumoperitoneum with the use of a Veress needle. After withdrawing the pneumoperitoneum needle, 6.0 mm trocar-cannula with pyramid tip was passed into the peritoneal cavity at its site (followed by removal of the trocar) to create the primary port. The

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Key words: Goat, laparoscopy, pelvic cavity, pneumoperitoneum, Trendelenburg.

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Contributions: MRF performed all the surgeries and prepared the manuscript; RAS assisted in surgeries; MHB, assisted in surgeries, photography; FAK, photography, postoperative care of the animals; AK, induction of anesthesia, its maintenance and periodic evaluation; SHY, preparation of the animals, maintenance of the optical system; NAN assisted in preparation of the manuscript, maintenance of the pneumoperitoneum; NAG, availability of the animals and drugs, critical evaluation of the manuscript.

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laparoscope was subsequently passed through the cannula into the peritoneal cavity. Systemic examination of the entire pelvic cavity was done by video camera connected to the eye piece of the laparoscope and the images were transferred through the control unit to the television monitor and recorded on a video tape. Secondary port was developed using a 6.0 mm threaded cannula unit in the right paramedian area (5.0 to 6.0 cm lateral to the first port). This port was used to pass the instruments for grasping and/or manipulation of the viscera. The laparoscope and the grasping forceps were then exchanged to visualize through the secondary port and manipulate through the primary port.

After completion of the visceral observation session, the accessory instrument and the laparoscope were retracted. The cannulas were removed only after evacuation of the abdominal air. The portal sites were closed with one subcuticular stitch using No 1 chromic catgut. Antiseptic was sprayed over the incision sites.

The animals were shifted from the cradle, placed in sternal recumbency on a level surface and allowed to regain complete motor power before leading them to their sheds. During this period, they were given a dose of amoxicillin-dicloxacillin (0.5 g) and meloxicam (0.5 mg/kg) intramuscularly. Antiseptic dressing of the portal sites was continued daily up to three days following suture removal on day 8. The animals were watched for complications if any for 10 days following laparoscopy.

Laparoscopy was repeated in does (n=15) after a variable period of two to five months while they were subjected to embryo transfer.

Results

Lumbosacral injection of lignocaine plus xylazine produced satisfactory analgesia of the abdominal area (up to the umbilical region) along with general sedation in all the goats. Supplementation or additional anesthetic administration was not required in these animals.

One of the animals that had not been fasted properly, showed regurgitation of the rumenal contents towards the end of the session. The animal however did not develop any complications later.

Preoperative fasting was found highly beneficial to reduce the size and motility of the gastrointestinal tract. It not only allowed satisfactory visualization but also prevented inadvertent puncture of the viscera in all the animals.

Pneumoperitoneum resulted in creation of sufficient space within the abdominal wall and in between the viscera; essential for satisfactory visualization and manipulation. Trendelenburg position encouraged abdominal organs to slide cranially exposing whole of the pelvic cavity. Occasionally, some of the animals had to be slightly rotated in the cradle to maintain perfect dorsal recumbency.

Insertion of the laparoscope at primary portal site allowed optimum visualization of the pelvic viscera in animals of both the sexes. By orienting the laparoscope in different directions and planes, various urogenital organs, segments of small and large intestines, ligaments supporting pelvic viscera, major blood vessels, internal inguinal ring and the muscles lining the pelvic cavity were identified.

Urinary bladder as a hollow, thin walled, ovoid organ having bluish semitransparent coloration and tortuous serosal vessels was

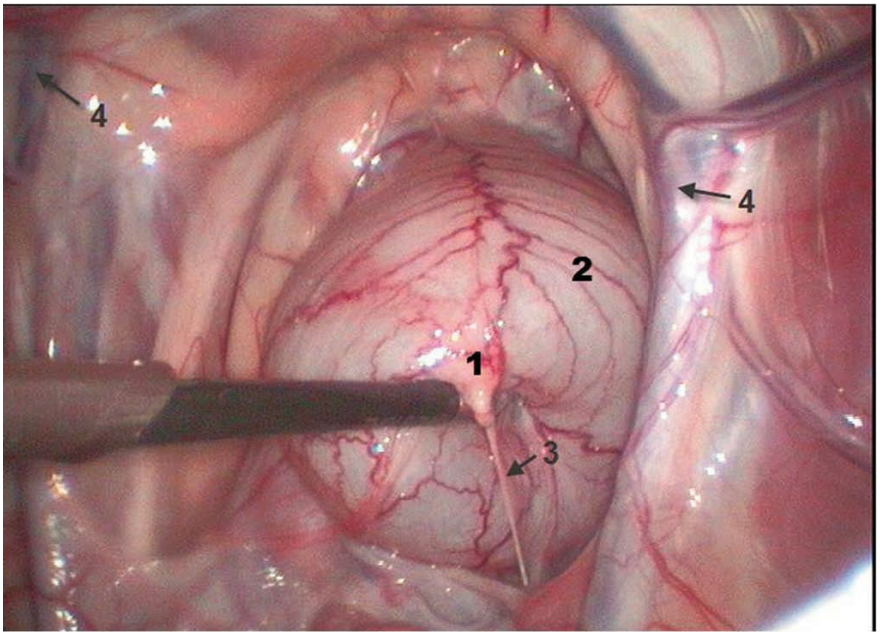

Figure 1. Photograph showing laparoscopic view of pelvic organs and structures in a goat: 1. remnant of urachus, 2. urinary bladder, 3. round liament of bladder, 4. internal iliac artery.

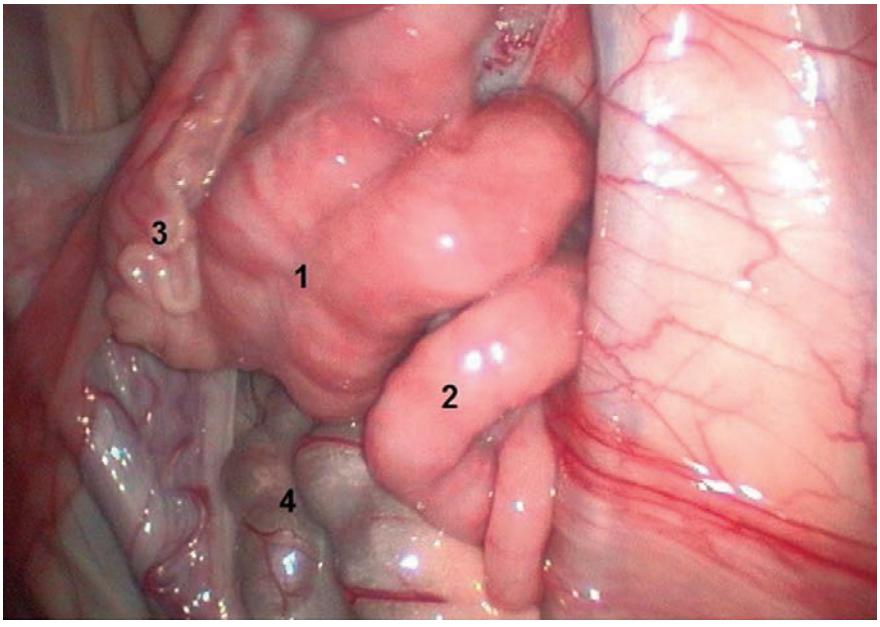

Figure 2. Photograph showing laparoscopic view of pelvic organs and structures in a goat: 1. uterine body, 2. uterine horn, 3. Fallopion tube, 4. distal colon.

easily located in the central area (Figure 1).

Most of the occasions, it was moderately filled with urine. Vestigial remnant of fetal urachus was visible at the vertex of the bladder. The round and lateral ligaments were detected (extending from both the sides of the bladder towards the corresponding lateral wall of the pelvic cavity) after elevation of the bladder by the accessory instrument (Figure 1). The elevation of the bladder from its vertex area towards the ventral pelvic wall was possible only in animals with minimal residual urine. The lateral ligaments prevented visualization of the dorsal neck area of the urinary bladder in animals of both the sexes and the accessory genital organs in male animals. In does the uterus (Figure 2) and broad ligaments and in bucks the rectum was located dorsally.

Cecum as a curved organ was located to its full length after it was pulled out of the supraomental recess or the later pushed cranially. Cecum appeared as a dilated, oval viscous of larger diameter than the small intestines showing caudally directed blind end (Figure 3). Its wall thickness was more but diameter lesser than that of the urinary bladder.

The descending colon was identified by its segmentation into the pellets and its fecal contents. The fecal pellets appeared darker bead like round objects within its lumen (Figure 2).

Small intestines were identified due to their mesenteric attachment and emanating vasculature, peristalsis, thin wall, smooth surface, smaller diameter and more closely coiled pattern than the colon and the cecum (Figure 3).

Segment of the uterine horns as pinkish, tubular, soft tissue, curved, smooth structures without peristalsis and without prominent superficial vasculature were visible just cranial to the bladder only in goats with mild to moderate distension of urinary bladder (Figure 2). The ovaries, their proper ligaments and mesovarium, fallopian tubes, broad ligaments and uterine horns were inspected in detail only after retraction of the overlying gastrointestinal structures, grasping and elevation of the reproductive tract by accessory instruments (Figure 2). Body of the uterus and rectogenital pouch was viewed easily after elevation of the uterus against the ventral abdominal wall by the grasping forceps. The fallopian tubes appeared tortuous with light pinkish color starting from the narrow tip of each uterine horn (Figure 2). The tubes were best exposed when the mesovarium was displaced or spread by accessory instrument. Surfaces of the ovaries were best viewed after the mesovarian ligaments were picked-up. The ovaries appeared as oval organs (Figure 4) and located craniolateral to the fimbria of the fallopian tube. Laparoscopy provided better understanding of the position of supporting structures of the uterus and ovaries. The ovaries were suspended in the abdominal cavity by proper and mesovarian ligaments. The mesovarium appeared as the cranial portion of the broad ligament suspending ovaries with the lateral abdominal wall and through which blood vessels, lymphatics and nerves passed to the ovary. The cyclic morphological alterations were detected on the ovaries, including ovarian follicle and corpus hemorrhagicum, corpus luteum and corpus albicans.

Internal inguinal ring as a slit like aperture in the lateral pelvic wall and the structures passing through it were visualized bilaterally (Figure 5). In male animals, the vas deferns was easily located as white cord like tubular structure passing from each internal inguinal ring towards the neck of the bladder (Figure 5).

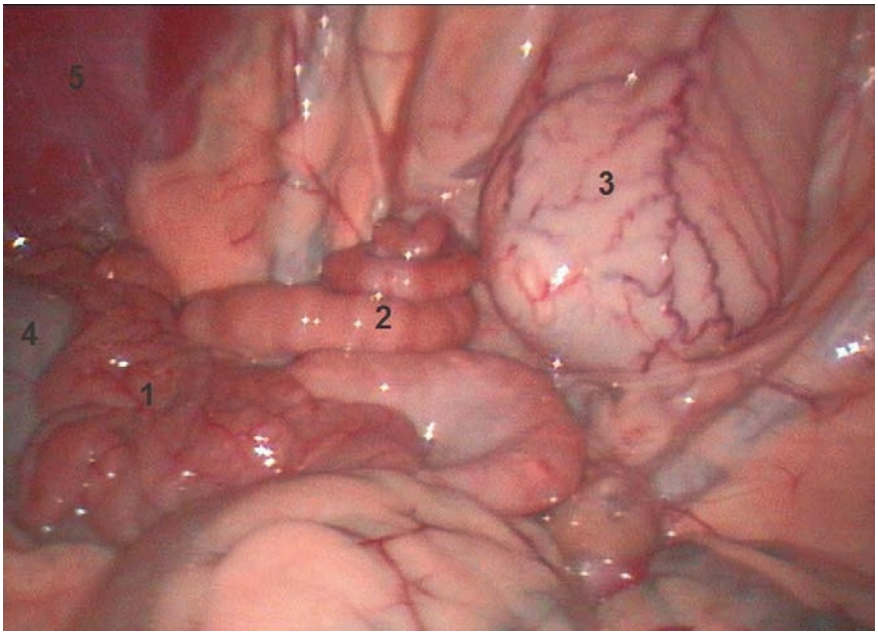

Figure 3. Photograph showing laparoscopic view of pelvic organs and structures in a goat: 1. small intestine, 2. uterine horn, 3. urinary bladder, 4. caecum, 5. internal abdominal obliques muscle.

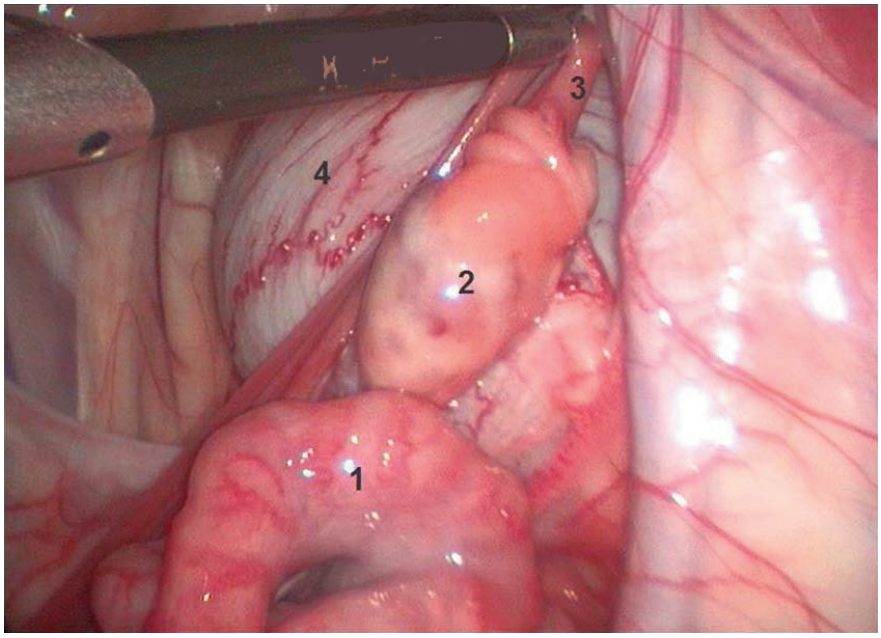

Figure 4. Photograph showing laparoscopic view of pelvic organs and structures in a goat: 1. uterine horn, 2. ovary, 3. mesovarium ligament, 4. urinary bladder.

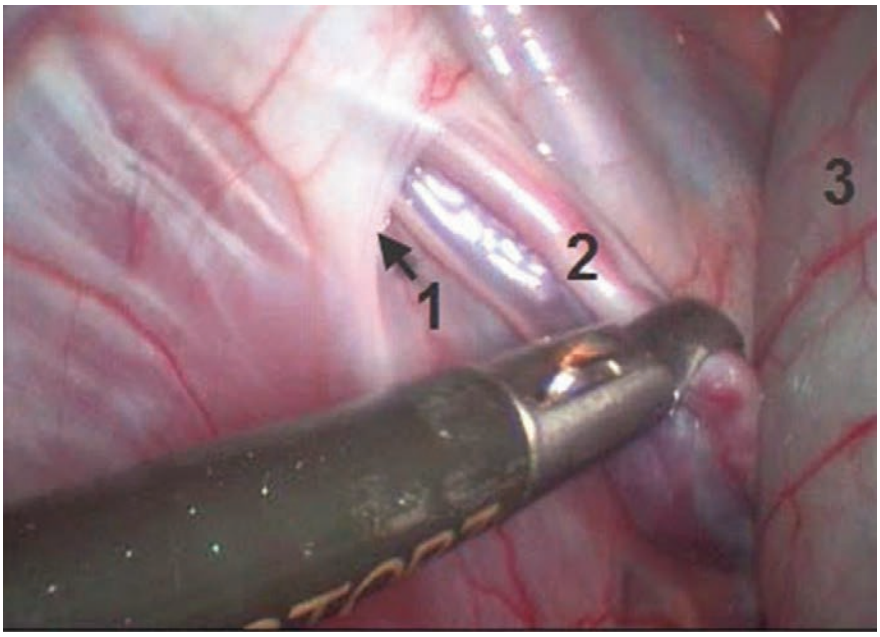

Figure 5. Photograph showing laparoscopic view of pelvic organs and structures in a male goat: 1. inguinal ring, 2. vas deferns, 3. urinary bladder.

The internal iliac artery and its bifurcation into cranial and caudal gluteal arteries were visible bilaterally near the caudal end of the pelvis (Figure 1).

All of the goats recovered normally within two hours from induction of the regional anesthesia. However mild subcutaneous emphysema around the portal sites in two goats subsided subsequently within three and four days respectively. On follow up laparoscopy, neither adhesions nor any other complication that could be attributed to the previous procedure was detected in the pelvic cavity of any animals.

Discussion and Conclusions

Laparoscopy offers the advantage of direct observation of internal anatomy of the abdominal and pelvic cavities. In addition to the several diagnostic and therapeutic advantages, the technique has particularly a very important pedagogic value. Magnification of the images and the organs being separated from each other (due to pneumoperitoneum), this technique helps in identification of even the smaller structures, rings, apertures and openings not seen in standard celiotomy approach.[12]

Laparoscopy in small ruminants has been performed either under general anesthesia or infiltration of the portal sites with the local anesthetic agent along with sedation.[6,8-12] However, we used lumbosacral epidural anesthesia satisfactorily without complications. In small ruminants, this technique has several advantages. Unlike local infiltration of the portal sites, lumbosacral epidural anesthesia results in abdominal wall relaxation which is particularly beneficial during laparoscopy.

In the present study, usage of 5.0 mm laparoscope provided good panoramic and close-up view of the pelvic cavity. Usage of 0^0 laparoscope allows satisfactory orientation and easier manipulation of the instruments.[13-15] It also maximizes light transmission compared with laparoscopes with an offset viewing angle.[1] Usage of cold light fountain xenon, with 175 Watt lamp and 4.5 mm fiber optic light cable provided satisfactory illumination for videoscopy. However, several workers have recommended 300-W xenon light source to perform laparoscopy.[1,15,16]

Preoperative fasting was mandatory before any laparoscopic procedure.[17-19] Fasting for 36 hours but not 24 hours was found sufficient to decrease the content of rumen and large intestine and reduce intestinal peristaltic motility in the goats included in this study. It not only reduced the risk of organ penetration during Veress needle introduction or undue pressure of the cranially displaced abdominal organs on diaphragm but also improved observation of abdominal/pelvic structures. In most of the animals, preoperative fasting is essential when a ventral surgical approach is used.[20]

In male animals the mid ventral area being occupied by penis and prepuce made it necessary for us to introduce the primary port at paramedian location. This however did not interfere in locating and visualizing different pelvic organs and structures. In the dogs and cats, insertion of the Veress needle caudolateral to the umbilicus and directed towards the pelvis to avoid falciform ligament and injury to the spleen has been reported.[21]

Trendelenburg position encourages abdominal organs to slide cranially, exposing caudal field.[1,20,22]

Laparoscopy provided a comprehensive description of the normal laparoscopic anatomy of the caprine pelvis in dorsal recumbent position.

The subcutaneous emphysema noticed in two goats resolved without intervention. This finding corroborates well with an earlier report.[23] Follow up in the female goats indicated that the exploratory laparoscopic procedure is safe. Repeated laparoscopy does not increase the risk of intra-abdominal complications.[24]

The minimal exposure of the abdominal/pelvic cavities to outside atmosphere and the least visceral handling was done during laparoscopy. Therefore, the systemic antibiotic and analgesic were administered only once postoperatively in all the animals. Such drugs are reportedly required only for 24 hours following laparoscopy in cattle.[1]

From this study, it is concluded that laparoscopy a minimally invasive procedure has several advantages over alternate methods of understanding anatomy, physiology and pathology of most of the intraperitoneal pelvic structures in goats. The procedure is also safe in experienced hands.

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The effects of aflatoxin B1 and silymarin-containing milk thistle seeds on ileal morphology and digestibility in broiler chickens

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Abstract

This study investigated the effects of aflatoxin B_1 (AFB1) and milk thistle seed (MTS) on some apparent ileal morphology and digestibility variables in the small intestines of broilers. A total of 216 Ross 308 male broiler chickens were allocated in a 3 3 factorial arrangement of the treatments with three concentration of AFB1 (0, 250, and 500 ppb) and three levels of MTS (0, 5, and 10 g kg^{-1}). On day 35, the birds that received diets with 500 ppb of AFB1 alone (main effect) showed significant decrease in apparent ileal digestibility [dry matter (DM; 72.46±0.27), calcium (Ca; 40.81±1.11), crude protein (CP; 29.42±1.89), apparent digestible energy (2653±58.82)], ileal morphology [villus length (VL; 822.5±7.47), villus width (VW; 90.16±2.17) and ratio of VL to crypt depth (VL/CD; 4.74±0.07)] in their ileum segments (P<0.01). However, the mean nitrogen (N; 61.39±0.48) and crypt depth (CD; 173.5±9.87), in the ileum were significantly greater for the birds that were fed with 500 ppb AFB1 alone in their diets when compared with the control (P<0.01). Also, thistle seeds can ameliorate the toxic effects of AFB1 on some ileal digestibility factors, that is, DM, N, Ca, and CP, in broiler chicks. Nevertheless, ileum morphology of VW and goblet cell numbers were not affected negatively by the AFB1 plus MTS in diets. The results of this study indicated that the use of MTS independently reduced the toxic effects of AFB1, facilitated the absorption of nutrients, and reduced the metabolic demands of the intestinal tract in broiler chickens.

Introduction

Aflatoxin B_1 (AFB1) is a secondary metabolite produced by *Aspergillus flavus* and *A. parasiticus*, and it has carcinogenic, mutagenic, hepatotoxic, and teratogenic effects.[1,2] Several diseases are associated with the human consumption of these toxins, including toxic hepatitis and even primary hepatocellular carcinomas.[1,2] Aflatoxin B_1 8,9-epoxide is the reactive form of the compound, and it binds to cellular macromolecules and causes periportal hepatic injury.[3] However, extrahepatic effects, namely, within the intestine, have not been studied thoroughly. Other researchers have documented the negative effects of AFB1 on total tract retention of energy, mean nitrogen (N), and amino acids in poultry.[4-7] It seems that AFB1 alone has a harmful effect on the metabolization of nutrients, a harmful effect on the intestine, or both, resulting in increased loss of endogenous nutrients, reduced digestibility of nutrients, or both.[8] From the aforementioned studies, it is difficult to discern a dose-effect relationship between AFB1 and histological changes in the gastrointestinal tract (GIT). AFB1 is widely believed to result in malabsorption syndrome regarding macronutrients and also to result in reduced activity of digestive enzymes.[9,10] Silymarin is a mixture of flavonoids extracted from milk thistle seed (MTS) (*Silybum marianum* L. Gaertn.), and it contains silybin, silydianin, and silychristin as the major fractions.[11] Silymarin acts in five different ways; as an antioxidant, absorber and regulator of the intracellular glutathione, as a stabilizer and regulator of cell membrane permeability that prevents the entering of hepatotoxic substances into hepatocytes, as the ribosomal ribonucleic acid (rRNA) synthesis promoter stimulating regeneration of the liver and an inhibitor of the transformation of liver stellate cells into myofibroblasts.[12] This suggests that silymarin may contribute to the prevention of aflatoxicosis-induced damage.[13-15] There has been no reports that have dealt with the effect of interactions of AFB1 combined with MTS on the ileal morphology and digestibility of broilers to date. Thus, this study was conducted to evaluate the effects of simultaneous supplementation of AFB1 and MTS on ileal morphology and digestibility in broiler chickens.

Materials and Methods

Plants collection

Milk thistle seeds were collected from Kashmar-Kohsorkh district (16.35° north latitude, 18.58° east longitude, about 1052 meters above sea level) in Khorasan-Razavi province, in the north-east of Iran, during autumn 2011 (Figure 1).

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Contaminating poultry feed

A. flavus was obtained from the Center of Scientific and Industrial Research Organization in Iran, PTCC NO: 5004 (IR111), and was cultured on potato dextrose agar (PDA) medium and used for *in vitro* studies. The AFB1 content in rice powder was analyzed by the method of Shotwell *et al.*[16] and measured on a thin layer chromatography (TLC) fluorometric densitometer (Camag-iII, Basel, Switzerland) on the TLC spots. The yield of AFB1 produced was 60 ppb gr^{-25} of sample from each flask.

Experimental design

Combinations of three levels of AFB1 (0, 250, and 500 ppb) with three levels of MTS (0, 5, and 10 g kg^{-1}) were incorporated into the basal diet (corn and soybean meal). A total of 216 one-day-old chicks (Ross 308) were allocated to nine treatments with four replicates based on a completely randomized design in a 3×3 factorial arrangement. There were nine experimental diets with four replicates of six birds in each replicate. All of the birds were fed a typical, commercial diet for the 35 days of the experiment. The birds were housed in wire cages with nipple waterers and 516 cm^2 of floor space per bird. Feed and water were provided ad libitum. A basal diet was formulated on

corn-soybean meal base for starter, grower, and finisher periods according to [National Research Council, NRC (1994)][17] the recommendations. The birds received the following diets with equal energy and protein levels: (T_1) control basal diet; (T_2) basal diet plus 250 ppb AFB_1; (T_3) basal diet plus 500 ppb AFB_1; (T_4) 5 g kg^{-1} of MTS; (T_5) 5 g kg^{-1} MTS plus 250 ppb AFB_1; (T_6) 5 g kg^{-1} MTS plus 500 ppb AFB_1; (T_7) 10 g kg^{-1} MTS; (T_8) 10 g kg^{-1} MTS plus 250 ppb AFB_1; and (T_9) 10g kg^{-1} MTS plus 500 ppb AFB_1. MTSs were acquired from the outskirts of the Birjand district in South Khorasan Province, Birjand, Iran. Also, all animals received humane care in compliance with the guidelines of animal science Dept. at Birjand University, Birjand, Iran.

Ileal digestibility

At the end of experiment (day 35), two birds per pen were sacrificed, and two-thirds from Meckel's diverticulum to the cecal junction was removed (about 10 cm), and the ileal digesta were flushed with distilled water. The ileal digesta was collected and stored at –20°C, freeze-dried, and ground with a mortar and pestle before the analyses. Feed, excreta, and ileal digesta were analyzed for determination of nutrient digestibility and retention. The dry matter (DM) content was determined on ground diets and freeze-dried ileal digesta and excreta by drying the samples at 100°C for 24 h. Titanium (Ti) was determined by the inductively coupled plasma atomic emission spectroscopy method (AOAC, 1995)[18] following nitric-perchloric acid wet ash digestion. Gross energy (N) determinations of feed and excreta samples were performed in a bomb calorimeter (Gallenkamp Autobomb, Loughborough, UK) with benzoic acid as a standard.[19] The apparent digestible energy (AMEn) (excreta) and AMEn (ileal digesta) of the diets were calculated using the index method (using Ti as the digestive marker) by using the formula of Meng and Slominski[20] as described by the NRC (1994).[17] The calcium (Ca) concentration of the feed and digesta were determined by Flame Atomic Absorption Spectrophotometer (A Analyst 100, Perkin-Elmer Inc., Waltham, MA, USA). The crude protein (CP) content (N 6.25) of the diet and individual samples of digesta were determined by the Kjeldahl method (AOAC, 1995).[18]

Ileal morphology

On day 35, two birds per pen were sacrificed by rupture of their carotid arteries and jugular veins. Then, two-thirds (about 5 cm) of the ileum was removed and flushed with distilled water. The mucosa was collected by scraping with a microscope slide and subsequently frozen in liquid nitrogen. A 3-cm section of the proximal ileum (Mecke's diverticulum to the cecal junction) was rinsed with 0.01 M phosphate buffered saline (PBS, pH 7.2) and placed in a 10% buffered, neutral formaldehyde (pH 7.2 to 7.4) solution. As a result, all samples were gradually dehydrated, sectioned at 6 mm thickness, and stained with hematoxylin and eosin. VL, CD, VW, and the thickness of the epithelium were measured at 100 magnification using computer software (Sigma Scan, Jandel Scientific, San Rafael, CA, USA). Also, the ratio of VL/CD was calculated (Figure 2). Two slides were made for each intestinal sample, and each slide from the ileum sample was stained with Periodic acid-Schiff (PAS) reagent (McManus, 1948).[21] The tissues were deparaffinized, hydrated, oxidized in periodic acid (6 g L^{-1}) for 5 min, rinsed in distilled water, and then placed in Coleman's Schiff's reagent (Polysciences, Inc.) for 30 min. After 15 min, the slides were rinsed in tap water, the tissues were counterstained in hematoxylin, rinsed, dehydrated, and mounted. The positively stained, PAS GCN were enumerated on six villi per sample, and the means were utilized for statistical analysis (Figure 2). Measurements of VL and VW were taken from the tip of the villus to the valley between the individual villi, and measurements for CD were taken from the valley between the individual villi to the basolateral membrane (AOAC, 1995).[18]

Statistical analysis

The data were statistically analyzed with the standard procedures of analysis of variance (ANOVA), using a 3 3 factorial with completely

Table 1. Effect of aflatoxin B1 (AFB1) and milk thistle seed (MTS) on apparent ileal digestibility in broilers at the end of the period (35 day).

AFB1 (ppb)	MTS (g kg^{-1})	Dry matter, %	Nitrogen, %	Apparent digestible energy, Kcal kg^{-1}	Calcium, %	Crude protein, %
Treatment						
0	0	77.36±2.12^{a}	56.33±1.97^{b}	2754±49.77	53.38±1.68^{a}	39.41±2.38^{a}
250	0	74.32±2.46^{ab}	57.42±1.83^{b}	2674±38.62	44.21±1.87^{b}	28.44±2.23^{bc}
500	0	72.39±2.34^{b}	69.52±2.18^{a}	2621±57.76	41.24±2.14^{c}	26.41±3.12^{c}
0	5	72.41±3.01^{b}	55.38±1.84^{b}	2762±67.64	53.31±1.68^{a}	41.46±3.75^{a}
250	5	73.29±2.57^{b}	54.36±2.39^{b}	2681±81.65	43.38±1.93^{a}	31.59±3.35^{b}
500	5	76.33±2.67^{ab}	55.51±1.83^{b}	2664±69.13	40.77±1.49^{a}	30.44±2.66^{b}
0	10	72.58±2.96^{b}	53.69±2.74^{b}	2809±84.77	51.33±2.25^{a}	38.51±2.96^{b}
250	10	72.35±2.65^{b}	54.76±1.67^{b}	2727±73.65	42.45±2.17^{a}	36.52±4.11^{b}
500	10	74.26±2.75^{b}	59.14±1.96^{b}	2675±77.71	40.42±1.69^{a}	31.43±3.68^{b}
Main effects						
0	-	75.98±0.27^{a}	55.13±0.48^{b}	2775±58.82^{a}	52.68±1.11^{a}	41.79±1.89^{a}
250	-	73.32±0.27^{b}	55.52±0.48^{ab}	2685±58.82^{b}	43.35±1.11^{b}	32.18±1.89^{b}
500	-	72.46±0.27^{b}	61.39±0.48^{a}	2653±58.82^{b}	40.81±1.11^{b}	29.42±1.89^{c}
-	0	73.12±0.27	61.11±0.48^{a}	2674±58.82	46.27±1.11	31.41±1.89^{b}
-	5	74.11±0.27	55.12±0.48^{b}	2702±58.82	45.82±1.11	34.51±1.89^{ab}
-	10	74.69±0.27	55.86±0.48^{b}	2737±58.82	44.73±1.11	37.49±1.89^{a}
Probabilities (P value)						
AFB1		0.01	0.01	0.05	0.01	0.01
MTS		Ns	0.01	Ns	Ns	0.05
AFB1 × MTS		0.05	0.05	Ns	0.05	0.05

$^{a-c}$Means within a column lacking a common superscript differ significantly (P<0.05 and P>0.05). Ns: not significant.

randomized design, as suggested by Macros software.[22] The data were compared with Tukey-Kramer *post hoc* test. Least squares means ± standard errors are reported and P≤0.05 and 0.01 indicates statistical significance. All of the care and procedures used in testing the birds in this experiment were conducted from 21 March to 24 May 2012 at University of Birjand (South Khorasan Province in 59° 13 east longitude and 32° 53 north latitude, East-Iran).

Results

The results of this study indicated that the interaction effects between AFB1 and MTS were significant for apparent ileal digestibility, that is, DM, N, Ca, and CP (P<0.05) (Table 1). In contrast, retention and digestibility of AMEn were unaffected by the combinations of AFB1 and MTS. The interaction effect from apparent N digestibility indicated that a quadratic increase occurred when the amount of AFB1 administered was increased from 250 (57.42±1.83 ppb) to 500 (69.52±2.18 ppb) (P<0.05). Also, different levels of AFB1 did not cause significant changes in the Ca and CP of diets that contained 5 or 10 g kg^{-1} of MTS (intraction effect) (Table 1). Feeding of 5 or 10 g kg^{-1} of MTS increased the CP (P<0.05) and decreased apparent N (P<0.01) digestibility (main effect). In contrast, apparent DM, AMEn, and Ca retention were unaffected by different levels of MTS alone. However, the average apparent ileal digestibility of CP and Ca that contained 5 and 10 g kg^{-1} of MTS alone were higher than different levels of AFB1 (250 and 500 ppb). Also, interaction and the main effect from ileal morphology indicated that there was a linear increase in CD and a linear decrease in VL when using diets contaminated with AFB1 compared to the control animals that were not fed the contaminated food (Table 2). Also, VL/CD ratio in the ileum was decreased significantly (P<0.05 and 0.01) at the end of study (day 35). In contrast, interaction from VW and GCN was unaffected by consumption of AFB1 plus MTS (Table 2). Also, for the broilers that were fed with the contaminated diet, a main effect was a decrease in VW (90.67±2.17 to 90.16±2.17) (P<0.01).

Discussion

The mechanism of action of MTS in apparent ileal digestibility on animals is not clearly understood. Currently, it seems that this plant can be referred to AFB1 absorbent on apparent ileal digestibility in broiler chicks. Diaz *et al.*[23] reported that low levels of AFB1 in the diet did not affect DM and N digestibility in birds. Verma *et al.*[24] reported a reduction in net protein utilization and AMEn when 1 to 2 mg kg^{-1} of AFB1 was fed to broiler chicks. The results of this study were in agreement with those of previous studies when the levels of AFB1 alone were increased from 250 to 500 ppb (main effect). Kermanshahi *et al.*[7] also noted differences in energy and protein utilization with low-level inclusion of AFB1 in the feed given to broiler chicks. In this report, feeding of 0.8 to 1.2 mg kg^{-1} of AFB1 reduced AMEn and apparent N retention.[7] Also, when apparent N retention was corrected for uric acid excretion, the differences were negated, suggesting a reduction in uric acid excretion and, plausibly, a reduction in amino acid digestibility.[25] Although the interactions between aflatoxin and MTSs are not clear, there are two possibilities, that is, first, MTSs may increase protein absorption by increasing its solubility in digesta and, as a result, by prolonging the transfer time in the small intestine and second, MTSs may provide better conditions for the action of ileal enzymes by acidification of the diet and the digestive fluids.[26] The effects of higher dosages of AFB1 in broilers on these variables are not known. Contrary to the observations in broilers, other authors noted a linear increase in the crypt depth in the distal jejunum with increasing levels of AFB1 in the diet, that is, 0, 0.6, 1.2, and 2.5 mg kg^{-1}, but they observed no effects of the toxin on villus height or the number of goblet cells.[27] From the recent studies of broilers by Kana *et al.*[28], Yunus *et al.*[27] and Kumar and Balachandran,[8] it appeared that the unit absorptive surface of the small intestine

Table 2. Effect of aflatoxin B1 (AFB1) and Milk thistle seed (MTS) on ileal morphology variables in broilers at the end of the period (35 day).

AFB1 (ppb)	MTS (g kg^{-1})	Villus length, µm	Villus width, µm	Crypt depth, µm	Ratio*	Goblet cell number**
Treatment						
0	0	844.4±8.22^{a}	93.23±3.34	147.3±18.34^{b}	5.74±0.13^{a}	10.93±1.23
250	0	824.6±8.17^{b}	90.24±3.62	153.5±18.68^{b}	5.37±0.12^{b}	11.92±1.12
500	0	819.6±8.51^{c}	89.61±4.11	177.3±19.66^{a}	4.62±0.14^{c}	14.45±1.38
0	5	841.5±9.12^{a}	93.18±5.75	146.3±19.58^{b}	5.76±0.14^{a}	11.54±1.62
250	5	825.4±9.28^{b}	90.34±5.95	150.3±14.55^{b}	5.51±0.14^{a}	11.35±1.18
500	5	822.3±9.61^{b}	88.44±4.47	169.6±15.71^{ab}	5.85±0.12^{a}	11.69±1.25
0	10	842.3±8.44^{a}	93.38±4.44	144.6±19.77^{b}	5.84±0.15^{a}	11.42±1.46
250	10	827.2±9.98^{b}	91.45±4.46	151.3±19.78^{b}	5.47±0.13^{a}	11.53±1.52
500	10	825.7±9.39^{b}	92.14±4.89	173.5±16.87^{ab}	4.75±0.17^{a}	11.73±1.22
Main effects						
0	-	842.7±7.47^{a}	93.26±2.17^{a}	146.1±9.87^{c}	5.74±0.07^{a}	11.31±0.43
250	-	825.7±7.47^{b}	90.67±2.17^{b}	151.7±9.87^{b}	5.45±0.07^{b}	11.62±0.43
500	-	822.5±7.47^{b}	90.16±2.17^{b}	173.5±9.87^{a}	4.74±0.07^{a}	12.62±0.43
-	0	829.5±7.47	91.13±2.17	159.3±9.87	5.24±0.07	12.44±0.43
-	5	829.7±7.47	90.65±2.17	155.4±9.87	5.37±0.07	11.53±0.43
-	10	831.7±7.47	92.32± 2.17	156.4±9.87	5.35±0.07	11.56±0.43
Probabilities (P value)						
AFB1		0.01	0.01	0.01	0.01	Ns
MTS		Ns	Ns	Ns	Ns	Ns
AFB1 × MTS		0.05	Ns	0.05	0.05	Ns

$^{a-c}$Means within a column lacking a common superscript differ significantly (P<0.05). *Ratio of villus length to crypt depth. **Numbers in area of epithelial cells. Ns: not significant.

Figure 1. Milk thistle plant collected from the outskirts of Kashmar-Kohsorkh district in Khorasan-Razavi province, Iran.

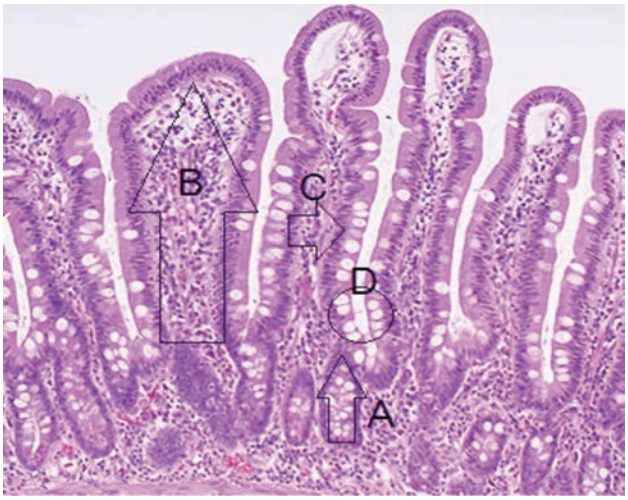

Figure 2. Ileal morphology variables measured at 100 X magnification: A) Crypt depth; B) Villus length; C) Villus width; D) Goblet cells.

deteriorated during chronic exposures to low levels of AFB1. Administration of AFB1 resulted in a reduction of T cells and alkaline phosphatase activity in the intestine.[29] Also, enterocytes and other ileal enzymes must differentiate during their time along the axis of the crypt-villus to fully express these digestive functions.[25-30] However, intestinal mucin production and secretion is a dynamic process that is continually degraded and renewed. It also has an effect on ileal morphology factors, especially villus length and the number of goblet cells.[31,32] Previous studies have not identified any positive effects of MTS on ileal digestibility and morphology. MTSs potentially are protective against intestinal diseases. However, the mechanisms of their action are not fully understood. Bean et al.[33] reported that silymarin has a good safety record, but some reports have indicated that it causes gastrointestinal disturbances and skin allergies.

Conclusions

In conclusion, these results suggest that MTSs might be used in chickens to prevent the effects of AFB1 in contaminated feed. This information provides a basis for further studies for the establishment of the mechanisms existing between MTS and protection against AFB1 toxicity. However, more research on this topic especially on the farm and field condition needs to be done to improve the safety and quality of poultry products.

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The effects of aflatoxin B1 and silymarin-containing milk thistle seeds on ileal morphology and digestibility in broiler chickens

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Abstract

This study investigated the effects of aflatoxin B_1 (AFB1) and milk thistle seed (MTS) on some apparent ileal morphology and digestibility variables in the small intestines of broilers. A total of 216 Ross 308 male broiler chickens were allocated in a 3 3 factorial arrangement of the treatments with three concentration of AFB1 (0, 250, and 500 ppb) and three levels of MTS (0, 5, and 10 g kg^{-1}). On day 35, the birds that received diets with 500 ppb of AFB1 alone (main effect) showed significant decrease in apparent ileal digestibility [dry matter (DM; 72.46±0.27), calcium (Ca; 40.81±1.11), crude protein (CP; 29.42±1.89), apparent digestible energy (2653±58.82)], ileal morphology [villus length (VL; 822.5±7.47), villus width (VW; 90.16±2.17) and ratio of VL to crypt depth (VL/CD; 4.74±0.07)] in their ileum segments (P<0.01). However, the mean nitrogen (N; 61.39±0.48) and crypt depth (CD; 173.5±9.87), in the ileum were significantly greater for the birds that were fed with 500 ppb AFB1 alone in their diets when compared with the control (P<0.01). Also, thistle seeds can ameliorate the toxic effects of AFB1 on some ileal digestibility factors, that is, DM, N, Ca, and CP, in broiler chicks. Nevertheless, ileum morphology of VW and goblet cell numbers were not affected negatively by the AFB1 plus MTS in diets. The results of this study indicated that the use of MTS independently reduced the toxic effects of AFB1, facilitated the absorption of nutrients, and reduced the metabolic demands of the intestinal tract in broiler chickens.

Introduction

Aflatoxin B_1 (AFB1) is a secondary metabolite produced by *Aspergillus flavus* and *A. parasiticus*, and it has carcinogenic, mutagenic, hepatotoxic, and teratogenic effects.[1,2] Several diseases are associated with the human consumption of these toxins, including toxic hepatitis and even primary hepatocellular carcinomas.[1,2] Aflatoxin B_1 8,9-epoxide is the reactive form of the compound, and it binds to cellular macromolecules and causes periportal hepatic injury.[3] However, extrahepatic effects, namely, within the intestine, have not been studied thoroughly. Other researchers have documented the negative effects of AFB1 on total tract retention of energy, mean nitrogen (N), and amino acids in poultry.[4-7] It seems that AFB1 alone has a harmful effect on the metabolization of nutrients, a harmful effect on the intestine, or both, resulting in increased loss of endogenous nutrients, reduced digestibility of nutrients, or both.[8] From the aforementioned studies, it is difficult to discern a dose-effect relationship between AFB1 and histological changes in the gastrointestinal tract (GIT). AFB1 is widely believed to result in malabsorption syndrome regarding macronutrients and also to result in reduced activity of digestive enzymes.[9,10] Silymarin is a mixture of flavonoids extracted from milk thistle seed (MTS) (*Silybum marianum* L. Gaertn.), and it contains silybin, silydianin, and silychristin as the major fractions.[11] Silymarin acts in five different ways; as an antioxidant, absorber and regulator of the intracellular glutathione, as a stabilizer and regulator of cell membrane permeability that prevents the entering of hepatotoxic substances into hepatocytes, as the ribosomal ribonucleic acid (rRNA) synthesis promoter stimulating regeneration of the liver and an inhibitor of the transformation of liver stellate cells into myofibroblasts.[12] This suggests that silymarin may contribute to the prevention of aflatoxicosis-induced damage.[13-15] There has been no reports that have dealt with the effect of interactions of AFB1 combined with MTS on the ileal morphology and digestibility of broilers to date. Thus, this study was conducted to evaluate the effects of simultaneous supplementation of AFB1 and MTS on ileal morphology and digestibility in broiler chickens.

Materials and Methods

Plants collection

Milk thistle seeds were collected from Kashmar-Kohsorkh district (16.35° north latitude, 18.58° east longitude, about 1052 meters above sea level) in Khorasan-Razavi province, in the north-east of Iran, during autumn 2011 (Figure 1).

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Contaminating poultry feed

A. flavus was obtained from the Center of Scientific and Industrial Research Organization in Iran, PTCC NO: 5004 (IR111), and was cultured on potato dextrose agar (PDA) medium and used for *in vitro* studies. The AFB1 content in rice powder was analyzed by the method of Shotwell *et al.*[16] and measured on a thin layer chromatography (TLC) fluorometric densitometer (Camag-iII, Basel, Switzerland) on the TLC spots. The yield of AFB1 produced was 60 ppb gr^{-25} of sample from each flask.

Experimental design

Combinations of three levels of AFB1 (0, 250, and 500 ppb) with three levels of MTS (0, 5, and 10 g kg^{-1}) were incorporated into the basal diet (corn and soybean meal). A total of 216 one-day-old chicks (Ross 308) were allocated to nine treatments with four replicates based on a completely randomized design in a 3×3 factorial arrangement. There were nine experimental diets with four replicates of six birds in each replicate. All of the birds were fed a typical, commercial diet for the 35 days of the experiment. The birds were housed in wire cages with nipple waterers and 516 cm^2 of floor space per bird. Feed and water were provided ad libitum. A basal diet was formulated on

corn-soybean meal base for starter, grower, and finisher periods according to [National Research Council, NRC (1994)][17] the recommendations. The birds received the following diets with equal energy and protein levels: (T_1) control basal diet; (T_2) basal diet plus 250 ppb AFB_1; (T_3) basal diet plus 500 ppb AFB_1; (T_4) 5 g kg^{-1} of MTS; (T_5) 5 g kg^{-1} MTS plus 250 ppb AFB_1; (T_6) 5 g kg^{-1} MTS plus 500 ppb AFB_1; (T_7) 10 g kg^{-1} MTS; (T_8) 10 g kg^{-1} MTS plus 250 ppb AFB_1; and (T_9) 10g kg^{-1} MTS plus 500 ppb AFB_1. MTSs were acquired from the outskirts of the Birjand district in South Khorasan Province, Birjand, Iran. Also, all animals received humane care in compliance with the guidelines of animal science Dept. at Birjand University, Birjand, Iran.

Ileal digestibility

At the end of experiment (day 35), two birds per pen were sacrificed, and two-thirds from Meckel's diverticulum to the cecal junction was removed (about 10 cm), and the ileal digesta were flushed with distilled water. The ileal digesta was collected and stored at –20°C, freeze-dried, and ground with a mortar and pestle before the analyses. Feed, excreta, and ileal digesta were analyzed for determination of nutrient digestibility and retention. The dry matter (DM) content was determined on ground diets and freeze-dried ileal digesta and excreta by drying the samples at 100°C for 24 h. Titanium (Ti) was determined by the inductively coupled plasma atomic emission spectroscopy method (AOAC, 1995)[18] following nitric-perchloric acid wet ash digestion. Gross energy (N) determinations of feed and excreta samples were performed in a bomb calorimeter (Gallenkamp Autobomb, Loughborough, UK) with benzoic acid as a standard.[19] The apparent digestible energy (AMEn) (excreta) and AMEn (ileal digesta) of the diets were calculated using the index method (using Ti as the digestive marker) by using the formula of Meng and Slominski[20] as described by the NRC (1994).[17] The calcium (Ca) concentration of the feed and digesta were determined by Flame Atomic Absorption Spectrophotometer (A Analyst 100, Perkin-Elmer Inc., Waltham, MA, USA). The crude protein (CP) content (N 6.25) of the diet and individual samples of digesta were determined by the Kjeldahl method (AOAC, 1995).[18]

Ileal morphology

On day 35, two birds per pen were sacrificed by rupture of their carotid arteries and jugular veins. Then, two-thirds (about 5 cm) of the ileum was removed and flushed with distilled water. The mucosa was collected by scraping with a microscope slide and subsequently frozen in liquid nitrogen. A 3-cm section of the proximal ileum (Mecke's diverticulum to the cecal junction) was rinsed with 0.01 M phosphate buffered saline (PBS, pH 7.2) and placed in a 10% buffered, neutral formaldehyde (pH 7.2 to 7.4) solution. As a result, all samples were gradually dehydrated, sectioned at 6 mm thickness, and stained with hematoxylin and eosin. VL, CD, VW, and the thickness of the epithelium were measured at 100 magnification using computer software (Sigma Scan, Jandel Scientific, San Rafael, CA, USA). Also, the ratio of VL/CD was calculated (Figure 2). Two slides were made for each intestinal sample, and each slide from the ileum sample was stained with Periodic acid-Schiff (PAS) reagent (McManus, 1948).[21] The tissues were deparaffinized, hydrated, oxidized in periodic acid (6 g L^{-1}) for 5 min, rinsed in distilled water, and then placed in Coleman's Schiff's reagent (Polysciences, Inc.) for 30 min. After 15 min, the slides were rinsed in tap water, the tissues were counterstained in hematoxylin, rinsed, dehydrated, and mounted. The positively stained, PAS GCN were enumerated on six villi per sample, and the means were utilized for statistical analysis (Figure 2). Measurements of VL and VW were taken from the tip of the villus to the valley between the individual villi, and measurements for CD were taken from the valley between the individual villi to the basolateral membrane (AOAC, 1995).[18]

Statistical analysis

The data were statistically analyzed with the standard procedures of analysis of variance (ANOVA), using a 3 3 factorial with completely

Table 1. Effect of aflatoxin B1 (AFB1) and milk thistle seed (MTS) on apparent ileal digestibility in broilers at the end of the period (35 day).

AFB1 (ppb)	MTS (g kg^{-1})	Dry matter, %	Nitrogen, %	Apparent digestible energy, Kcal kg^{-1}	Calcium, %	Crude protein, %
Treatment						
0	0	77.36±2.12^{a}	56.33±1.97^{b}	2754±49.77	53.38±1.68^{a}	39.41±2.38^{a}
250	0	74.32±2.46^{ab}	57.42±1.83^{b}	2674±38.62	44.21±1.87^{b}	28.44±2.23^{bc}
500	0	72.39±2.34^{b}	69.52±2.18^{a}	2621±57.76	41.24±2.14^{c}	26.41±3.12^{c}
0	5	72.41±3.01^{b}	55.38±1.84^{b}	2762±67.64	53.31±1.68^{a}	41.46±3.75^{a}
250	5	73.29±2.57^{b}	54.36±2.39^{b}	2681±81.65	43.38±1.93^{a}	31.59±3.35^{b}
500	5	76.33±2.67^{ab}	55.51±1.83^{b}	2664±69.13	40.77±1.49^{a}	30.44±2.66^{b}
0	10	72.58±2.96^{b}	53.69±2.74^{b}	2809±84.77	51.33±2.25^{a}	38.51±2.96^{b}
250	10	72.35±2.65^{b}	54.76±1.67^{b}	2727±73.65	42.45±2.17^{a}	36.52±4.11^{b}
500	10	74.26±2.75^{b}	59.14±1.96^{b}	2675±77.71	40.42±1.69^{a}	31.43±3.68^{b}
Main effects						
0	-	75.98±0.27^{a}	55.13±0.48^{b}	2775±58.82^{a}	52.68±1.11^{a}	41.79±1.89^{a}
250	-	73.32±0.27^{b}	55.52±0.48^{ab}	2685±58.82^{b}	43.35±1.11^{b}	32.18±1.89^{b}
500	-	72.46±0.27^{b}	61.39±0.48^{a}	2653±58.82^{b}	40.81±1.11^{b}	29.42±1.89^{c}
-	0	73.12±0.27	61.11±0.48^{a}	2674±58.82	46.27±1.11	31.41±1.89^{b}
-	5	74.11±0.27	55.12±0.48^{b}	2702±58.82	45.82±1.11	34.51±1.89^{ab}
-	10	74.69±0.27	55.86±0.48^{b}	2737±58.82	44.73±1.11	37.49±1.89^{a}
Probabilities (P value)						
AFB1		0.01	0.01	0.05	0.01	0.01
MTS		Ns	0.01	Ns	Ns	0.05
AFB1 × MTS		0.05	0.05	Ns	0.05	0.05

$^{a-c}$Means within a column lacking a common superscript differ significantly (P<0.05 and P>0.05). Ns: not significant.

randomized design, as suggested by Macros software.[22] The data were compared with Tukey-Kramer *post hoc* test. Least squares means ± standard errors are reported and P≤0.05 and 0.01 indicates statistical significance. All of the care and procedures used in testing the birds in this experiment were conducted from 21 March to 24 May 2012 at University of Birjand (South Khorasan Province in 59° 13 east longitude and 32° 53 north latitude, East-Iran).

Results

The results of this study indicated that the interaction effects between AFB1 and MTS were significant for apparent ileal digestibility, that is, DM, N, Ca, and CP (P<0.05) (Table 1). In contrast, retention and digestibility of AMEn were unaffected by the combinations of AFB1 and MTS. The interaction effect from apparent N digestibility indicated that a quadratic increase occurred when the amount of AFB1 administered was increased from 250 (57.42±1.83 ppb) to 500 (69.52±2.18 ppb) (P<0.05). Also, different levels of AFB1 did not cause significant changes in the Ca and CP of diets that contained 5 or 10 g kg^{-1} of MTS (intraction effect) (Table 1). Feeding of 5 or 10 g kg^{-1} of MTS increased the CP (P<0.05) and decreased apparent N (P<0.01) digestibility (main effect). In contrast, apparent DM, AMEn, and Ca retention were unaffected by different levels of MTS alone. However, the average apparent ileal digestibility of CP and Ca that contained 5 and 10 g kg^{-1} of MTS alone were higher than different levels of AFB1 (250 and 500 ppb). Also, interaction and the main effect from ileal morphology indicated that there was a linear increase in CD and a linear decrease in VL when using diets contaminated with AFB1 compared to the control animals that were not fed the contaminated food (Table 2). Also, VL/CD ratio in the ileum was decreased significantly (P<0.05 and 0.01) at the end of study (day 35). In contrast, interaction from VW and GCN was unaffected by consumption of AFB1 plus MTS (Table 2). Also, for the broilers that were fed with the contaminated diet, a main effect was a decrease in VW (90.67±2.17 to 90.16±2.17) (P<0.01).

Discussion

The mechanism of action of MTS in apparent ileal digestibility on animals is not clearly understood. Currently, it seems that this plant can be referred to AFB1 absorbent on apparent ileal digestibility in broiler chicks. Diaz *et al.*[23] reported that low levels of AFB1 in the diet did not affect DM and N digestibility in birds. Verma *et al.*[24] reported a reduction in net protein utilization and AMEn when 1 to 2 mg kg^{-1} of AFB1 was fed to broiler chicks. The results of this study were in agreement with those of previous studies when the levels of AFB1 alone were increased from 250 to 500 ppb (main effect). Kermanshahi *et al.*[7] also noted differences in energy and protein utilization with low-level inclusion of AFB1 in the feed given to broiler chicks. In this report, feeding of 0.8 to 1.2 mg kg^{-1} of AFB1 reduced AMEn and apparent N retention.[7] Also, when apparent N retention was corrected for uric acid excretion, the differences were negated, suggesting a reduction in uric acid excretion and, plausibly, a reduction in amino acid digestibility.[25] Although the interactions between aflatoxin and MTSs are not clear, there are two possibilities, that is, first, MTSs may increase protein absorption by increasing its solubility in digesta and, as a result, by prolonging the transfer time in the small intestine and second, MTSs may provide better conditions for the action of ileal enzymes by acidification of the diet and the digestive fluids.[26] The effects of higher dosages of AFB1 in broilers on these variables are not known. Contrary to the observations in broilers, other authors noted a linear increase in the crypt depth in the distal jejunum with increasing levels of AFB1 in the diet, that is, 0, 0.6, 1.2, and 2.5 mg kg^{-1}, but they observed no effects of the toxin on villus height or the number of goblet cells.[27] From the recent studies of broilers by Kana *et al.*[28], Yunus *et al.*[27] and Kumar and Balachandran,[8] it appeared that the unit absorptive surface of the small intestine

Table 2. Effect of aflatoxin B1 (AFB1) and Milk thistle seed (MTS) on ileal morphology variables in broilers at the end of the period (35 day).

AFB1 (ppb)	MTS (g kg^{-1})	Villus length, µm	Villus width, µm	Crypt depth, µm	Ratio*	Goblet cell number**
Treatment						
0	0	844.4±8.22^{a}	93.23±3.34	147.3±18.34^{b}	5.74±0.13^{a}	10.93±1.23
250	0	824.6±8.17^{b}	90.24±3.62	153.5±18.68^{b}	5.37±0.12^{b}	11.92±1.12
500	0	819.6±8.51^{c}	89.61±4.11	177.3±19.66^{a}	4.62±0.14^{c}	14.45±1.38
0	5	841.5±9.12^{a}	93.18±5.75	146.3±19.58^{b}	5.76±0.14^{a}	11.54±1.62
250	5	825.4±9.28^{b}	90.34±5.95	150.3±14.55^{b}	5.51±0.14^{a}	11.35±1.18
500	5	822.3±9.61^{b}	88.44±4.47	169.6±15.71^{ab}	5.85±0.12^{a}	11.69±1.25
0	10	842.3±8.44^{a}	93.38±4.44	144.6±19.77^{b}	5.84±0.15^{a}	11.42±1.46
250	10	827.2±9.98^{b}	91.45±4.46	151.3±19.78^{b}	5.47±0.13^{a}	11.53±1.52
500	10	825.7±9.39^{b}	92.14±4.89	173.5±16.87^{ab}	4.75±0.17^{a}	11.73±1.22
Main effects						
0	-	842.7±7.47^{a}	93.26±2.17^{a}	146.1±9.87^{c}	5.74±0.07^{a}	11.31±0.43
250	-	825.7±7.47^{b}	90.67±2.17^{b}	151.7±9.87^{b}	5.45±0.07^{b}	11.62±0.43
500	-	822.5±7.47^{b}	90.16±2.17^{b}	173.5±9.87^{a}	4.74±0.07^{a}	12.62±0.43
-	0	829.5±7.47	91.13±2.17	159.3±9.87	5.24±0.07	12.44±0.43
-	5	829.7±7.47	90.65±2.17	155.4±9.87	5.37±0.07	11.53±0.43
-	10	831.7±7.47	92.32± 2.17	156.4±9.87	5.35±0.07	11.56±0.43
Probabilities (P value)						
AFB1		0.01	0.01	0.01	0.01	Ns
MTS		Ns	Ns	Ns	Ns	Ns
AFB1 × MTS		0.05	Ns	0.05	0.05	Ns

$^{a-c}$Means within a column lacking a common superscript differ significantly (P<0.05). *Ratio of villus length to crypt depth. **Numbers in area of epithelial cells. Ns: not significant.

Figure 1. Milk thistle plant collected from the outskirts of Kashmar-Kohsorkh district in Khorasan-Razavi province, Iran.

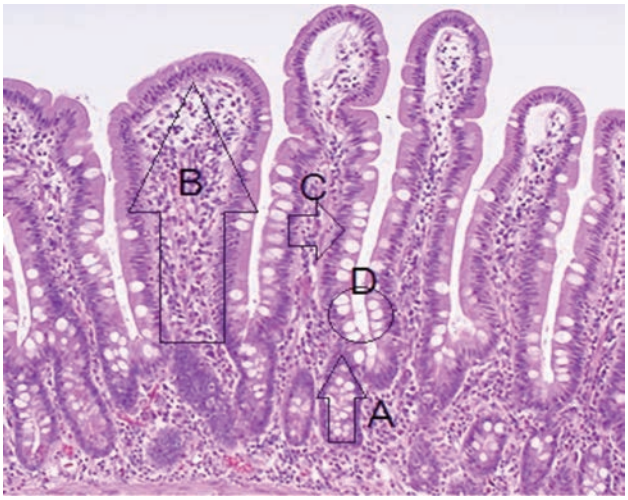

Figure 2. Ileal morphology variables measured at 100 X magnification: A) Crypt depth; B) Villus length; C) Villus width; D) Goblet cells.

deteriorated during chronic exposures to low levels of AFB1. Administration of AFB1 resulted in a reduction of T cells and alkaline phosphatase activity in the intestine.[29] Also, enterocytes and other ileal enzymes must differentiate during their time along the axis of the crypt-villus to fully express these digestive functions.[25-30] However, intestinal mucin production and secretion is a dynamic process that is continually degraded and renewed. It also has an effect on ileal morphology factors, especially villus length and the number of goblet cells.[31,32] Previous studies have not identified any positive effects of MTS on ileal digestibility and morphology. MTSs potentially are protective against intestinal diseases. However, the mechanisms of their action are not fully understood. Bean et al.[33] reported that silymarin has a good safety record, but some reports have indicated that it causes gastrointestinal disturbances and skin allergies.

Conclusions

In conclusion, these results suggest that MTSs might be used in chickens to prevent the effects of AFB1 in contaminated feed. This information provides a basis for further studies for the establishment of the mechanisms existing between MTS and protection against AFB1 toxicity. However, more research on this topic especially on the farm and field condition needs to be done to improve the safety and quality of poultry products.

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Juvenile sterile granulomatous dermatitis (puppy strangle) in Pekingese and German shepherd puppies

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Abstract

Juvenile sterile granulomatous dermatitis is an uncommon granulomatous and pustular disorder of the face, pinnae, and submandibular lymph nodes of puppies. A 10-week-old male Pekingese and a 8-week-old female German shepherd presented with submandibular lymphadenomegaly, skin lesions on muzzle and periocular area (Papules, crusts and pustules). The case did not respond to antibiotic therapy. Results of a hemogram, biochemical panel, and urinalysis were normal. Due to skin scraping, cytology examination (impression smear), fungal and bacterial culture and response to therapy puppy strangle (juvenile cellulitis) was diagnosed. The puppies made a full recovery on glucocorticoid therapy. The present case report describes the first report of juvenile sterile granulomatous dermatitis in Iran.

Introduction

Juvenile cellulitis (juvenile pyoderma, puppy strangles, juvenile sterile granulomatous dermatitis and lymphadenitis) is an uncommon granulomatous and pustular disorder of the face, pinnae, and submandibular lymph nodes, usually of puppies.[1-3]

Puppies can be febrile, depressed, and anorexic. There is an acute swelling of the muzzle, lips, and eyelids. Sterile pustules often develop in the skin of these areas as well as on the inner surface of the pinnae. Otitis externa is common, and pinnae are frequently thickened and edematous.[3,4]

After the pustules rupture, small ulcers, draining tracts, seropurulent exudates, or crusts can develop. Submandibular lymphadenopathy occurs and, occasionally, lymph nodes will abscessate and drain. Nodules over the trunk, preputial, and perineal areas due to pyogranulomatous panniculitis, as well as sterile suppurative arthritis, have been reported in a small number of cases. Permanent areas of alopecia and scarring may result if the lesions are extensive.[3]

Diagnosis is based on history, physical examination, and result of skin biopsy of early lesions.[4-6]

Reported treatments are glucocorticoids and antibiotics, administrated daily until response, then tapered.[3,4,6]

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Contributions: the authors contributed equally.

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Case Report #1

A 10-week-old, 1 kg body weight, male Pekingese dog was presented to the clinic of Faculty of Veterinary Medicine, Shiraz University, Iran with clinical signs of depression, anorexia and lesions on muzzle and eyelids. The dog had been treated with Penicillin G (30 mg/kg, IM, q24h for 5 days) by the referring veterinarian, but this treatment did not lead to any significant improvement in the dog's condition. On physical examination, the puppy was pyrexic (39.6°C), and heart and respiratory rates were within normal range. Submandibular lymphadenomegaly was observed. Papules, crusts and pustular lesions localized on the chin, muzzle and periocular area of this dog were detected (Figure 1A,B). Additionally, otitis externa was present (Figure 1C). Results of a hemogram, biochemical panel, and urinalysis were normal. Impression smears from the exudative lesions from skin or the ear revealed macrophages, with non-degenerate neutrophils. Microorganisms were not seen (Figure 2). No significant parasites, bacteria, and fungi were found on deep and superficial skin scraping and cytology examination. Culture results for bacteria and dermatophytes were negative. Diagnosis of juvenile sterile granulomatous dermatitis was made according to the clinical signs, cytology

Figure 1. Case #1: A) lesions in facial region before treatment and B) 7 days after treatment (improvement). C) Otitis externa and purulent exudates in ear of the dog with juvenile cellulitis.

finding and the results of fungal and bacterial culture. Prednisolone (2 mg/kg, po, q24h) for 14 days and continuing for another one week (1 mg/kg, po, 24h) was prescribed. Significant improvement was seen during the first 24-48 hours of treatment (Figure 1A,B). The three-month follow up showed no regression.

Case Report #2

A female 8-week-old German shepherd was referred to clinic with similar signs of case A (Papules, pustules and crusts). In contrast to case 1, there was no otitis or pyrexia. The lesions did not respond to topical treatment with Tetracycline (q12h) and Co-amoxiclav (25 mg/kg q12h). Similar examinations (cytology, skin scraping, fungal and bacterial culture) were made and the same results were obtained. Treatment and signs of improvement were similar (to case 1). Skin lesions before and 7 days after treatment are shown in Figure 3.

Results and Discussion

The present case report describes the first report of juvenile sterile granulomatous dermatitis in Iran. Juvenile cellulitis is an uncommon acute dermatitis of dogs. Usually, puppies are affected between the ages of 3 weeks and 4 months, and one or several puppies in a litter may have the condition. The etiology and pathogenesis of this condition are unknown. An immunologic abnormality may be involved, as glucocorticoid therapy results in resolution of lesions. There is some evidence for a hereditary factor, as some breeds as well as particular lines within a breed, are predisposed. Predisposed breeds include the golden retriever, dachshund, beagle, pointer and Gordon setter.[3-5] In the present cases, the dogs were Pekingese and German shepherd puppies and both were non predisposed breeds. Surprisingly, although the sibling of Pekingese puppy (case 1) was kept elsewhere, he had the similar lesions (he was not referred to the mentioned clinic for further investigation). It could be attributed to the hereditary nature of the disease.

The main differential diagnoses include angioedema, staphylococcal dermatitis, demodicosis, and adverse cutaneous drug reaction.[3] Due to marked regional lymphadenopathy, systemic illness, impression smear and skin scraping results and no history of medication and vaccination, these possibilities (diagnosis) were ruled out. Besides, response to treatment with no regression revealed no possibility of demodicosis, pyoderma or any autoimmune disease (like *Pemphigus foliaceus*).[4,5] As in these cases, cytologic examination of papulopustular lesions of juvenile cellulitis reveals pyogranulomatous inflammation with no microorganisms and bacterial or fungal cultures are negative. Biopsies of early lesions reveal multiple discrete or confluent granulomas and pyogranulomas consisting of clusters of large epithelioid macrophages with variably sized cores of neutrophils.[3]

These two cases were the only puppy strangles cases in 2014 and among 2100 cases that referred to Shiraz Veterinary School Clinic during this time, the prevalence of this disease is 0.09%.

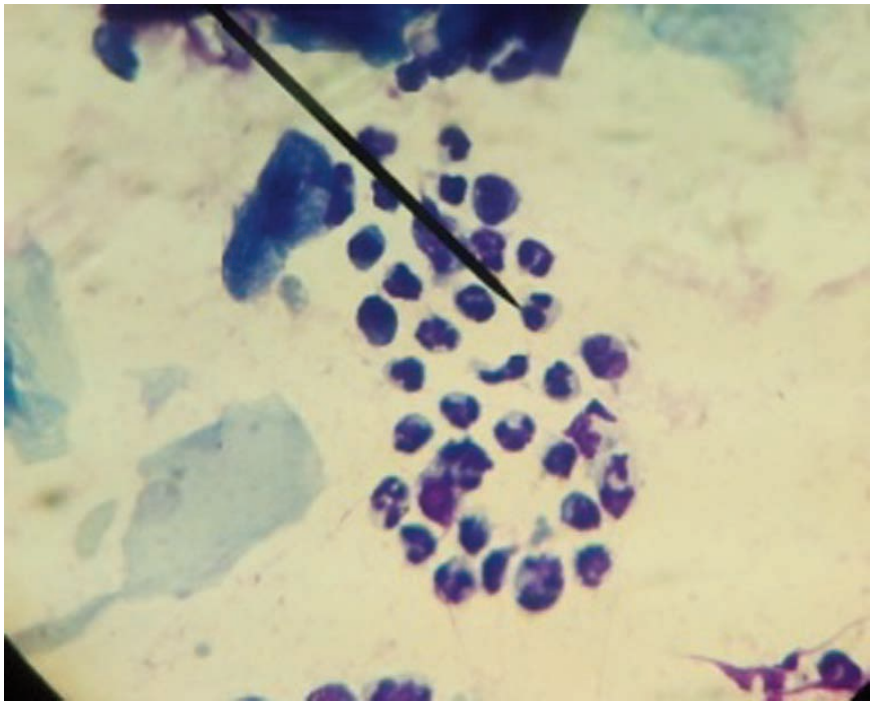

Figure 2. Non-degenerate neutrophils (pointer) in the dog with juvenile cellulitis.

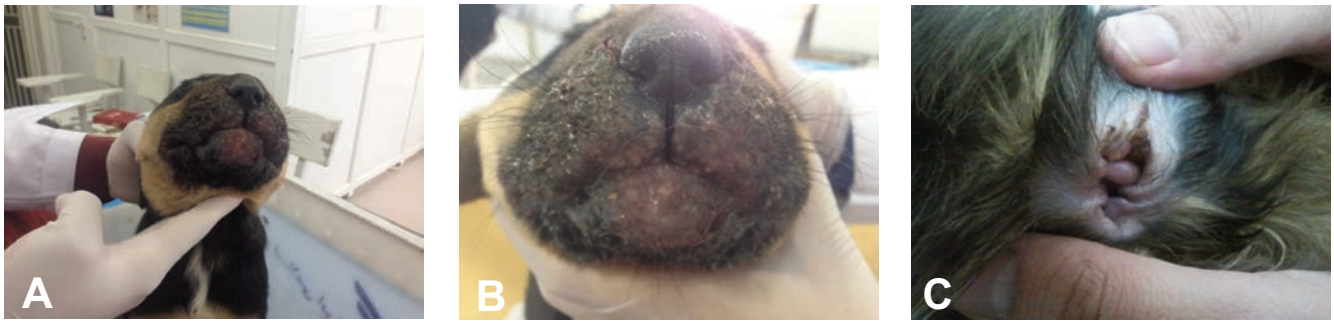

Figure 3. Case #2, A,B) lesions in facial region before treatment and C) 7 days after treat

Conclusions

The juvenile cellulitis could be fatal if not treated. The prognosis is good and recovery without scarring is possible if early treatment is undertaken.[5] Prednisolone (with or without cyclosporine), oral dexamethasone and topical therapy, especially wet soaks with aluminum acetate or magnesium sulfate have been mentioned in treatment of this disease.[3-5] The present cases were successfully treated with oral prednisolone.

When puppies are first presented with what appears to be staphylococcal pyoderma, juvenile cellulitis, a relatively rare condition, may not be considered. However, it is important that the possibility of juvenile cellulitis be explored early,[1,6] allowing for initiation of glucocorticoid therapy, which is contraindicated for treatment of bacterial pyoderma.[1,3,6]

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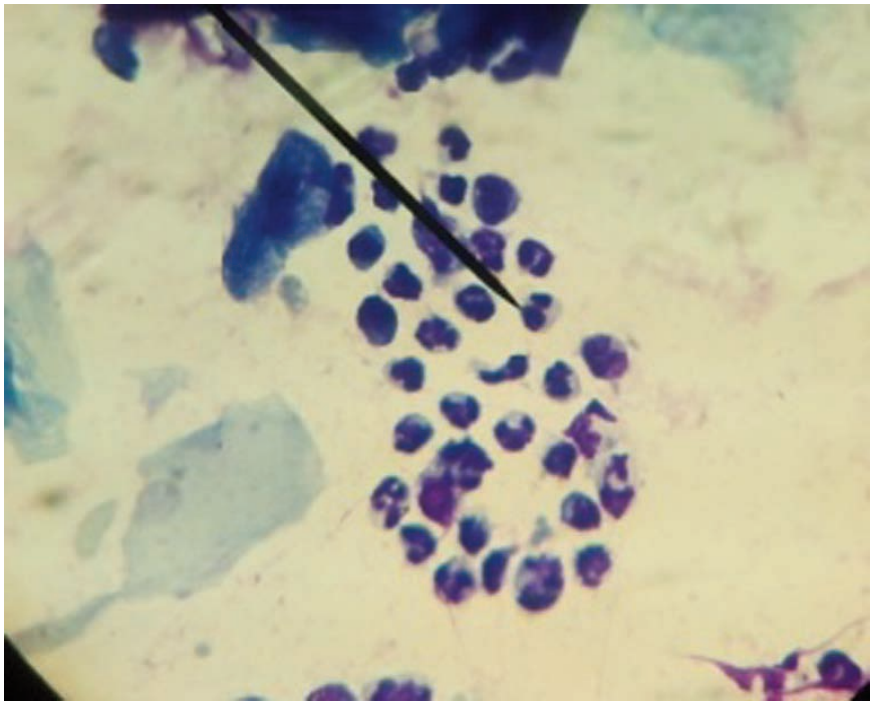

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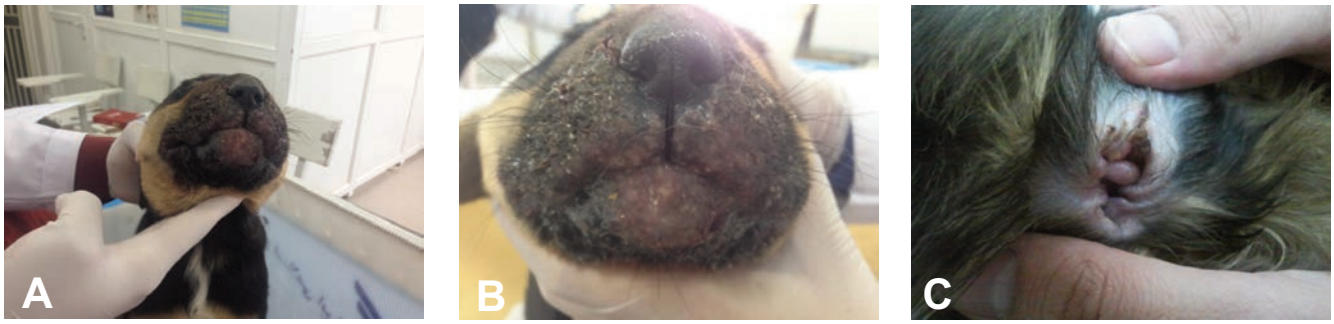

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Cd and Hg significantly (P<0.05) decreased the total anaerobic bacteria both in day 21 and 42 of sampling. Also, our data indicated that usage of probiotics significantly (P<0.05) affected the anaerobic count and increased the number of bacteria from 7.32 Log CFU/g to 7.54 Log CFU/g.

Figure 3 represents the effect of synbiotic diet (probiotic and prebiotic) on Lactic Acid Bacteria (LAB) count of stool in rats exposed to Cd and Hg. Data showed that there was significant change in LAB counts in heavy metal groups compared with control group which caused reduction in the LAB microbiota population from 8.83 Log CFU/g to 7.16 Log CFU/g. Both Cd and Hg significantly decreased the total LAB in day 21 and 42 of sampling. The presence of probiotics in heavy metal treated-rat diets significantly affected the total LAB and increased the population to 8.77 Log CFU/g compared with heavy metal group. Both *L. plantarum* and *B. coagulans* produced an equal effect on total LAB counts. Statistically significant differences were observed for the count of *L. plantarum* in heavy metals-treated groups and synbiotic groups (Table 2). Prescription of *L. plantarum* in rat diets caused significant increase (P<0.05) in the *L. plantarum* populations in rat intestine and faeces in day 21 and 42 of sampling that the number of the bacterium from 7.26 and 6.93 Log CFU/g in control group increased to 8.17 and 7.90 Log CFU/g in *L. plantarum* group, respectively. Cd and Hg significantly decreased the *L. plantarum* count in rats that did not receive this bacteria both in day 21 and 42 of sampling. Particularly, feeding *L. plantarum* in Cd and Hg treated groups cause significant increase in *L. plantarum* count (Table 2).

Table 3 shows the effect of synbiotic supplement on *B. coagulans* count of stool in rats exposed to Cd and Hg. The data revealed that application of *B. coagulans* in rat diets caused significant increase (P<0.05) in the *B. coagulans* populations in rat stool in day 21 and 42 of sampling that the number of *B. coagulans* from 4.47 Log CFU/g in control group increased to 7.36 and 7.84 Log CFU/g in *B. coagulans* group, respectively. Cd and Hg significantly decreased the *B. coagulans* count in rats that did not receive this bacteria both in day 21 and 42 of sampling. Feeding *B. coagulans* in Cd and Hg treated groups cause significant increase in *B. coagulans* count that raise the number (Table 3).

Table 3. Effect of synbiotic diets on *Bacillus coagulans* count of stool in rats exposed to cadmium and mercury.

Treatments	*Bacillus coagulans* count (Log CFU/g)	
	Day 21	Day 42
Control	4.47±0.07[a]	4.47±0.10[a]
Lp	7.36±0.04[b]	7.84±0.07[b]
Cd	2.45±0.30[c]	2.41±0.25[c]
Lp+Cd	5.49±0.06[d]	5.40±0.03[d]
Hg	2.63±0.11[c]	2.55±0.14[c]
Lp+Hg	5.70±0.05[d]	5.65±0.05[d]

Results are mean ± SD. Bc: *B. coagulans*, Cd: cadmium, Hg: mercury.Different letters indicate significant differences between treatments (P<0.05)

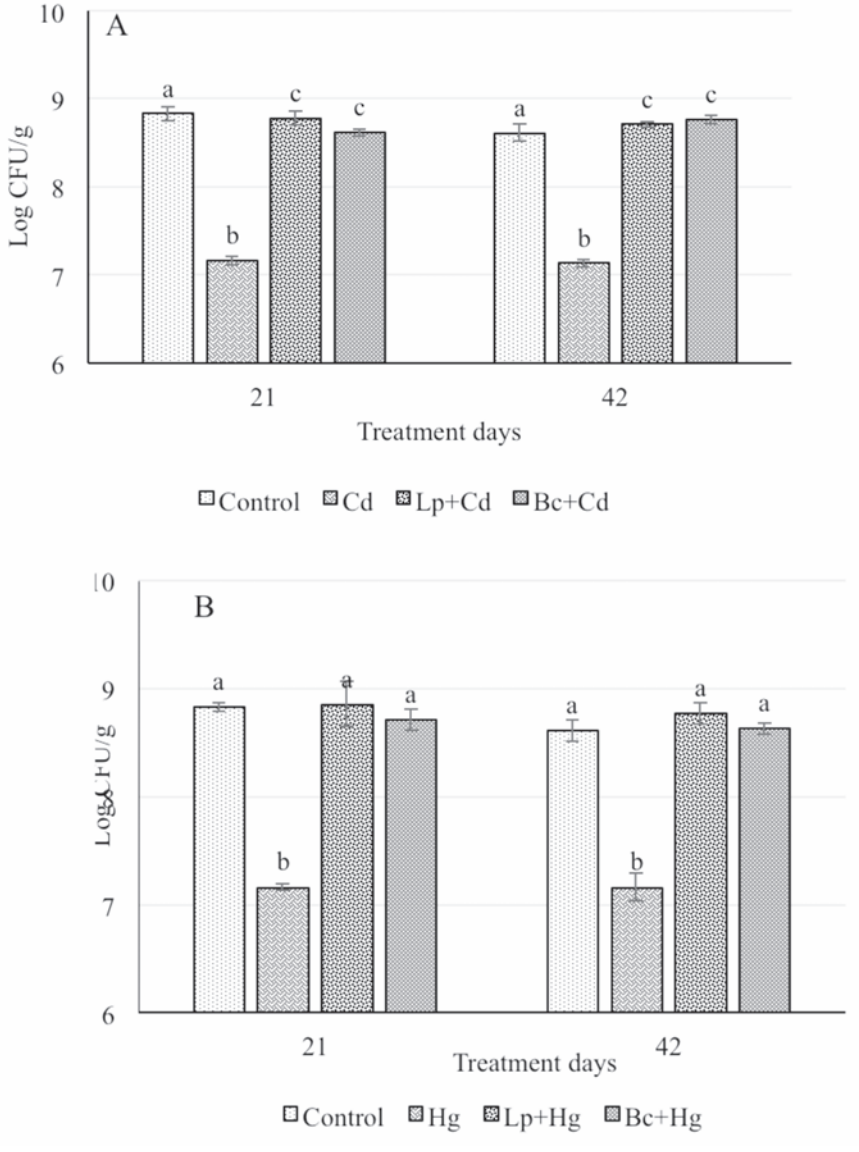

Figure 3. Effect of synbiotic diets on lactic acid bacteria count of stool in rats exposed to cadmium (A) and mercury (B). Cd: cadmium, Hg: mercury, Lp: *L. plantarum*, Bc: *B. coagulans*. The different letters indicate statistically significant differences between groups in each day of sampling (P<0.05).

Discussion

The heavy metals poisoning has become a major concern in industrialized countries. Here, a murine model was used to examine the effect of synbiotic supplementation on the intestinal microbiota of rats that had been poi-

Impact of synbiotic diets including inulin, *Bacillus coagulans* and *Lactobacillus plantarum* on intestinal microbiota of rat exposed to cadmium and mercury

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Abstract

The aim of this study was to investigate the efficacy of two probiotics and a prebiotic (inulin) on intestinal microbiota of rats exposed to cadmium and mercury. Fifty-four male Wistar rats were randomly divided into nine groups. All groups except control group were fed standard rat chow with 5% inulin and treated as follows: i) control (standard diet), ii) *Lactobacillus plantarum*-treated group (1×10^9 CFU/day), iii) *Bacillus coagulans*-treated group (1×10^9 spores/day), iv) cadmium-treated group (200 µg/rat/day), v) *L. plantarum* and cadmium-treated group, vi) *B. coagulans* and cadmium-treated group, vii) mercury-treated group (10 µg/rat/day), viii) *L. plantarum* and mercury-treated group, ix) *B. coagulans* and mercury-treated group. Cadmium, mercury and probiotics were daily gavaged to individual rats for 42 days. Treatment effects on intestinal microbiota composition of rats were determined. Data showed that cadmium and mercury accumulation in rat intestine affected the gastrointestinal tract and had a reduction effect on all microbial counts (total aerobic bacteria, total anaerobic bacteria, total Lactic acid bacteria, *L. plantarum* and *B. coagulans* counts) compared to the control group. It was also observed that application of synbiotics in synbiotic and heavy metals-treated groups had a significant effect and increased the number of fecal bacteria compared to the heavy metals groups. Based on our study, it can be concluded that *L. plantarum* and *B. coagulans* along with prebiotic inulin play a role in protection against cadmium and mercury inhibitory effect and have the potential to be a beneficial supplement in rats' diets.

Introduction

Gastrointestinal microbiota consists of a complex of microorganism species that live in the digestive tracts of human and other mammals. Bacteria make up most of the flora in the colon and up to 60% of the dry mass of feces.[1] Bacteria in the gut fulfill a host of useful physiological functions and have a direct impact on human health, including digestion of unutilized energy substrates,[2] repressing the growth of harmful microorganisms, helping with the production of some vitamins (B and K), training the immune system to respond only to pathogens and defending against some diseases.[3,4] Gut microbiota's balance can be affected through some conditions due to their high sensitivity to physiochemical and environmental factors. These factors consist of antimicrobial agents, disorders of peristalsis, inflammatory bowel diseases, cancer, stress, redox potential, drugs, temperature and nutrients.[5]

Heavy metals are other factors that have toxic effects on the gut ecosystem. Cadmium (Cd) and mercury (Hg) are such heavy metals that have become a major concern for public health. Cd is present at low concentrations in soil, rock and drinking water.[6] Because of its highly soluble nature compared to other metals, Cd is taken up by plants and is stored in food and feed production.[7] Dietary exposure to large Cd doses has been reported to result in adverse health effects in the kidneys, liver, bone, mammary gland, breast, pancreas and colon.[8-10] Liu *et al*.[11] reported that Cd exposure has toxic effects on microbiota of the intestinal tract in mice. Hg can also be found in air, water and soil. Fish and shellfish are main sources of this toxic element. Hg exposure at high levels can harm the brain, heart, kidneys lungs, and immune system.[12,13] Many studies investigated how early gut development may be stimulated and overall efficiency of the intestinal microbiota progressed by accumulation of synbiotics (probiotic and prebiotic) with feed.[14] Lactic acid bacteria (LAB) such as *L. acidophilus*, *L. plantarum* and *Bifidobacterium* are the most claimed probiotics. These probiotics are very sensitive to normal physiological conditions such as the very low pH of the stomach and bile salts.[15,16] Hence, a novel beneficial probiotics is introduced that can survive under extreme status. Some strains of *B. coagulans* are able to stand through the gastrointestinal tract and continue their metabolic activities via spore production.[17,18] Prebiotics are typically non-digestible fibre compounds that pass undigested through the gastrointestinal tract and stimulate the growth and activity of advantageous bacteria like probiotics.[19] Accordingly, the purpose of the present study is to investigate the influence of two probiotics (*L. plantarum* and *B. coagulans*) and a prebiotic (inulin) on gut microbiota of rats exposed to Cd and Hg.

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Materials and Methods

Preparing suspension of probiotic bacteria

The bacterial strains used in this study were *L. plantarum* (NCDO 1193) and probiotic strain of *B. coagulans*. Lyophilized *L. plantarum* CNR273 was obtained from the culture collection of the Department of Food Science and Technology, Shiraz University, Iran and was plated aerobically on the De Man Rogosa Sharpe (MRS) agar (Difco, Detroit, MI, USA) at 37°C for 48 h. Then, a single colony was inoculated into 500 ml of MRS broth and incubated at 37°C with shaking at 250 rpm for 48 h. The *L. plantarum* pellets were achieved by centrifugation at 3000×*g* for 20 min and washed with sterile normal saline. To determine the viable bacterial cells per ml of suspension, appropriate serial dilution was done and plated in MRS agar. Lyophilized probiotic *B. coagulans* were donated by the Pardis Roshd Mehregan Company, Iran. Spore suspension of the bacterium (1×10^9 spore/mL of sterile saline) was prepared according to the method of Abhari *et al.*[20]

Preparation of rat diets

The experimental diets were based on the standard diets for rats plus 5% chicory based inulin (Roosendaal, The Netherlands) and contained 24.5% protein, 6.2% ash, 52.2% starch, 6.6% sugar, 6.5% fat and 4% moisture. Regarding micronutrients, the rat chow contained 0.72% calcium, 0.6% phosphorus, 0.25% chloride and 0.23% magnesium among others. The inulin content in the rat's diet was calculated based on food intake. The food intake of each rat with mean of 200 g body weight is 10 g/day, so feedstuff was mixed with 5% inulin; it means each rat received 0.5 g inulin/day.

Preparation of heavy metal solutions

The preparation of Cd and Hg solutions was done according to the method of Nwokocha *et al*.[21] The $CdCl_2$ (Merck, Darmstadt, Germany) solution (200 µg/mL) was administered continuously at a dose of 200 µg/rat/day. The $HgCl_2$ (Merck) solution (10 µg/mL) was performed continuously at a dose of 10 µg/rat/day. Both metals were fed to each rat using a special gavage needle.

Animals and treatment

Fifty-four male Wistar rats weighing 170±10 g were purchased from the Razi Vaccine and Serum Research Institute, Shiraz, Iran. Rats were kept in stainless steel cages under standardized conditions at temperature of 23±2°C, relative humidity of 60±5% and exposure to a 12 h light/dark cycle with ad libitum access to diet and tap water.

After an acclimatization period of 1 week, as shown in detail in Table 1, the animals were randomly divided into nine groups (n=6/group) and treated for 42 days. All the experimental procedures were done following the ethical guidelines of the animal welfare (approved by Shiraz University animal welfare laws, guidelines and policies in Iran).

Culturing of fecal microbiota

Fresh fecal samples were collected from each rat on two mentioned days of the experimental period (21 and 42) by gently handling their tails to induce defecation. Then, samples were immediately brought to the laboratory, accurately weighed and diluted in a ratio of 9:1 with sterile saline. After homogenizing for 90 s using a stomacher (Model BA6021, Steward Lab., UK), specimens were diluted in 10-fold dilution solution (saline) to count the bacterial load.

Total aerobic bacteria count

Plate count agar (PCA, Merck, Germany) was used for detection of total aerobic bacterial. Plates were incubated aerobically at 37°C for 24 h.

Table 1. Treatment groups used in the experimental study.

Treatment groups	Feeding	Gavaging (1 mL volume, once daily)
Control	Standard diet	Normal saline
Lp	Standard diet + 5% inulin	*L. plantarum* (1×10^9 CFU/mL)
Bc	Standard diet + 5% inulin	*B. coagulans* (1×10^9 spore/mL)
Cd	Standard diet + 5% inulin	Cadmium (200 mg/L)
Lp+Cd	Standard diet + 5% inulin	Cadmium + *L. plantarum*
Bc+Cd	Standard diet + 5% inulin	Cadmium + *B. coagulans*
Hg	Standard diet + 5% inulin	Mercury (10 mg/L)
Lp+Hg	Standard diet + 5% inulin	Mercury + *L. plantarum*
Bc+Hg	Standard diet + 5% inulin	Mercury + *B. coagulans*

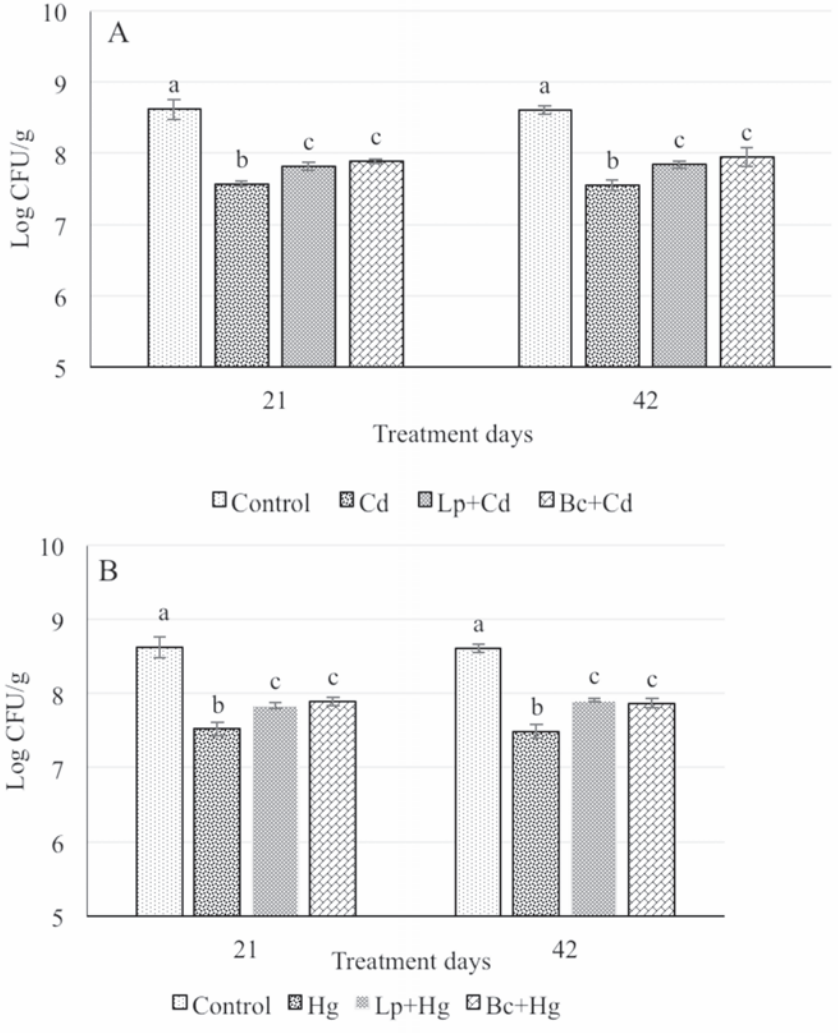

Figure 1. Effect of synbiotic diets on total aerobic count of stool in rats exposed to cadmium (A) and mercury (B). Cd: cadmium, Hg: mercury, Lp: *L. plantarum*, Bc: *B. coagulans*. The different letters indicate statistically significant differences between groups in each day of sampling (P<0.05).

Total anaerobic bacteria count

Total anaerobic bacteria were counted on PCA with anaerobic condition (Anaerocult A®, Merck, Germany). Incubation was done at 37°C for 24 h.

Total lactic acid bacteria count

MRS agar was used for the enumeration of total LAB. All plates were aerobically incubated at 37°C for 48 h.

Lactobacillus plantarum count

The number of *L. plantarum* was determined by applying appropriate dilutions on the MRS agar supplemented with 4 mg/L ciprofloxacin (Sigma, USA). The plates were aerobically incubated at 37°C for 48 h.

Bacillus coagulans count

NYSM agar (0.5% NaCl, 0.5% pepton, 0.3% beef extract, 0.05% yeast extract, 0.01% $CaCl_2$, 0.02% $MgCl_2$, 0.001% $MnCl_2$, 1% glucose and 1.5% agar) was used for the enumeration of *B. coagulans*. NYSM agar plates were aerobically incubated at 37°C for 24 h.

Statistical analysis

The results are expressed as mean ± SD. Statistical analysis for significant differences among group means was tested by one-way analysis of variance (ANOVA), followed by Duncan's post hoc test with the help of a software SPSS 16.0 windows. P<0.05 was considered significance level.

Results

Figure 1 shows the effect of synbiotic diet (probiotic and prebiotic) on total aerobic counts in rats exposed to Cd and Hg. The number of Log Total Aerobic Count (TAC) in the control group was 8.61 CFU/g. Cd and Hg significantly decrease the total aerobic bacteria both in day 21 and 42 of sampling. Addition of synbiotic in heavy metal groups caused changes in Log TVC level. The results showed that feeding two probiotics, *L. plantarum* and *B. coagulans* to Cd and Hg- treated rats significantly (P<0.05) increased the total aerobic counts. For example in Hg- treated rats the Log TVC at day 42 was 7.49 CFU/g but applying *L. plantarum* increased the Log TVC to 7.91 CFU/g.

The effect of synbiotic diet (probiotic and prebiotic) on total anaerobic counts in rats exposed to Cd and Hg is shown in Figure 2. The number of log total anaerobic count in the control group was 8.84 Log CFU/g. Consumption of Cd in treated rats decreased the number to 7.26 CFU/g and in Hg-treated rats to 7.32 Log CFU/g. The data indicated that

Table 2. Effect of synbiotic diets on *Lactobacillus plantarum* count of stool in rats exposed to cadmium and mercury.

Treatments	*Lactobacillus plantarum* count (Log CFU/g)	
	Day 21	Day 42
Control	7.26±0.02[a]	6.93±0.15[a]
Lp	8.17±0.06[b]	7.90±0.04[b]
Cd	4.82±0.10[c]	4.37±0.06[c]
Lp+Cd	7.26±0.10[a]	7.22±0.05[d]
Hg	5.01±0.01[d]	5.06±0.09[e]
Lp+Hg	7.30±0.06[a]	7.39±0.02[d]

Results are mean ± SD. Lp: *L. plantarum*, Cd: cadmium, Hg: mercury. Different letters indicate significant differences between treatments (P<0.05)

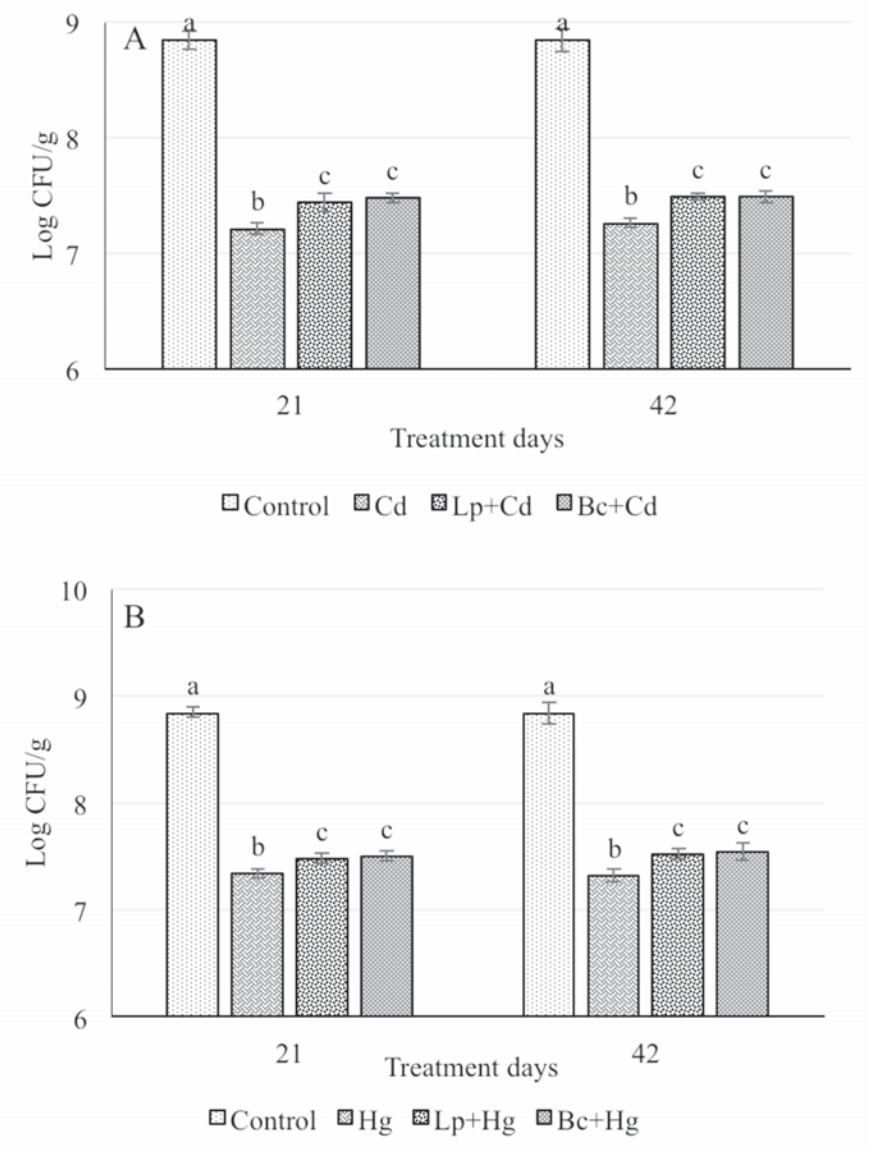

Figure 2. Effect of synbiotic diets on total anaerobic count of stool in rats exposed to cadmium (A) and mercury (B). Cd: cadmium, Hg: mercury, Lp: *L. plantarum*, Bc: *B. coagulans*. The different letters indicate statistically significant differences between groups in each day of sampling (P<0.05).

soned with two heavy metals, cadmium and mercury. In this study, we focused on the microbiota and its response to host consumption of Cd and Hg along with synbiotic supplement.

Two probiotic bacteria (*B. coagulans* and *L. plantarum*) and prebiotic (inulin) were applied as synbiotic diets. It was observed that cadmium and mercury accumulation in rat intestine affected the gastrointestinal tract and impaired the gut barrier. Diets containing heavy metals (Cd or Hg) were investigated to determine whether they have a reduction effect on all microbial counts in treated groups. Many reports indicated that heavy metals (Cd, Zn, Cs) have an inhibitory effect on bacteria by delaying the initiation of bacterial growth and inhibiting their growth.[22-24] Ghorbani *et al.*[25] expressed that sufficient metal exposure will result in immediate death of microorganisms due to disruption of essential functions, and to more gradual changes in population sizes due to changes in viability or competitive ability. Probiotic bacteria *B. coagulans* and *L. plantarum* received significant stress from the two mentioned heavy metals (Cd and Hg) compared to the non-exposed groups. Our result is confirmed by the report of Liu *et al.*[11] that Cd treatment could decrease the population of gut bacteria and the thickness of mice inner mucus layer was also attenuated by Cd treatment.

In contrast, it was found that in treated synbiotic + heavy metals groups the microbial counts were not so reduced compared with heavy metals groups. It seems that when the number of bacteria is low the heavy metals affect them, but in synbiotic + heavy metals groups by increasing the probiotic bacteria heavy metals' effect is reduced. In recent years studies have demonstrated that some bacteria such as probiotics have the largest role in binding metals, preventing their entry to the body and, thus, protecting the host.[26,27] It was found that inulin and probiotics had a considerable effect on fecal microbiota. According to Van Heugten *et al.*[28] dietary supplement with probiotics can potentially alter gut microbiota by selectively stimulating the growth of beneficial bacteria while suppressing the growth of pathogenic bacteria. Also, Lin *et al.*[29] reported that the use of probiotics in the feed enhanced Lactobacillus number and reduced *E. coli* population indicating that dietary supplements are efficient to improve broiler intestinal microbiota balance.

Moreover, the utilization of inulin in rat diet as a prebiotic ingredient demonstrated a remarkable effect on the ecosystem of intestinal tract by increasing the total lactic acid bacteria population in rats. This result is supported by the report of Roberfroid that fermented non-digestible food ingredients, such as inulin, beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon by acting as substrate for them.[30]

Conclusions

In conclusion, this study provided substantial insight in illustrating the inhibitory effect of cadmium and mercury and the impact of synbiotic to rat gut microbiota. This work reveals the potential of synbiotics to decrease the cadmium and mercury repressive effect on the population of gut bacteria.

These interesting findings demonstrate that since *B. coagulans* and *L. plantarum* have the potential to supply the gut microbiota to the normal condition in Cd and Hg treated rats, it can be used as a supplementary component in treatment diets.

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