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ON THE COVER

Cross section of a sarcocyst of *S. tenella* in the skeletal muscle of a sheep.
Villar protrusions on the sarcocyst wall enclose intensely stained bradyzoites and pale staining metrocytes arranged peripherally. 1 µm section, toluidine blue stain. See page 1.

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Histopathological, ultrastructural and molecular examination of *Sarcocystis* spp. in sheep of Mashhad area, Khorasan Razavi province, Iran

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ABSTRACT

This study aimed to determine and identify *Sarcocystis* spp. infection in sheep of Mashhad city, Iran. From October 2018 to May 2019, the entire esophagus and diaphragm from 100 slaughtered sheep were collected from the Mashhad abattoir. Initially, samples were inspected by the naked eye for the presence of macrocysts. Also, all samples were examined for *Sarcocystis* spp. by tissue impression smear, histopathology, and PCR tests. Additionally, eight samples were inspected by transmission electron microscopy (TEM) and gene sequencing to confirm species identification. The infection rate of sarcocystosis by impression smear, histopathology, and PCR methods were 69%, 96%, and 100%, respectively. Histopathological examination revealed the existence of *S. gigantea* macrocyst with PAS-positive secondary cyst wall in 26% of sheep. Also based on cyst wall morphology, two types of microcysts including *S. tenella* with striated thick cyst wall and *S. arieticanis* with smooth thin cyst wall were identified in 47% and 11% of sheep, respectively. By TEM, the cyst wall of *S. gigantea* had cauliflower-like, *S. tenella* had finger-like and *S. arieticanis* had hair-like villar protrusions. Comparative analyses of the sequencing of the 18S rRNA gene revealed *S. gigantea*, *S. tenella*, and *S. arieticanis* in PCR samples. The results showed that the infection rate of *Sarcocystis* spp. was very high by the PCR method. Also, the existence of *S. gigantea*, *S. tenella*, and *S. arietcanis* species was confirmed by histopathology, TEM, and DNA sequencing methods in sheep of this area.

Keywords

sheep, sarcocystis, morphology, ultrastructure, 18S rRNA, Mashhad

Number of Figures: 6
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Abbreviations

CNS: central nervous system
TEM: transmission electron microscopy
PCR: polymerase chain reaction
spp.: species
HE: hematoxylin eosin
PAS: periodic acid schiff
µm: micrometer
nm: nanometer
M: molar
h: hour

Introduction

Sarcocystis spp. are cyst-forming tissue parasites that can infect the striated muscle of humans and numerous domestic and wild herbivores. This protozoan is a heterogeneous and two-host prey-predator life cycle. The carnivores as predator animals are definitive hosts and herbivores as prey animals are intermediate hosts. Sheep (*Ovis aries*) can act as intermediate hosts for four *Sarcocystis* spp. Among *Sarococystis* species, *S. tenella* and *S. arieticanis* are pathogenic and create microscopic cyst, and transmitted by canids whereas *S. gigantea* and *S. medusiformis* are non-pathogenic and create macroscopic cysts and transmitted by felids [1,2].

Although sarcocystosis is asymptomatic and sporadic in infected sheep with clinical signs, the presence of macroscopic cysts in sheep has a major economic impact due to condemnation of part or the whole infected carcasses in slaughterhouses [3]. The Khorasan Razavi province with more than 6 million sheep has the largest population of sheep in Iran [4]. In Iran, many studies have been reported the high prevalence of *Sarcocystis* spp. infection in sheep by conventional methods such as parasitological, pathological, and molecular methods [5–14]. However, to the best of our knowledge the infection rate of *Sarcocystis* spp. and identify species that infect sheep in Khorasan Razavi Province is unknown. Therefore, the major aims of the current study were to determine the frequency of infection and identify *Sarcocystis* species in sheep of Mashhad city, Khorasan Razavi province, Iran.

Results

Macroscopic study

At the gross inspection, macrocysts were found in 26 of 100 sheep (26%). The highest frequency of macrocysts was detected in the esophagus (26%) (Table 1). Macroscopic sarcocysts were milky white, elongated, and spindle-shaped in the diaphragm and round to oval-shaped in the esophagus (Figure 1). *Sarcocystis gigantea* was identified by histological evaluation of *Sarcocystis* macrocysts in 26 sheep (26%).

Microscopic examination

Out of 100 sheep with or without macrocysts that were examined with tissue impression smear technique and routine histopathology technique (HE), the bradyzoites of *Sarcocystis* were detected in 69% and 96% of examined sheep, respectively (Table 1). The morphological features of *S. gigantea* had a thin cyst wall as primary cyst wall surrounded by a thick PAS positive secondary cyst wall. The cyst was separated by septa to different compartments and contained ovoid-shaped metrocytes and numerous bradyzoites (Figure 2).

Histopathological examination of esophagus and diaphragm sections showed slender or oval-shaped *Sarcocystis* microcysts with numerous bradyzoites that were separated by septa and had variable sizes. These variations were dependent on the angle of tissue sections. A higher rate of positive sheep with microcyst was found using histopathology compared to the tissue impression smear ($p < 0.05$). Two types of microcysts were identified according to the thickness of the cyst wall. One of them had radially-striated thick walls that were identified as *S. tenella* and the other had smooth thin-wall microcysts that were identified as *S. arieticanis* (Figures 3 and 4). The morphological wall details could not be accurately visualized by light microscopy. Microcysts of *S. tenella* and *S. arieticanis* were found in 47 sheep (47%) and 11 sheep (11%), respectively. In addition, 38 sheep (38%) were infected with both types of microscopic sarcocyst.

Transmission electron microscopy

Identification of *Sarcocystis* spp. in tissues was mainly based on ultrastructural features of villar protrusions in cysts walls according to the classification by Dubey et al. [2]. Tissue cysts collected from eight animals were tested by Transmission electron microscopy (TEM). Three different types of villar protrusions were observed for cyst walls which were

Table 1.
The frequencies of *Sarcocystis* spp. infection in the esophagus and diaphragm of one hundred slaughtered sheep.

The examined organ muscles	No. of infected animals (%)				
	Macroscopic examination	Impression smear	Histopathology	PCR	Total
Esophagus	26 (26)	69 (69)	96 (96)	100(100)	100
Diaphragm	21 (21)	60 (60)	92 (92)	100(100)	100

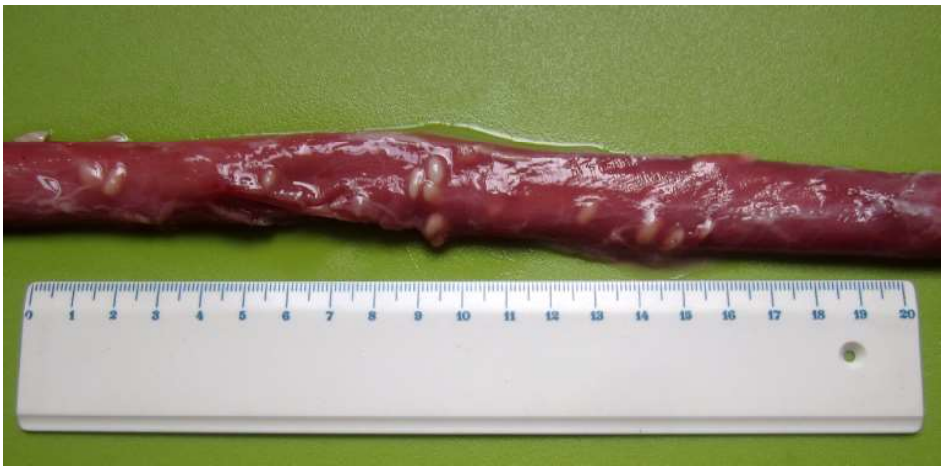

Figure 1.
Esophagus of a sheep showing several macroscopic sarcocysts. The scale is in cm.

identified as *S. gigantea, S. tenella, and S. arieticanis.* The ultrastructural features of *S. gigantea* macrocysts showed cauliflower-like villar protrusions containing numerous microtubules and the ground substance inside the cyst wall with homogeneous in appearance and 2-3 µm in thickness. The cysts were filled with bradyzoites that were internally separated in different chambers by septa (Figure 5, A and B). The cyst walls of *S. tenella* had finger-like villar protrusions with average lengths up to 1 µm. Finger-like protrusions were positioned closely side by side and distinguished by the electron dense plaques in their apex. Microtubules were absent in the protrusions. A layer of ground substances measuring 0.3-0.6 µm in thickness was located underneath the wall. Bradyzoiets were present in the cysts and their amylopectin granules were evident (Figure 5C). The cysts of *S. arieticanis* were identified as having invaginations of cyst wall with hair-like villar protrusions. The cyst wall was thin and measured 0.2-0.3 µm in thickness and the protrusions were parallel to the direction of the cyst wall surface. The ground substance ranged from 0.4 to 0.6 µm in thickness and was located immediately under the primary cyst wall. The villar protrusions appeared bone-like structures in some locations of the cyst wall (Figure 5D).

Molecular analysis

In this study, all muscle samples were positive for the *Sarcocystis* spp. by PCR. DNA amplified of eight PCR positive samples were purified from the agarose gel and sequenced (Figure 6). The sequences were blasted and submitted to GenBank. PCR alignment of the nucleotide sequences showed 100% similarity with *S. gigantea* accession number MT026574, 99% similarity with *S. tenella* accession number MT026575, and 97% similarity with *S. arieticanis* accession number MT729808 in 18S rRNA sequences in the GenBank database.

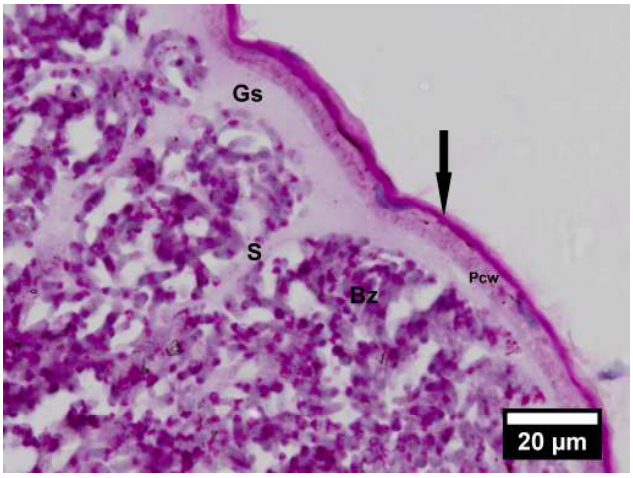

Figure 2.
Sarcocystis gigantea macrocyst in esophagus muscle. Histological section stained with periodic acid Schiff. Magnification: 100×. Note secondary thick cyst wall (arrow) that surrounds Primary cyst wall (Pcw). Ground substance (Gc), Septa (S), Bradyzoiets (Bz).

Figure 3.
Histological section of esophagus muscle showing thick-walled microcyst of *S. tenella* with prominent radial striations (arrow) stained with hematoxylin and eosin (HE). Magnification: 100×. Note Bradyzoiets (Bz), Host cell (Hc), Adipose cell (Ac).

Figure 4.
Histological section of diaphragm muscle showing smooth thin-walled microcyst of *S. arieticanis* (arrow) stained with hematoxylin and eosin (HE). Magnification: 100×. Bradyzoiets (Bz), Host cell (Hc).

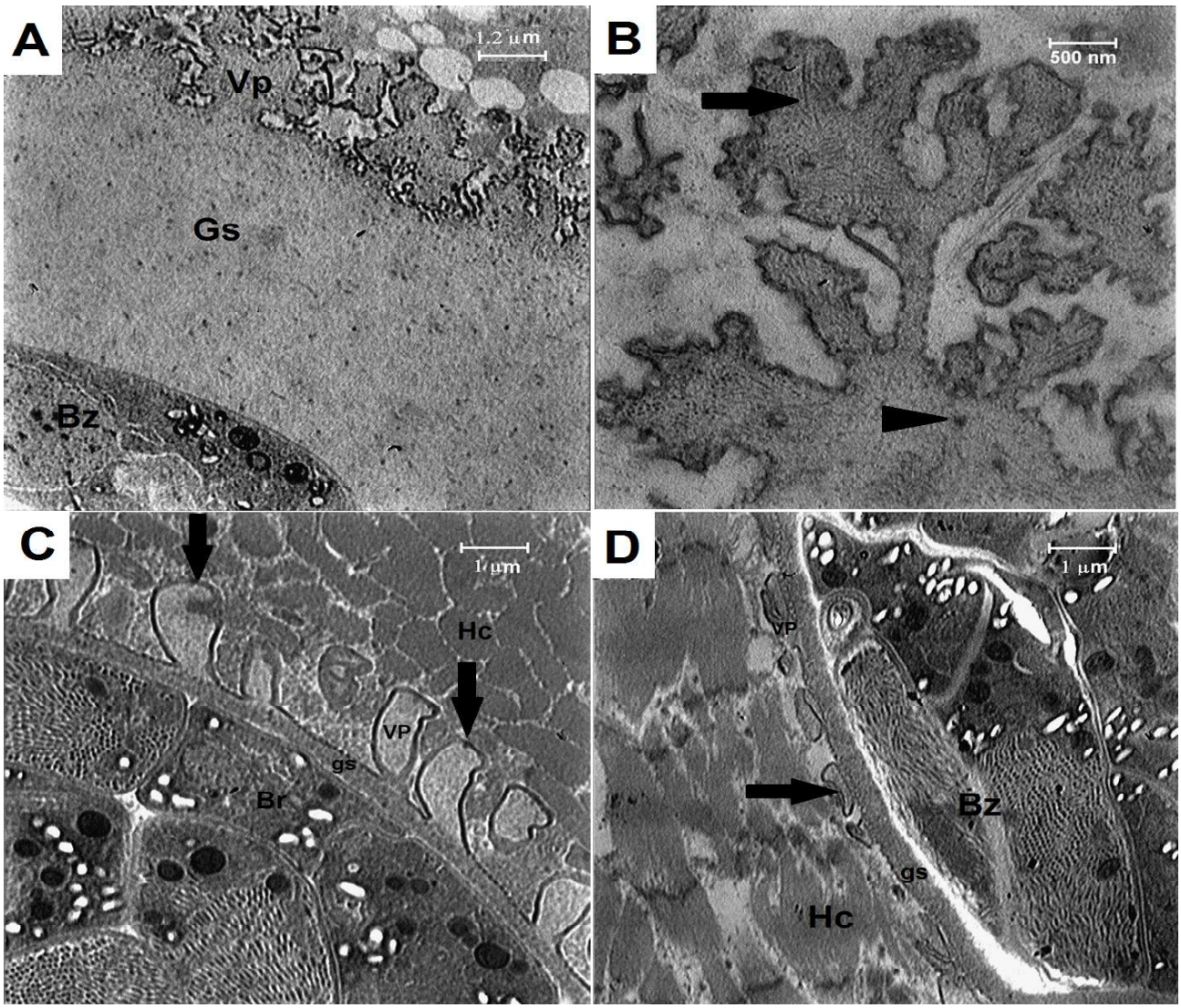

Figure 5.
Transmission electron micrographs showing a comparison of villar protrusions on the sarcocyst wall of three species of *Sarcocystis* in sheep. Uranyl acetate and lead citrate stain. A) *S. gigantea* with cauliflower-like villar protrusion. B) Cauliflower-like villar protrusion of *S. gigantea* containing microtubules (arrow) and fine or coarse granules (arrowhead) inside the protrusion. C) *S. tenella* with finger-like villar protrusion. electron-dense plaques are visible (arrows) at the apex of villar protrusions. D) *S. arieticanis* with short hair-like villar protrusions. The villar protrusions appeared bone-like structures in some locations of the cyst wall (arrow). Note Villar protrusion (VP), Ground substance (Gs), Bradyzoiets (Bz), Host cell (Hc).

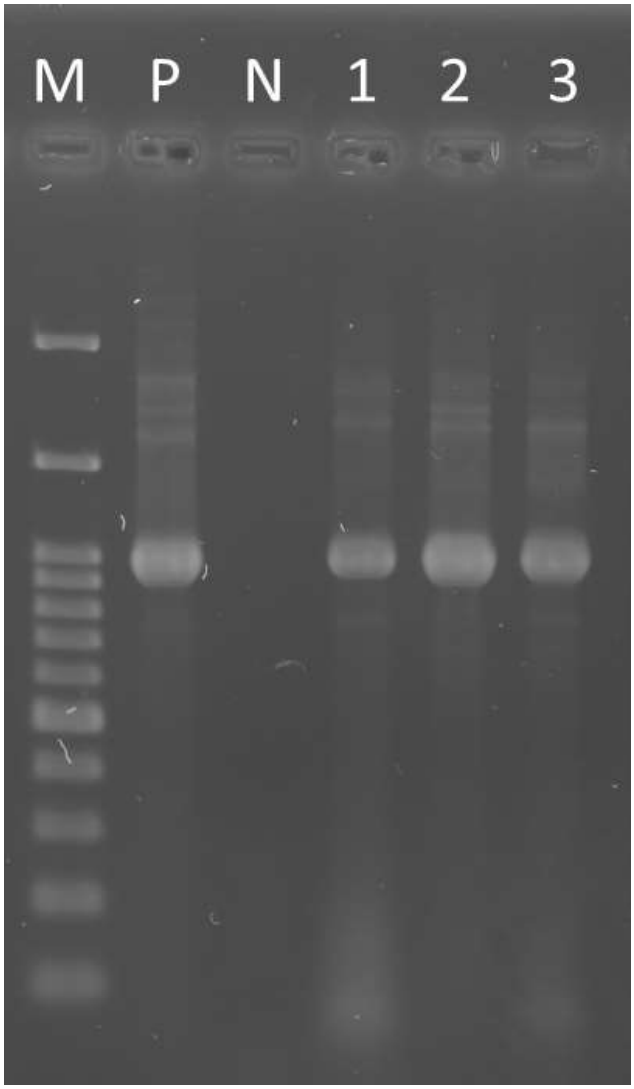

Figure 6.
PCR amplification products of *Sarcocystis* spp. in muscle samples. M= Molecular weight marker (100bp ladder); P= Positive control; N= negative control; 1,2,3 positive samples.

Discussion

In this study, *Sarcocystis* spp. cysts were microscopically found via tissue impression smear and routine histopathology techniques. The tissue impression smear as a fast, simple, and applied technique was compared with routine histopathology technique; the latter technique found 27% more infected sheep than the tissue impression smear technique. Although the routine histopathology method is more sensitive than the tissue impression smear and tissue squash methods, it has been shown that the digestive method is more sensitive than the pathology method [15,16]. In addition, the PCR technique has been used to determine the frequency and identify of *Sarcocystis* spp. in tissue samples. All sheep were found infected with *Sarcocystis* spp. by PCR. This finding revealed that PCR is the most accurate and sensitive method for determining the frequency of infection. A regional survey from the Americas in retail beef samples showed that PCR assays may increase the detection sensitivity of *Sarcocystis* spp. and contribute to diagnostic precision [17].

In the present study, macroscopic cysts were found in both esophageal and diaphragmatic muscles. In Iran, the prevalence rates of macrocysts in sheep have ranged from 3.58 % to 36.83 % in abattoir studies carried out in different provinces [8,11,12,18,19]. The highest prevalence of macroscopic cysts incorporated in the esophagus was similar to the studies of other researchers [8,20–22]. In contrast, Farhang-Pajuh et al. [11] found the highest prevalence of macrocysts in the diaphragm samples of slaughtered sheep of Urmia city, Iran. In this study, similar results about infection rate of microcysts were reported in other studies as follows: 97% in Iraq [20], 96.9% in Mongolia [23], 95.8% in Brazil [24], 93% in Ethiopia [25] and 91.9% in China [26]. Ovine microscopic sarcocysts have been reported to be more prevalent than macroscopic *Sarcocystis* species in Iran [8,12,19] and other countries [21,22,27,28].

The histopathologic examinations of macroscopic cysts by light microscopy allowed the identification of *S. gigantea* with PAS-positive secondary cyst wall. This finding is following the results of other studies [22,29,30]. Many studies have shown that *S. gigantea* was the predominant macroscopic species in sheep [28,31]. The use of light microscopy in histological examination permitted the recognition of two types of microcysts based on the thickness of the cyst wall, as described by Dubey et al. [31] and Beyazit et al. [22]. Thick radially striated walled microcysts were identified as *S. tenella* and thin-walled microcysts were identified as *S. arieticanis*. Similar results have been reported by O'Donoghue and Ford [32]. In the present study, the frequency of *S. tenella* microcysts was more than *S. arieticanis*. Some researchers have found that *S. tenella* was the predominant species in sheep [1,15,28,31]. A study showed that 80% of slaughtered sheep were infected with *S. tenella* in the southwest of Iran [14].

The use of TEM to study the ultrastructure of sarcocyst walls is a crucial taxonomic criterion for the identification and differentiation of species of *Sarcocystis* in infected animals [24,33]. The ultrastructural features of sarcocyst wall in our samples were consistent with the cyst wall ultrastructure *S. gigantea, S. tenella, and S.arieticanis* that classified by Dubey et al. [2]. The observation of cauliflower-like villar protrusions in macroscopic cyst samples permitted the identification of *S. gigantea* cysts and was similar to that described previously for this species in sheep [30,34,35]. The villar protrusions in microcyst with

finger-like or palisade-like protrusions allowed the identification of *S. tenella* and the villar protrusions in other microcyst with hair-like morphology were confirmed as *S. arieticanis*. These findings were similar to those described by Odening et al. [34] and El-Morsey et al. [1].

In the present study, nucleotide sequencing of the 18s rRNA genetic marker of PCR products was done in eight samples. The DNA acquired from individual cysts allowed the identification of *S. gigantea, S. tenella, S. arieticanis* in selected samples.

In conclusion, the current investigation showed a high frequency of infection by *Sarcocystis* spp. that form macroscopic and microscopic cysts in sheep slaughtered for human consumption in the Mashhad area. We report *S. tenella* and *S. arieticanis* by TEM for the first time in Iran. The high frequency of *Sarcocystis* spp. indicates the importance of infection in sheep as intermediate hosts. On the other hand, the frequency of microscopic sarcocysts was determined more than macroscopic sarcocysts infection. The result showed the important role of sentinel dogs to induce sarcocystis infection in sheep.

Materials & Methods

Samples collection

From October 2018 to May 2019, tissue samples of the esophagus and diaphragm were collected from sheep (> 2 years) in a slaughterhouse in Mashhad city, the capital of Khorasan Razavi Province where is located at 36.18˚ north latitude and 59.36˚ east longitude, Iran. The collected samples of each sheep were separately kept in sealed plastic bags and transported in boxes containing ice packs to the laboratory of pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran. For calculating sample size with an expected infection frequency of 90% and confidence interval of 95 % and desired absolute accuracy of 5 %, one hundred sheep were randomly selected [36]. All specimens were obtained from the sheep carcasses after they were slain in the slaughterhouse under instructions of the Iran Veterinary Organization and its regular commercial routine for meat production for human use in the internal Iranian market. So, there was not required specific ethics approval.

Macroscopic examinations

In the laboratory, all tissue samples were grossly examined by the naked eye for the existence of macroscopic cysts. For better visualization of the macroscopic cysts in muscle depth, in diaphragm samples at least 15 transversal sections were made with a scalpel and in esophagus samples longitudinally cuts were done with the scissor.

Tissue impression smears

The cut surface of each fresh tissue sample was pressed on a glass slide, then fixed with absolute methanol, and finally stained with Giemsa and examined for the existence of free bradyzoites by a light microscope at × 40 and × 100 magnification [37].

Histopathologic examination

First, two pieces from each esophagus and diaphragm samples, with or without macrocysts were fixed in 10 % neutral buffered formalin, then dehydrated in graded ethanol alcohol series, finally embedded in paraffin and made the paraffin wax blocks. The tissue sections were prepared from the paraffin wax blocks by tissue processor and stained conventionally with hematoxylin and eosin (HE). Selected tissue sections with macrocysts were also stained with periodic acid Schiff (PAS). Histological slides were examined for sarcocysts and their wall features by light microscopy.

Transmission electron microscopy

Samples from eight sheep included two esophagus samples that contained macrocyst, two esophagus samples contained microcyst, two diaphragm samples contained macrocyst and two diaphragm samples contained microcyst were prepared for TEM. The Specimens were fixed with 2.5 % glutaraldehyde solution in 0.1 M sodium cacodylate buffer (pH 7.4) at least for 2 h at room temperature and stored refrigerated overnight. After fixation, the samples were washed three times in sodium cacodylate buffer 0.1 M (pH 7.4), then post-fixed in 1 % osmium tetroxide for 2 h at room temperature, dehydrated in graded ethanol [30, 40, 50, 70, 90, and 100 %), transferred to propylene oxide for 1 h and finally embedded in Epoxy Resin then polymerization at 60 °C for 72 h in an oven. After polymerization, semithin sections (1 µm) were cut with an ultramicrotome (Ultracut R, Leica, Austria) and stained with toluidine blue to determine the area of *Sarcocystis* spp. cysts. Also, the ultrathin sections (80 nm) from the cyst area were stained with uranyl acetate and lead citrate and examined using a Zeiss Leo 912 AB transmission electron microscope at 120 kV[24,38].

DNA extraction and PCR method

Genomic DNA of each sheep sample was extracted via commercial MBST Blood and Tissue Genomic DNA isolation kit (Molecular Biological System Transfer, Iran) followed according to manufacturer's instructions. The extracted DNA was stored at -20°C until use. Each reaction was carried out in a total volume of 25µL, containing 1 µL of the sample DNA, 0.5 µL of each primer, and 12.5 µL of PCR Master Mix. The conventional PCR was performed to amplify a fragment of the subunit 18S ribosomal RNA (18S rRNA) gene of *Sarcocystis* spp. with the forward (1L) and reverse (3H) primer pair (Table 2). The PCR was performed in a thermal cycler (Bio-Rad, USA) as follows: 95°C for 2 min, followed by 40 cycles of 94°C for 40 s, 50-57°C for 1 min, 72°C for 1 min, followed by 72°C for 6 min [39]. The PCR products were electrophoresed in 1.5 % agarose gels, then stained with ethidium bromide and observed under ultraviolet light. DNA was extracted from macrocyst of *Sarcocystis* spp. from an infected sheep as positive control and ultrapure water as negative control were included in each experiment.

Gene sequencing

The amplicons were purified from the gel bands using the Gel extraction kit (Dena Zist Asia, Iran) as per manufacturers' instructions. Concentrations of the PCR products were evaluated by spectrophotometry (Nanodrop ND1000). The purified PCR products were submitted for sequencing (Faza Pajooh Co. Tehran, Iran). The 18S rRNA gene nucleotide sequences were assembled and edited using CLC bio software (CLC Main Workbench, Qiagen, Aarhus). The nucleotide sequences in the present study were aligned with previously described sequences of *Sarcocystis* spp. in GeneBank (NCBI) using the Clustal W method (Mega software version 6). The nucleotide sequences obtained in this study were deposited in GenBank (NCBI).

Table 2.
PCR primers used for amplification of 18S rRNA genes in *Sarcocystis* spp.

Primer name	Orientation	Sequence (5'→3')	Length (bp)	Product size (bp)	Annealing temperature (°C)	Reference
Primer 1L	Forward	CCATGCATGTCTAAGTATAAGC	22	900	55	39
Primer 3H	Reverse	GGCAAATGCTTTCGCAGTAG	20			

Statistical analysis

To compare two microscopic methods the tissue impression smear technique and routine histopathology technique, the McNemar test was used. The SPSS version 25.0 (IBM Crop., USA) was used for statistical analysis. Values of $p < 0.05$ was considered

Authors' Contributions

Conceived and designed the experiments and revised the manuscript draft: H.N., G.R.R. performed the experiments, analysed the data and drafted the manuscript: H.R. All authors approved the final version of the manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Estradiol benzoate priming during induction of estrus with Vitex-castus extract in dogs

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ABSTRACT

This study compared two methods of estrus induction between dogs (using vitagnus and vitagnus-estradiol). A total of 10 adult cyclic female Shih tzu Terrier mix breed dogs at anestrus stage were selected and divided into two groups. The first group (VAC) received vitagnus for five weeks (90 mg daily, PO). The second group (VAC-E2) was treated with vitagnus and estradiol benzoate. Estradiol benzoate was injected at the beginning of each week (0.01 mg/kg, IM). Blood sampling for evaluation of steroid hormones and vaginal smears were taken weekly. The sings and return to the estrus with the number of puppies were recorded. In the VAC group, 60%, and in the VAC-E2 group, 80% of dogs returned to the follicular phase after five weeks. In the VAC-E2 group, signs of estrus appeared 7 to 10 days and a mating process started 4 to 6 days earlier than those in the VAC group. The average number of delivered puppies was 4. These symptoms were confirmed by the cytology of the vagina. There was no significant difference in the estradiol and progesterone levels between groups. The mean concentration of estradiol significantly changed between weeks 1 and 4, 1 and 5, and 2 and 5 ($p < 0.05$). The progesterone level in the VAC-E2 group on week five was higher than that in other weeks. In conclusion, the administration of estradiol benzoate before vitagnus improved induction of estrus in dogs.

Keywords

estrus induction; estradiol benzoate; Vitex-castus extract; dog

Number of Figures: 4
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Number of References: 19
Pages: 10-18

Abbreviations

VAC: *Vitex agnus castus*
E2: Estradiol
P4: Progesterone
PMDD: Premenstrual dysphoric disorder
PCOS: Poly cystic ovarian syndrome
PMS: Premenstrual syndrome

Introduction

Training various breeds of dogs for rescuing people, police surveillance, diagnosis of diseases, helping the disabled people and keeping them as pets is increasingly expanded [1]. Dogs are monoestrus and experience an obligate anestrus of 2–10 months following the luteal phase, having an average duration of about 75 days [2]. The anestrous period of the cycle begins when the progesterone plasma concentration falls below 1 ng/ml. Bitches in long anestrus should be treated after the factor leading to anestrus is identified [3]. Therefore, several drugs used to induce estrus in bitches in various programs include bromocriptine, estrogen, GnRH, LH, FSH, hCG, eCG, cabergoline, and herbal ingredients such as *Vitex-castus* extract [1,4,5].

All methods of inducing estrus have advantages and disadvantages. For instance, the use of cabergoline as a dopaminergic compound is not cost-effective. On the other hand, since the drugs are designed for human beings, it is difficult to determine the dosage of the drug for animals. It should also be noted that this drug can affect the central nervous system and leads to nausea and vomiting. The change in the color of the outer skin is another side effect of cabergoline [1].

Administration of deslorelin, PGF2α, and estradiol benzoate can lead to the induction of estrus in dogs, but ovulation is not guaranteed in all of them [6]. Injection of eCG can induce oxidative stress and increase the amount of oxidation in the ovary, hence, it can affect fertility in the long run [7].

The use of GnRH analogues (e.g. Deslorelin, Leuprolide) through hypodermic osmotic mini pumps or implants can lead to estrus induction and pregnancy, however it has some limitations: 1) high cost and lack of availability of implants, 2) formation of immature corpus luteum which shortens diestrus period and reduces fertility rate, and 3) long-term administration of GnRH agonists leads to over stimulation in pituitary and reduced expression of GnRH receptors, which is followed by suppression of LH secretion and in some species it can reduce FSH [8-10].

Considering the adverse effects of hormonal compounds, it is essential to use a safe method. *Vitex agnus castus* (VAC) extract is widely used in the treatment of patients suffering from premenstrual dysphoric disorder (PMDD), menstrual cycle irregularities, polycystic ovarian syndrome (PCOS), lactation difficulties, hyperprolactinemia, cyclical breast pain, menopause-related complaints, premenstrual syndrome (PMS), and associated cyclic mastalgia, acne, digestive complaints, infertility, anxiety, and as antimicrobial, antioxidant, and antifungal [11-13].

Although *Vitex agnus castus* (VAC) extract reduces prolactin secretion by binding to dopamine receptors in the pituitary gland, administration of different dosages of this extract can either stimulate or suppress prolactin secretion [12]. VAC compounds (i.e. linoleic acid) induce expression of ER-α and ER-β receptor genes. Furthermore, VAC stimulates the synthesis of progesterone and presenelin-2 mRNA receptors. This extract can change uterine weight and cytology of the vagina [14]. Estrogenic compounds of the VAC extract, such as flavonoids, penduletin and apigenin, have been identified. This compound normalizes and balances hormone levels. Due to the existence of these compounds, levels of prolactin and cholesterol are diminished [13].

Generally, VAC extracts reduce FSH secretion and increase the secretion of LH. Increasing LH secretion leads to corpus luteum maturation and increases in progesterone levels. On the other hand, reduction in prolactin production leads to the maintenance of corpus luteum structure and function, and it creates a hormonal balance between progesterone and estrogen. However, since FSH is reduced, reaching the end of anestrus and high intake of VAC extract, FSH increased and estrus is engendered through folliculogenesis in dog [1,15]. The VAC extract has several side effects, including gastrointestinal disorders, urticaria, fatigue, dizziness, headache, dry mouth, tachycardia, nausea, and agitation. These side effects are found in less than 2% of the patients. Also, due to a lower cost and higher availability of this compound, it can be used as an alternative for hormonal compounds to induce estrus in dogs [11].

This study aimed to evaluate the effects of a safe and inexpensive herbal extract in order to induce estrus in dogs with the least side-effects compared to hormonal compounds. Since VAC reduces FSH and subsequently reduces estrogen synthesis, this study investigates the effects of combining estradiol and VAC to expedite estrus induction in dogs.

Results

Clinical evaluation

At the end of the fifth week in the VAC group, of the 5 dogs studied, 3 dogs entered their follicular phase. Of these 3 dogs, 2 were in the proestrus phase and 1 was in the estrus phase. At the end of the fifth week in the VAC-E2 group, out of the 5 dogs studied, only one dog was still in the anestrus phase. In the VAC-E2 group, dogs showed signs of estrus and bloody vaginal discharge seven to ten days earlier than the first group. One of the dogs in the group was in the diestrus phase at the end of the fifth week. Dogs were

also monitored for up to three months after the study and their pregnancy and parturition were recorded. In the VAC group, 2 dogs that were in proestrus at the end of the fifth week, mated after 10 ± 2 days (Figure 1). One dog in estrus at the end of the fifth week, mated twice. Of the 3 dogs that were in anestrus, 2 dogs showed bloody discharge after 3 weeks, and 1 dog was still in anestrus. Of the 3 dogs in the VAC group that were in the follicular phase at the end of the fifth week, all 3 gave birth to healthy puppies after average of 62 days. The average number of delivered puppies was 4.

In the VAC-E2 group, 2 dogs that were in proestrus at the end of the fifth week, mated after 6 ± 2 days. One dog that was in estrus, mated twice at the end of the fifth week. One dog that was in diestrus, mated twice from the end of the fourth week until the middle of the fifth week. The anestrus dog did not reveal any mating signs until three months after the study.

Vaginal Cytology

Results related to cellular changes of the vagina are shown in Table 1. As you can see, estrus induction in one of the dogs is performed from the third week and it is performed from the fifth week in the other (Cornified cells were dominant in these dogs). It should be noted that in three other cases of the population, parabasal cells were still dominant at the end of the fifth week, thus, the dogs were in their anestrus phase. It seems that the dogs of the VAC-E2 group, which were in the same sexual cycle as that of the dogs in the first group (sexual non-acceptance), returned to estrus earlier than the first group, since four dogs of this group entered their follicular phase earlier than the fifth week. It seems that estradiol along with Vitagnus can hasten the sexual cycles of the dog. Out of the 10 dogs studied in two groups, only two dogs were in the anestrus phase at the end of the study. We can conclude that administration of Vitagnus alone or along with estradiol can help induce estrus in dogs (Figure 1).

Estradiol Concentration of the Serum

After measuring estradiol by ELISA method, the changes of the hormones were compared in two groups. In statistical terms, in the whole five weeks of the study, there was no significant difference between two VAC and VAC-E2 groups ($p > 0.05$). It seems that these results indicate that Vitagnus-estradiol shortens anestrus period, but it does not have any advantage in increasing estradiol level during the first five weeks compared to Vitagnus group. Hence, Vitagnus alone can help follicular growth induction and subsequent estradiol synthesis at the end of the anestrus. Furthermore, a slight increase in estrogen level of dogs can lead to resurgence of estrus and improving estrus signs. There was no significant difference in estrogen level in the VAC group during different weeks ($p > 0.05$). It seems that, the duration of Vitagnus usage does not affect the amount of estradiol synthesis. But there is a significant difference in estrogen level between the first week and weeks four and five, and between the second week and week five in the VAC-E2 group ($p < 0.05$). Thus, the increase in estradiol level during the last week compared to the initial week

Table 1.
Cyclic changes of dog in two VAC (Vitagnus) and VAC-E2 (Vitagnus+E2) groups based on cytological changes of the vagina.

Drug \ Weeks	1	2	3	4	5
Vitagnus	An	An	An	An	An
Vitagnus	An	An	An	An	An
Vitagnus	An	An	An	An	pro
Vitagnus	An	An	An	An	pro
Vitagnus	An	An	pro	ES	ES
Vitagnus + E2	An	An	An	pro	pro
Vitagnus + E2	An	An	An	An	pro
Vitagnus + E2	An	An	An	An	An
Vitagnus + E2	An	An	pro	ES	Di
Vitagnus + E2	An	An	An	pro	ES

The mating date was set 2 times at 24-hour intervals after entering the estrus.
Anestrus (An); proestrus (pro); Estrus (ES); Diestrus (Di); estradiol benzoate (E2)

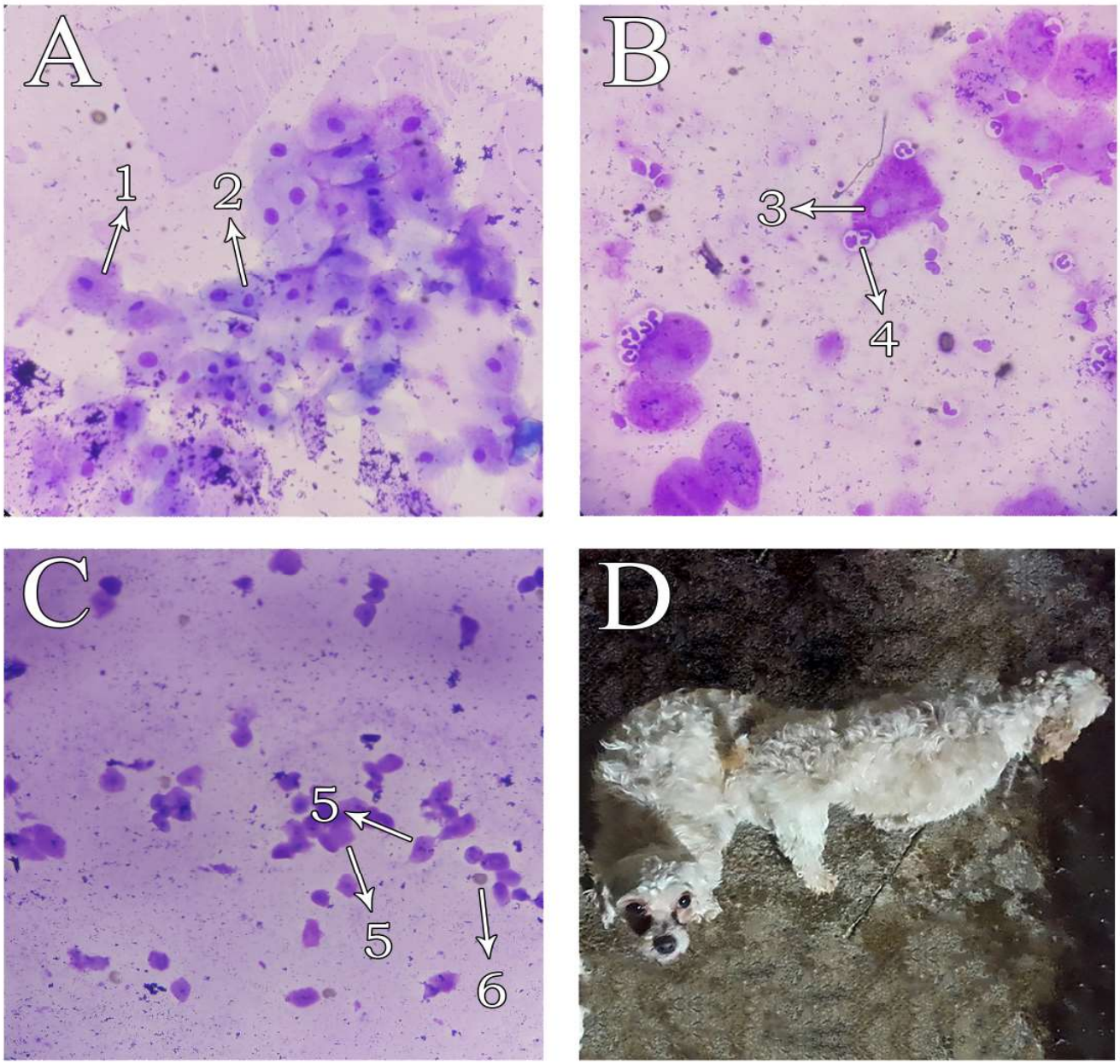

Figure 1.
A-C) Samples of vaginal cytology in the studied groups after Giemsa staining. Numbers 1 to 6 denote the large intermediate cells, parabasal cells, superficial cells, neutrophils, squamous cells, and Red blood cells, rescpectively. Dominant cells in vaginal cytology analysis during proestrus are RBCs and superficial cells, superficial and squamous cells in estrus, intermediate cells and neutrophils in diestrus, and parabasal cells in anestrus. D) Mating of one of the dogs in the VAC group (The copulatory tie in the dog).

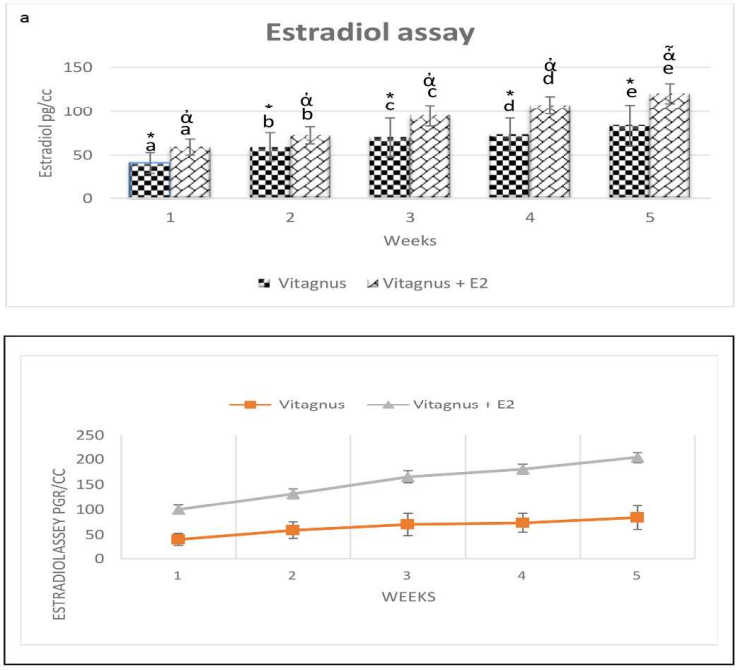

Figure 2.
Evaluation of estradiol changes in the VAC and VAC-E2 groups. The letters a-e show the estradiol changes between the VAC and VAC-E2 groups during the study weeks. There was no significant differences between groups ($p > 0.05$). The asterisk shows the comparison of estradiol changes in the VAC group in different weeks. There was no significant differences between groups ($p > 0.05$). The ȧ and ă show the estradiol changes in the VAC-E2 group in different weeks. There was a significant difference between the first and fifth week ($p < 0.05$).

shows that the duration of estradiol consumption is effective in increasing this hormone, which is quite natural. It can be concluded that since Vitagnus alone can raise estradiol level during the late anestrus phase, hence there is no justification for using it along with estradiol. Returning to estrus is seen earlier in VAC-E2 group, which leads to estrus induction earlier that the VAC group in the studied dogs. Statistical evaluations show that the drugs and the weeks can separately affect the estrogen level, but the reaction of the drug and week on estrogen level is not significant.(Figure 2)

Serum Progesterone Concentration: After measuring progesterone by ELISA method, the changes of this hormone were compared in weekly manner between two groups. From the statistical standpoint, there was no significant difference in progesterone level in the VAC and VAC-E2 groups ($p > 0.05$). From the analysis of progesterone level in two Vitagnus groups, it can be concluded that, at the onset of the estrus, in spite of an increase in progesterone in proestrus and anestrus, there is no significant difference, but passage of the weeks after beginning of estrus, progesterone level increases to a degree that it can lead to significant differences between groups. Analysis of progesterone in different weeks in Vitagnus and estradiol group, it can be concluded that there is a significant difference between progesterone level and five initial weeks of the study ($p < 0.05$). Therefore, it seems that estradiol injection at the onset of each week can help induce estrus in the animal earlier than the VAC group which only received Vitagnus. Early estrus leads to luteinization of the follicular wall. In fact, the procedure of increasing progesterone level in the first groups takes more than five weeks, however it can be reduced to less than five weeks in the second group. As can be seen in the mean rank table, the progesterone level in the fifth week is more considerable in the second group compared to other weeks. In fact, this result is consistent with the increase in estradiol in the final weeks of the VAC-E2 group, since luteinization of the follicular wall happens before the ovulation in dog (Figure 3).

Discussion

After five weeks of treatment in two VAC and VAC-E2 groups, of 10 dogs, 6 dogs entered the follicular phase and only one was in the luteal phase. The results indicate that Vitagnus along with estradiol or alone can help return to estrus in 60% of female dogs. Various compounds with differing dosages have been suggested for returning the female dog to estrus. Dopaminergic compounds are among the main ones. Cabergoline is one of the important compounds in re-

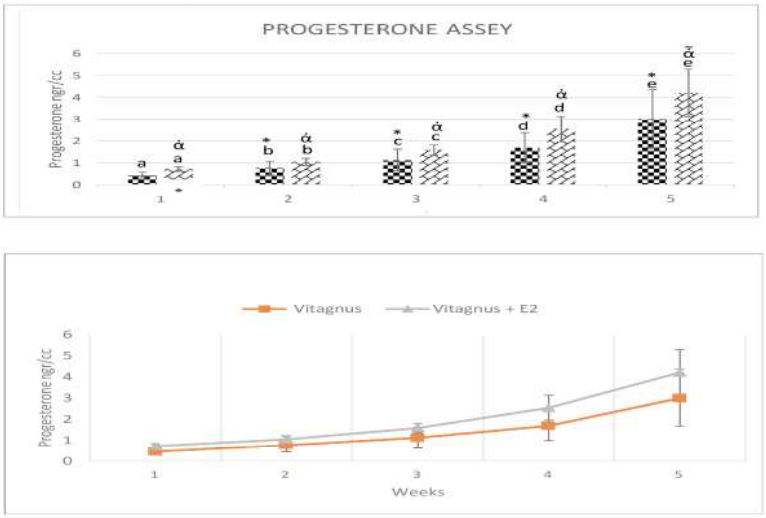

Figure 3.
Evaluation of progesterone changes in the VAC and VAC-E2 group s. The letters a-e show the progesterone changes between the VAC and VAC-E2 groups during the study weeks. There was no significant differences between groups ($p > 0.05$). The asterisk shows the comparison of progesterone changes in the VAC group in different weeks. There was no significant differences between groups ($p > 0.05$). The letters ȧ-ă show the progesterone changes in the VAC-E2 group in different weeks. There was a significant difference between the fifth and first week ($p < 0.05$).

turning the dog to estrus. Mogheiseh et al. (2017) reported that vitagnus can be as successful in returning the dog to estrus just as cabergoline and leads to cytological changes of the vagina and hormonal changes [1]. The results of the current study are in line with the previous studies. An issue that has to be considered in using Vitagnus is that the dog should be evaluated in terms of its reproductive cycle [16].

As stated in the results section, the estradiol level does not increase after Vitagnus, and it only sufficiently increases at the end of the estrus to show signs of the estrus. These results are confirmed in a report provided by Zahid et al. in 2016 [12]. They reported that Vitagnus has a higher tendency to combine with alpha and beta estrogenic receptors. On the other hand, there is a decrease in FSH after administering vitagnus, hence a lack of considerable increase in estradiol especially in the first group (Vitagnus) is seen [12].

There is a considerable difference between progesterone in different weeks of Vitagnus. On the other hand, as Rashidi et al., (2017) reported Vitagnus increases the LH levels [11]. We expect that increasing LH to increase progesterone, but the progesterone level in different weeks showed a significant difference in the VAC group. This seems natural since FSH is low and folliculogenesis is affected. On the other hand, since luteinization of the follicular wall happens before ovulation in the dog, thus no increase in the progesterone level in the first group was observed. However, in the second group, since the return to estrus occurred quickly, and since FSH increases at the end of the anestrus following Vitagnus administration, progesterone level increases considerably in the final weeks.

EEstradiol injection at the beginning of each week led to returning to estrus faster than that in the VAC group. Estrus length was the same in both groups. Estrus signs were more obvious in the VAC-E2 group. Mogheiseh et al. (2017) reported in another study that estradiol injection one week before cabergoline can speed up the dogs returning to estrus compared to the control group [1]. In this study, returning to estrus was faster in the group which received estradiol compared to the VAC group. According to the report provided by Mogheiseh et al. (2017) and the results of the current study, it seems that estradiol can speed up the return to estrus in anestrus dogs [16].

In the anestrus period, due to a decrease in FSH, Inhibin, and lack of negative feedback, there is an amount of estradiol in the serum of the dogs, but the level of alpha and beta receptors in the hypothalamus, pituitary, and ovary is low, hence the estrogen in the anestrus period cannot induce estrus. On the other hand, the main phytoestrogens of Vitagnus are

apigenin, vitexin, and Pendleton, which are bound with ERα and ERβ receptors. Apiginin is the most important due to its higher combination tendency with ERβ. Hence estrogen can bound to its receptors on the hypothalamus, pituitary, and ovary. The result of this bounding is enabling the hypothalamus-pituitary-ovary axis (HPO). With the increase in sensitivity of the pituitary in response to GnRH hormone, LH increases, and progesterone is synthesized. In this study, increasing progesterone level occurs at the end of diestrus and immediately before estrus induction and entrance to proestrus, and as Hatoya et al. (2003) reported, the increase in progesterone level is justifiable and it is in line with the studies conducted by Mogheise et al.[1, 18].

Niroumand et al. (2018) reported that Vitagnus extract can increase estrogen and progesterone level compared to the control group and it reduced prolactin levels. The results of the current study are consistent with that of Niroumand et al. (2018) [14].

The cytological changes of the vagina following hormonal changes especially in the final weeks of the study are obvious. Haji et al. (2018) reported that cytological changes of the vagina are moving toward epithelial and cornified cells following the increase in estrogen level of the blood and it leads to a decrease in neutrophils and parabasal cells. The increase in progesterone level leads to an increase in interstitial cells. In the current study, the follicular phase in anestrus dogs led to a decrease in parabasal cells and increased cornified cells [19].

Various protocols have been used to induce estrus in dogs. Compounds such as Gonadotropin-releasing hormone, gonadotropins, prostaglandins, steroid hormones, dopaminergic compounds, and plant compounds have been used by researchers. All methods of estrus induction have their advantages and disadvantages. For instance, although cabergoline is an effective compound for inducing estrus in the dog, since it is not for veterinary usage, the determination of the exact dose for the dog is difficult [1]. Rodas-Ruiz et al. (2015) used three methods of deslorelin injection, prostaglandin F2α and estradiol benzoate in anestrus dogs and the dogs in the luteal phase and concluded that these methods can induce estrus in dogs, but unlike Vitagnus, they are not able to induce ovulation and pregnancy, hence their study is not in line with the current study [6].

The results of this study show that using the Vitagnus plant alone can induce estrus in female dogs. On the other hand, this compound has fewer side effects than cabergoline and prostaglandin. Due to its availability in the country and also its low cost, this compound can be a suitable alternative for inducing estrus in anestrus dogs which is due to containing dopaminergic compounds. Also administration of estradiol benzoate before Vitagnus improved induction of estrus in dogs. To expedite the estrus in dogs, it is possible to use vitagnus and estradiol. A summary of the function of Vitagnus and Vitagnus-estradiol can be seen in Figure 4.can be seen in Figure 4.

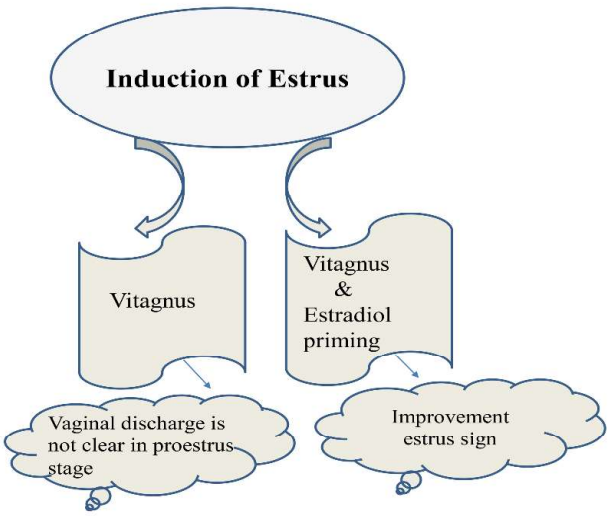

Figure 4.
A summary of the function of Vitagnus and Vitagnus-estradiol.

Materials & Methods

Animals

10 adult non-pregnant dogs with the age of 3-4 years and an average weight of 4 kg were chosen from the Shih tzu Terrier mix breed. Dogs were obtained from a dog training center in Urmia, Iran. Before the initiation of the study, the health status of the obtained dogs was investigated by referral to the Faculty of Veterinary Medicine of Urmia University. Weight and specification were recorded. The dogs were evaluated for their reproductive history. All dogs were in the mid-anestrus stage based on the date of the last estrus signs and observation of vaginal smear by gram staining and serum progesterone assays. Finally, the dogs were given an anti-parasite tablet (Cestal; 1 tablet per 10 kg of body weight). Animal's ID card was evaluated in terms of vaccination status and age. All dogs were kept in Urmia Pet House, Iran.

Study Groups

The animals were divided into two groups: Vitagnus (VAC) and Vitagnus-Estradiol (VAC-E2). Vitagnus is the extract of a plant that is commercially available. In each group, there were five dogs. Before the study, from all dogs, a vaginal smear was prepared to determine the sexual cycle. Serum samples were obtained for the evaluation of steroid hormones.

Estrus induction

To induce estrus in the VAC group, all dogs received oral Vitagnus (Poursina Pharmaceutical Company, Iran) at a daily dose of 90 mg [1]. In the VAC-E2 group, in addition to Vitagnus, Estradiol Benzoate (Abureyhan Pharmaceutical Company, Iran)

was intramuscularly administered at the beginning of the week at a dose of 0.01 mg kg^{-1} of body weight [16]. Meanwhile, the date of starting to use the drug and also the date of observing estrus signs and estrus confirmation based on vaginal cytology were recorded [17]. The occurrence of adverse behavior or side-effects of the drugs were recorded. The duration of the treatment was 5 weeks. At the end of each week, vaginal cytology and evaluation of estrogen and progesterone were conducted in both groups. After the fifth week, the mating date and their delivery time along with the number of puppies were recorded.

Preparation of Vaginal Smear

First, the vaginal area and its surroundings were washed, and then were disinfected with alcohol. After binding the animal, the tip of the sterile swab was smeared with normal saline and entered the dorsal commissure vulva by a tilt of 45 degrees. It was pushed gently in order not to enter the clitoral duct. After passing the ischial arch, by rubbing the swab cotton tip to the vaginal wall taking the contents and wall cells by swab, they were placed on the slide and after drying and fixing with ethanol, they were stained with Giemsa. All of the slides were registered with the name of the case and the sample number and they were investigated with a microscope with a 40X objective. Finally, cytological changes were recorded and the cycle of each animal was estimated [17].

Measuring the Concentration of Steroid Hormones

To measure the progesterone, blood samples were taken from each case while making vaginal smear. The blood samples were placed at a temperature of 37°C for 10-20 minutes to be clotted. It was centrifugated for 10 minutes at 2500 rpm to separate the serum. After serum separation and labeling, the obtained serum was stored at -70 °C. Finally, the ELISA kits (IBL International Germany, Hamburg, GmbH) were used to measure progesterone and estradiol concentrations according to the instructions provided by the manufacturer. After recording the data obtained from vaginal smears and analysis of the data obtained on the estradiol and progesterone concentrations, the VAC and VAC-E2 groups were compared.

Clinical Evaluation

DDogs were evaluated in two groups in terms of returning to estrus, clinical symptoms of estrus, mating after estrus, delivery process, and the number of puppies. The experiments were performed on animals following the guidelines of the ethical committee for research on animals of Urmia University (IR-UU-AEC-1357/DA3-2019).

Statistical Analysis

Statistical analyses were carried out using a mixed design (within and between compared groups). The ANOVA analyses were performed with 95% confidence intervals, independent t-test, and Kruskal Wallis test using SPSS software (version 22.0; SPSS Inc., Chicago, USA). All data are presented as means ± SEM and $p < 0.05$ was considered to be statistically significant.Chicago, USA). All data are presented as means ± SEM and $p < 0.05$ was considered to be statistically significant.

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Authors' Contributions

AG provided some of the materials. EA and AK performed the experiments and wrote the paper. AK analyzed the data.

Conflict of interest

The authors declare no conflict of interest.

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Serum biochemical and oxidative status in Holstein cattle affected with foot and mouth disease

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ABSTRACT

Foot and mouth disease (FMD) is a severe, highly contagious viral disease of cloven-hoofed ruminants caused by an aphthovirus of the family Picornaviridae. The disease in cattle is clinically characterized by fever and vesicles on the foot, in the oral cavity and on the mammary gland.This study was carried out to determine the changes in some serum biochemical parameters of cattle naturally infected with FMD O in Shahrekord district, Iran. For this purpose, blood samples were obtained from 23 Holsteins with clinical signs of FMD, as well as 22 blood samples from healthy animals. Serum analysis revealed significantly higher levels of AST, CK, CK-MB and LDH activities as well as MDA, troponin I, glucose and triglycerides concentrations in FMD-affected cattle compared to healthy control group ($p < 0.05$). Serum GPx and SOD activities in cattle with FMD were significantly lower than those in normal animals ($p < 0.05$), while there was no significant difference in serum CAT activity between 2 groups of animals. It is concluded that oxidative stress and some degrees of myocardial and pancreatic lesions develop in FMD-affected cattle. These findings provided information to better understand the pathogenesis of the disease and gives further insight to improve supportive treatment procedures in FMD virus infection in cattle.

Keywords

Foot and mouth disease, cattle, Picornaviridae, serum biochemistry, pancreas

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Number of Tables: 3
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Abbreviations

FMD: Foot and mouth disease
AST: Aspartate aminotransferase
CK: Ceatinine Kinase
CK-MB: Creatinine kinase myocardial band
LDH: Lactate dehydrogenase
MDA: Malondialdehyde
SOD: Superoxide dismutase
GPx: Glutathione peroxidase

Introduction

FMD is a severe, highly contagious viral disease of cloven-hoofed ruminants. The causative agent of FMD is an *aphthovirus* of the family *Picornaviridae* with seven strains (A, O, C, SAT1, SAT2, SAT3, and Asia1) which are endemic in different countries worldwide including several parts of Asia and in most of Africa and the Middle East [1].The causative agent can be found in all secretions and excretions from acutely infected animals and spreads rapidly by various direct and indirect contacts and airborne routes. FMD in cattle is clinically characterized by high fever, profuse salivation, and vesicles at the interdigital cleft in the oral cavity and on the mammary gland [2]. The disease is rarely fatal in adult animals, but there is often high mortality, up to 50%, in young animals due to myocarditis [3].

There have been few studies conducted on pathogenesis of FMD in domestic animals [4,5]. These studies generally conducted on virus kinetics in the host rather than the development of lesions in tissues and following organ failure. On the other hands , much of the basic knowledge about FMDV virus–host interactions is derived from in vitro studies under controlled cell culture conditions. These studies, while important, cannot fully address the complexity of the virus–host interaction at the tissue, organ and systemic levels in the natural hosts[6]. Additionally, there is limited information in the literature about the serum biochemical findings of cattle naturally infected with the FMD virus [7]. Serum biochemical analysis can be a useful tool for assessing animal health and helps better understanding the pathogenesis of the disease. Therefore, the aim of this study was to determine biochemical and oxidant-antioxidant status by evaluating some oxidative stress parameters and serum biochemical profile in Holstein cattle naturally affected with FMD.

Results

FMDV partial gene sequence was detected using RT-PCR test specific for VP1 nucleotide fragment. The FMDV-specific band with the size of 108 bp was detected in the tested sample and FMDV positive control. The positive PCR product band was the same as the positive control. No band was observed in the negative control (Fig. 1).

After sequencing of the RT- PCR products and preforming BLAST analysis, the FMDV VP1 gene fragment sequence was confirmed. Furthermore, alignment of the read sequences with the published sequences in the NCBI gene bank confirmed the presence of FMDV serotype O in the sample (99.8% identity).

The serum activities of CK, CK-MB, AST and LDH of FMD-affected cattle were significantly higher than healthy animals ($p < 0.001$) (Table 1). Serum amylase and lipase activities of FMD-affected cattle were also significantly higher than those in normal animals ($p < 0.05$) (Table 2). Serum concentration of troponin I, glucose and triglycerides of FMD-affected cattle were significantly elevated in comparison to healthy animals ($p < 0.05$) (Tables 1 and 2). The result of oxidative stress indices measurement is shown in Table 3. Serum activities of SOD and GPx of FMD-affected cattle were significantly lower than those in healthy animals ($p < 0.05$), while serum catalase activity showed no significant difference between FMD-affected and healthy cattle ($p > 0.05$). The serum concentration of MDA of FMD-affected cattle was significantly higher than that in the control group ($p < 0.001$) (Table 3).

Discussion

Serum biochemical references of cattle naturally infected with FMD virus are not known. The results of the present study revealed that serum activities of CK and AST, biomarkers of muscle degeneration and necrosis, in cattle with clinical FMD were increased and were significantly higher than those of healthy cattle. Furthermore, serum CK-MB activity and concentration of troponin I, biomarkers of myocardial damage, in cattle with FMD were also significantly higher than those in normal animals. Myocarditis with high mortality in neonate calves and lambs is a well known feature of FMD [1]. It has been suggested that after initial replication of FMD virus in the oropharynx, it disseminates to the secondary sites including cardiac muscle in suckling ruminants, and virus replication

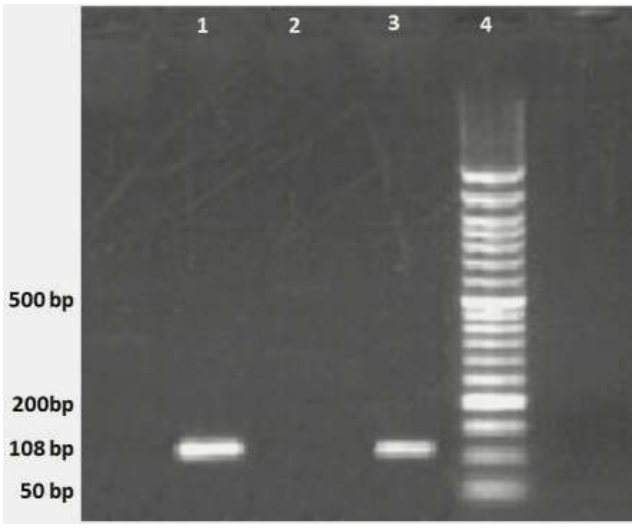

Figure 1
Electrophoresis of RT-PCR products. lane 1: positive control (108 bp). Lane 2: negative control. Lane 3: positive sample (108 bp). Lane 4: ExcelBand™ 50 bp DNA ladder.

in this tissue results in cardiac degeneration and necrosis [8]. Following cardiac cell damage, CK-MB and troponin I is released to the blood stream and their serum levels elevates, as indicators of myocarditis [9,10].

AST is present in all tissues except bone, with the highest levels in liver and skeletal muscle. CK activity is also greatest in skeletal muscle and rapidly increases in serum following muscle injuries. On the other hand, serum AST activity in cows with muscle injury slowly increases and has longer half-life than of CK [11]. AST is also present in high levels in the hepatocytes and its serum elevation is routinely considered as a sensitive indicator of hepatocyte damage, even if the damage is of a subclinical nature [12]. However, concurrent serum elevation of AST with CK is considered as inthe dicators of muscular damage [13]. The results of the present study showed that during the course of FMD in non-suckling cattle, some degrees of injuries occurs in cardiac and skeletal muscles. Although the myocardial degeneration and necrosis and associated mortality is a common finding in the suckling calves, lambs and pigs, the myotropism and the mechanism of muscular damage of FMDV are not fully understood. LDH is an enzyme involved in energy production that is found in almost all cells of the body, with the highest levels found in the cells of the heart, liver, muscles, kidneys, lungs, and in blood cells [12]. Although determination of serum LDH activity is one of

Table 1.
Parameters associated with cardiac and skeletal muscle damage in the FMD group and control group. Data are expressed as mean and standard error of the mean (Mean ± SE)

Parameters	FMD	Control	*p*
CK (U/L)	267.40 ± 11.01	140.37 ± 8.47	0.001
CK-MB (U/L)	127.86 ± 6.78	71.73 ±3.28	0.001
AST (U/L)	54.50 ± 3.85	31.00 ±1.02	0.001
LDH (U/L)	1484.58 ± 72.67	709.26 ± 42.53	0.001
Troponin I (ng/mL)	0.020 ± 0.0013	0.016 ± 0.0006	0.033

Table 2.
Parameters associated with pancreatic damage in the FMD group and control group. Data are expressed as Mean ± SE

Parameters	FMD	Control	*p*
Amylase (U/L)	81.37 ± 2.50	52.70 ± 2.32	0.001
Lipase (U/L)	39.00 ± 2.03	33.26 ± 1.60	0.031
Glucose (mg/dL)	73.73 ± 12.57	44.33 ± 11.59	0.001
Triglycerides (mg/dL)	22.76 ± 1.53	16.33 ± 1.08	0.033

Table 3.
Parameters of oxidative status in the FMD group and control group. Data are expressed as Mean ± SE

Parameters	FMD	Control	*p*
MDA (ng/mL)	4.58 ± 0.24	2.27 ± 0.14	0.001
GPx (U/L)	11.70 ± 0.04	14.52 ± 0.77	0.002
CAT (Ku/L)	1.21± 0.04	1.18 ± 0.20	0.323
SOD (% inhibition)	27.53 ± 1.23	30.79 ± 1.37	0.047

the most frequently performed assays as an aid in the diagnosis of myocardial and pulmonary infarction, it suffers from lack of specificity for cardiac disease [1]. Thus, serum elevation of LDH as was observed in the cattle affected by FMD indicates the involvement of various tissues. Elevation of serum CK-MB, AST and LDH activities have been reported in buffaloes infected with FMD virus, serotype O [14]. High levels of serum troponin I concentration has also been detected in calves and lambs suffered from FMD [9,10]. Although significantly higher levels of aforementioned parameters in cases of FMD in comparison to control animals is noticeable, and shows some degrees of myocardial damage, but its clinical importance is unknown for authors. Very high levels of serum CK and troponin I has been reported in clinical cases of FMD in lambs and calves [9, 10].

The present study demonstrated that FMD in cattle increases lipid peroxidation end products which was indicated by the elevation of the MDA concentration in the serum of affected animals compared to the healthy subjects. Polyunsaturated lipids are susceptible substrates to free radical oxidative damage and biomarkers of lipid peroxidation including MDA, are considered as a reliable marker for oxidative stress [15].This study revealed decreased activities of SOD and GPx in FMD-affected cattle compared with the control group. There were also no significant differences between the mean activities of CAT in the control group compared with the infected group. These are in agreement with the findings of Khoshvaghti et al. (2014) that reported low serum activities of SOD and GPx with no changes in CAT in FMD cattle infected with FMD virus [7]. SOD, CAT and GPX are the first line defense antioxidant enzymes. These enzymes dismutate superoxide radicals, breakdown hydrogen peroxides, and hydroperoxides to harmless molecules (H2O2/alcohol and O2), respectively [16]. It has been suggested that lower antioxidant enzyme activity might be caused by the depletion of antioxidant defense system occurring as the consequence of overproduction of free radicals induced by various factors, including virus infections [17]. During oxidative stress, production of highly reactive oxygen species (ROS) beyond the scavenging capacity of antioxidant defense mechanisms is directly involved in the oxidative damage of macromolecules including lipids, proteins and nucleic acids in tissues. Accumulating studies have indicated that excessive ROS production plays important roles in the pathogenesis of inflammatory diseases. For example, ROS contributes to virus replication and the activation of inflammatory cytokines during the infection of both influenza virus and bovine herpes virus -1 [18].

In the present study serum amylase and lipase activity in the FMD-affected cattle were found to be higher in comparison to healthy animals. Increased serum amylase and lipase activity is used as a reliable biomarker for the diagnosis of acute pancreatitis in animals (Hoffman and Solter2008). Serum activity of these enzymes increases due to the leakage from the inflamed pancreas into the blood stream. Amylase and lipase have relatively short blood half-time and so when rise rapidly after pancreas damage, return to normal range within five days [19]. On the other hand, high serum concentrations of glucose and triglycerides in the FMD-affected cattle in parallel to high serum activities of amylase and lipase found in the present study indicate pancreatic injuries [12] cause by FMD virus infection. Pancreatic acinar necrosis, inflammation and regeneration are manifestations of the acute form of FMD and diabetes mellitus has been observed in both experimental and natural cases of the disease [20]. Pancreatic degeneration and necrosis has been reported in a gazelle naturally infected by FMD virus serotype O1 [21]. It has also been suggested that viral replication in the myocardium and pancreas, and their associated pathologies are two common FMD virus infection features in the mouse model [22]

Enough data is not available for the serum biochemistry of FMD affected cattle. As a conclusion, serum analysis elucidates oxidative stress and some degrees of myocardial and pancreatic lesions in FMD-affected cattle. These findings provided information to better understand the pathogenesis of the disease and gives further insight to improve supportive treatment procedures in FMD virus infection in cattle.

Materials & Methods

During an outbreak of FMD in the Shahrekord district, blood samples (10 mL) were obtained from affected 23 cattle by jugular venipuncture using vacutainer tubes. Sampled cattle were Holstein and aged between 2 and 19 months and of both sexes (7 males and 16 females). During the investigation the disease was observed within that age range. Blood samples without anticoagulant were also taken from healthy cattle (n = 21; Holstein bred and age and herd management similar to the first group) originating from a control farm where FMD was not reported. Blood samples were left to clot, centrifugated at 3000 r.p.m for 20 minutes and separated serum samples were stored at -70 °C until biochemical analysis.

Samples including vesicular fluids were collected from the cattle showed clinical signs of FMD. These samples were placed in glycerol saline and kept at -20 °C until subjected to trials of virus detection. RNA was isolated using QIAzol (QIAGEN, Germany, Catalog Number: 79306) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed with TaqMan Reverse Transcription kit (Invitrogen, Germany, Catalog Number: 8080234N) according to the manufacturer's instructions. PCR was carried out using the primers designed for

a part of VP1 gene of FMDV using GenScript Primer Design online tool. The sequences of primers were as follows: FMDVF: 5'-TGAGTGCAGGTACAGCAGAA-3' and FMDVR: 5'-ATGGCACCGTAGTTGAAGGA-3'. The PCR thermal cycle reactions consisted of denaturation at 95 ºC for 30 s followed by 30 cycles at 95 ºC for 20 s, 55 ºC for 30 s, and 72 ºC for 30 s, followed by a final extension at 72 ºC for 5 minutes. The positive and negative controls prepared from Razi Vaccine and Serum Research Institute was included in each test. Six µL of the amplified products were loaded on a 1.3% agarose gel; visualized by staining with Green viewer (Pishgam Co. Tehran, Iran) and compared to DNA markers (Excel Band™ 50 bp DNA ladder, SMOBIO, Taiwan). PCR-positive samples in a volume of 50 µL was sent to the Bioneer Company for sequencing. The sequencing procedure was performed on the ABI 3730XL DNA Analyzer, with a high quality of sequence analysis data (Phred Score (QV): ≥ 20, Guaranteed read lengths: ≥ 600 bp).

Serum activities of aspartate aminotransferase (AST), creatinine Kinase (CK), creatinine kinase myocardial band (CK-MB) and lactate dehydrogenase (LDH), amylase and lipase, and serum concentration of glucose and triglycerides were measured by an auto-analyzer (BT 1500, Italy) using standard diagnostic kits (Dialab, Austria). Serum troponin I concentration were determined by an enzyme-linked immune-absorbent assay (ELISA) kit (Monobind. Inc, Canada). The kit had a detection sensitivity limit of 50 pg/mL troponin I. The intra and inter-assay coefficients of variations were 0.70% and 1.00% for CK, for CK- MB 0.61% and 0.53%, for AST 3.06% and 1.38%, for LDH 2.01% and 2.30% for glucose 1.74% and 1.19%, for lipase 1.01% and 0.82%, for amylase 067% and 0.97%, for triglyceride 1.82% and 1.60%, for HDL 0.69% and 0.58%; and for cholesterol 0.61% and 1.22%, respectively.

Serum malondialdehyde (MD) concentrations, also known as thiobarbituric acid reactive substances (TBARS), were determined colorimetrically using Buege & Aust method (1978)[23]. The activity of superoxide dismutase (SOD) was measured by nitrobluetetrazolium (NBT). In this method the superoxide radicals change NBT to blue NBTH2. By adding the serum to the studied material, producing of blue color by superoxide dismutase is controlled [24]. Catalase (CAT) activity was determined by using the Goth method (1991)[25]. Glutathione peroxidase was determined using the method described by Paglia and Valentine (1967)[26].

Statistical analysis

All biochemical data were expressed as the mean ± standard error. Differences between the groups were tested by *t*- test. Statistical analysis was performed by using Sigma Plot 12 software. A *p*-value of less than 0.05 was considered statistically significant.

Authors' Contributions

All authors contributed to the design of study, data analysis and manuscript preparation.

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Competing Interests

The authors declare that there is no conflict of interest.

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Effects of single injection of vitamin D3 on some immune and oxidative stress characteristics in transition dairy cows

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ABSTRACT

Recent studies suggest that vitamin D may have preventive and therapeutic effects on autoimmune disease, cancer, and diabetes type 1 and 2 beyond the skeletal condition and calcium metabolism. To demonstrate the effects of an over-supplemented single 8 million I.U. vitamin D3 IM injection on the modulation of immune responses and oxidative/antioxidative variables in transition dairy cows, this study was conducted on a commercial dairy farm with about 1500 lactating cows in the Tehran province, Iran. Twenty-four multiparous Holstein cows were randomly categorized into control and treatment groups. In the treatment group, 12 cows received a single dose of 8,000,000 IU vitamin D intramuscularly. In the control group, a placebo (distilled water) was injected into 12 cows 2 to 8 days before the expected calving time. Blood samples were collected on 21 and 7 days before calving and 1,3,7,15, and 30 days after calving. 25(OH)vitamin D3, tumor necrosis factor- α (TNF-α), interferon-γ (INF-γ), haptoglobin, interleukin 6 (IL-6), ferric reducing the ability of plasma (FRAP), glutathione peroxidase (GPx), superoxide dismutase (SOD), and hemolysate GPx were measured. This study showed that the treatment group had significantly higher amounts of 25(OH) vitamin D3, hemolysate GPx, and IL-6 values than the control group. According to our results, vitamin D3 injection increased the amounts of IL-6 and hemolysate GPx activity and tended to affect serum GPx activity.

Keywords

Haptoglobin, immune regulation, superoxide dismutase, 25(OH) Vitamin D

Abbreviations

DMI: Dry matter intake
Mcal: Mega calorie
DCAD: Dietary anion cation difference

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Introduction

For many years, vitamin D has been known for its classical effects on calcium (Ca) metabolism and and skeletal turnover. Initial evidence for non-classical effects of the active form of vitamin D (1, 25-dihydroxy vitamin D) arose from studies in the early 1980s. Recent studies in humans suggest that beyond the skeletal effects and Ca metabolism, vitamin D may have preventive and therapeutic effects in cardiovascular and autoimmune diseases, common cancers, and type 1 and 2 diabetes [1, 2].

Vitamin D classical target organs include bones, intestine, kidney, and parathyroid glands. Vitamin D promotes active uptake of calcium to regulate calcium concentrations within normal limits. The epithelial calcium channel, transient receptor potential vanilloid 6 (TRPV6), and calbindin transport of calcium into the cells are upregulated by vitamin D [3]. Most studies in cattle are toward regulating calcium homeostasis by vitamin D linked to the onset of lactation and hypocalcemia after parturition (milk fever) [4, 5]. After parturition, most cows undergo negative calcium balance. Thus, transition period management is an important step to prevent many diseases in dairy cows, and maintaining calcium in a normal range is necessary [6].

Vitamin D receptor (VDR) is widely distributed in many organs such as the heart, stomach, pancreas, brain, skin, and glands. Activated T and B lymphocytes and macrophages also have nuclear receptors for Vitamin D. Many autoimmune diseases including type 1 diabetes, rheumatoid arthritis, and multiple sclerosis have all been successfully prevented by receiving 1,25 $[OH]_2$D3 early in life [7].

Vitamin D signaling in immune cells can improve innate and adaptive immune reactions. Vitamin D influences the adaptive immune system in cattle similar to that in humans, but the target of vitamin D in the innate immune system is somewhat different. Recent studies in cattle and humans showed that vitamin D inhibits the pro-inflammatory effect of interferon-γ (INF-γ) and interleukin -17 (IL- 17) secreted by antigen-specific T cells *in vivo* [8, 9]. Vitamin D also plays a role in cattle innate immune system mediated by Toll-like receptors that upregulate vitamin D receptors and CYP27B1 (1α[OH]ase), the enzyme that converts 25[OH]vitamin D3 to 1,25$[OH]_2$D3. Vitamin D induces the expression of VDR on macrophages and phagocytes. In cattle and humans, vitamin D is responsible for the upregulation of nitric oxide (NO) gene expression and increasing NO production [2, 8].

1, 25 $[OH]_2$D3 affects the activity of antigen-presenting cells (APCs), which act as a mediator between innate and adaptive immunity. 1,25$[OH]_2$D3 induces 'tolerogenic' dendritic cells, [DC] down-regulates CD40 (required for B cell activation), elevates IL-10 production in DC, and down-regulates MHC class II molecule expression on APCs [9].

Studies have shown that a high dose of vitamin D can prevent type 1 diabetes by immune regulation. Vitamin D is a potent blocker of dendritic cell differentiation that directly blocks IL-12 secretion [1, 10]. Vitamin D deficiency has long been linked to glucose intolerance in humans. Many studies in humans have shown that vitamin D plays an important role in the pathogenesis of type 2 diabetes by affecting either insulin sensitivity or β cell function, or both [10, 11].

The dose of vitamin D supplementation effective in dairy cow health is not well defined. The NRC (2001) recommends 21,000 IU supplemental vitamin D3/day (~800 to 1,000 IU/kg of DM) for lactating Holstein cows (calculated for 680 kg of BW). Instead, dairy producers typically provide lactating cows with 30,000 to 50,000 IU of vitamin D3 [12]. Nelson et al. clarified that 22% of cows supplemented with 20,000 IU/day vitamin D (NRC recommendation) had serum 25[OH] D below 30 ng/mL whereas, 95% of cows receiving 30,000 IU/d or more have serum 25[OH] D above 40 ng/mL [12]. Nelson et al. showed that most dairy cows that received vitamin D 1.5 to 2.5 times more than the recommended NRC vitamin D have an average serum vitamin D of 60 to 70 and in the range of 40 to 100 ng/ml [12]. Lean et al. suggested 40,000 IU vitamin D for appropriate vitamin D functional role but considering optimal time supplementation before calving is a vital step [13]. A recent study has shown that serum vitamin D concentration is diminished in the transition period, particularly in early lactation when dairy cows are most susceptible to disease and metabolic stress. Diminished vitamin D concentrations may enhance inflammatory reaction and oxidative stress [14]. Early studies have shown that NRC recommendation for vitamin D supplementation may not be enough for a proper immune function. Moreover, in dairy cows vitamin D concentrations have been reduced in critical periods just after calving [12, 14]. In early lactation, cows are vulnerable to metabolic diseases and oxidative stress, so the reduced serum vitamin D may play a role in this condition. The effects of vitamin D on immune system regulation and energy balance are well known in human medicine, but there is still a lack of knowledge in veterinary research in this field [9].

Dysregulated inflammation and adipose remodeling are recognized as the key components of the metabolic stress syndrome and cytokines secreted in the lipolysis process, particularly after parturition [15]. The present study aimed to demonstrate the effects of an over-supplemented single 8 million I.U. vitamin D3 IM injection is variable on the modulation

of immune responses and oxidative/antioxidative status during the transition period of dairy cows.

Results

This study showed that injection of vitamin D significantly increased 25(OH) vitamin D, IL-6, and hemolysate GPx values in the treatment group (Figurs 1 and 2, Table 1, $p \leq 0.05$) in comparison with control cows. Time of sampling (time) did not have a significant effect on any of the measured variables. There was not any treatment × time interaction for measured variables. BCS and number of parturition did not have significant effects as the covariate.

Pairwise comparisons for each sampling time showed that serum GPx at 7 days after calving, hemolysate GPx at 7 and 30 days after calving, and SOD at 15 days after calving caused a significant difference between trial groups (Figurs 1 and 2, Table 1, $p \leq 0.05$).

Discussion

Serum vitamin D concentration is reduced in early lactation due to increased demand in calcium homeostasis and consumption by the immune system. This condition may be associated with metabolic diseases after calving [14]. In the present study, the injection of vitamin D prevented the reduction of vitamin D in early lactation in the treatment group, compared to that in the control group.

Many studies proved that vitamin D enhances innate immunity by stimulating antimicrobial peptides, such as β-defensin, nitric oxide, cathelicidin, pattern recognition receptors, and cytokines [16,17]. Vitamin D may downregulate TLR-2 and TLR-4 expression and cause hyporesponsiveness to the pathogen-associated molecular pattern molecules (PAMPS), and the controlled immune responses [2]. In the present study, total globulin in the treatment group did

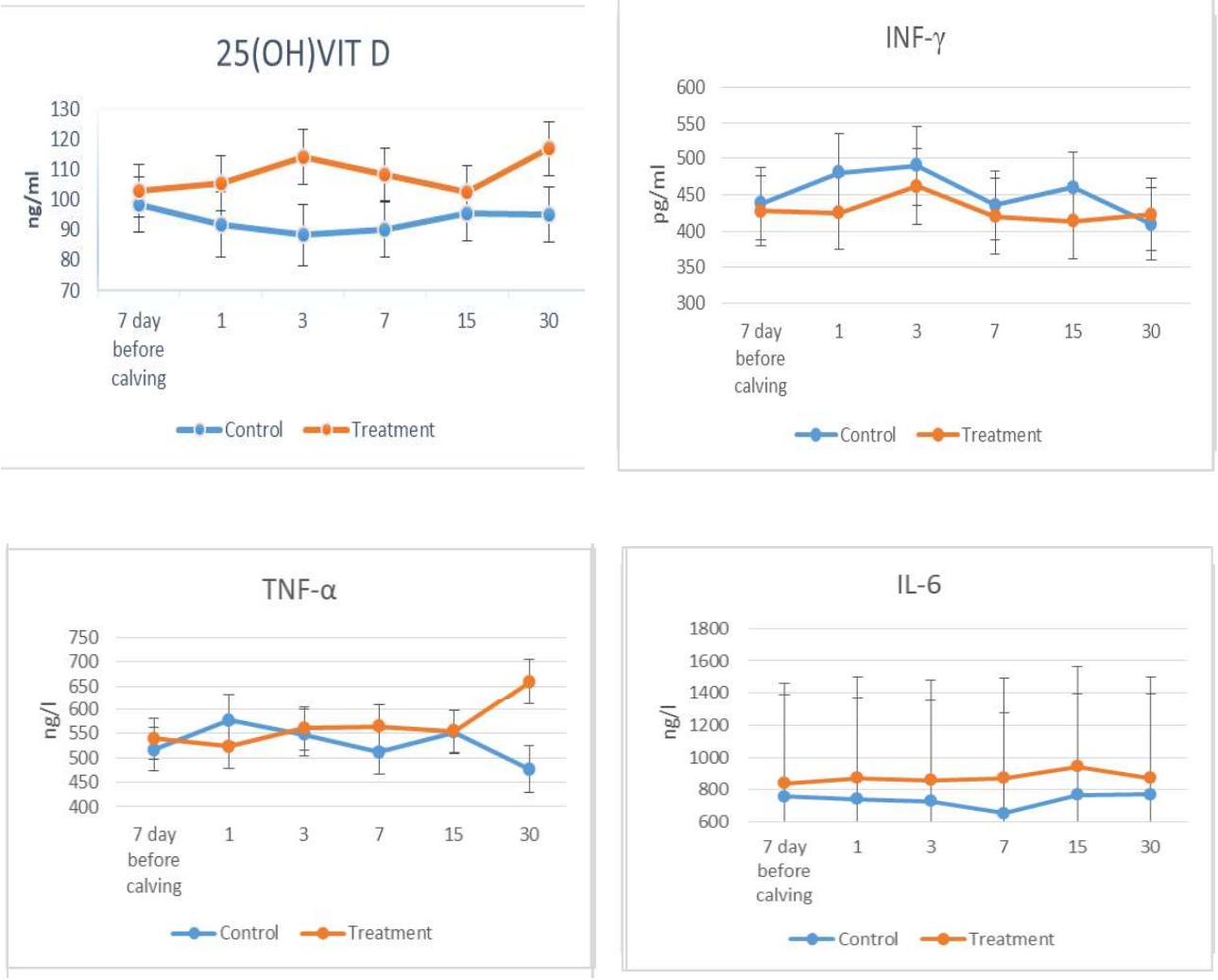

Figure 1.
Time related changes (LSM ± SE) and pairwise comparisons for the amounts of 25(OH) vit D, TNF- α, INF-γ, and IL-6 in trial groups.

Table 1.
Least square mean and SE of measured variables and the effects of time and treatment in trial groups

Variables	Control (n=12)	Treatment (n=12)	SE	Treatment effect	Time effect	Time*Treatment effect
25(OH)Vitamin D (ng/ml)	93.21	108.41	3.94	0.01	NS	NS
Total-globulin (g/dl)	3.81	3.99	0.08	NS	NS	NS
INF-γ (pg/ml)	452.10	428.39	20.9	NS	NS	NS
TNF-α (ng/l)	530.21	566.71	21.07	NS	NS	NS
IL-6 (ng/l)	736.07	875.62	618.07	0.04	NS	NS
Haptoglobin (μg/ml)	203.14	208.28	5.21	NS	NS	NS
GPx (U/ml)	948.07	1010.91	22.78	0.06	NS	NS
hemolysate GPx (U/mg Hb)	1385.79	1639.59	189.49	0.003	NS	NS
SOD (IU/ml)	293.15	295.67	4.46	NS	NS	NS
FRAP (mmol Fe^{2+}/L)	16.13	16.33	0.36	NS	NS	NS

S: significant difference ($p \leq 0.05$), NS: not significant

not show any significant difference with the control group. According to the results concerning the globulin amount, a previous study suggested that vitamin D has a greater tendency to promote cell-mediated T helper1 immunity than antibody-mediated T helper 2 immunity [2].

TNF-α, IL-6, INF-γ are the major cytokines in immune regulation. Borges et al. proposed that the immune-modulatory effects of vitamin D are associated with a reduction in the INF-γ level [18]. Adam and Hewison (2008) suggested that vitamin D could suppress T helper proliferation and also inhibit Th1 cytokine expression (INF- γ, TNF- α, IL-2) [2]. In this regard, our results for INF-γ are similar to the previous studies. The treatment group had lower INF-γ mean value than the control group, although it was not significant (control group =452.1, treatment group= 428.39). The results of the present study were in agreement with the previous studies showing that vitamin D supplementation could not significantly diminish INF-γ value [19, 20]. There are some possible explanations for non-significant INF-γ changes in the present study. Firstly, in the absence of active inflammation, the immunomodulatory effect of vitamin D on the levels of INF- γ did not occur [19]. Secondly, the small number of animals in each group may have reduced the statistical power for comparison to find a significant difference between the two groups. On the other hand, in the present study, TNF-α amount was numerically higher in the treatment group. In addition, the IL-6 levels were significantly higher in the treatment group. IL-6 and TNF-α are involved in innate immunity and are produced by T helper1 cells and macrophages [18]. The previous studies demonstrated that TNF-α and IL-6 were important cytokines produced in visceral adipose tissues [18,21,22]. The transition period in dairy cows is a critical time for the occurrence of inflammation and many inflammatory conditions experienced during this period, including metabolic and infectious diseases, stressful situations, trauma at calving time, energy excess or deficit, and digestive upsets [23]. In dairy cows, adipose tissue plays an endocrine role. There is a hypothesis that cows with higher (obese) body scores develop inflammation, which leads to insulin resistance and impaired insulin secretion [24]. Our results about IL-6 are different from the previous studies in humans. An earlier study revealed that vitamin D could diminish IL-6 and TNF-α concentrations [18]. Incubation of isolated monocytes with $1,25[OH]_2D3$ attenuates the expression of pro-inflammatory cytokines involved in insulin resistance such as IL-1, IL-6, and TNF-α in type 2 diabetes mellitus human patients [25]. Also, in dairy cows and other ruminants, macrophages of adipose tissue and lipolysis mechanism (adipose tissue remodeling) cause the activation of NF-κB signal transduction and induce IL-6, TNF-α, and IL-1 secretion, especially during the dairy cow transitional period [15]. Although IL-6 was significantly increased in the treatment group, there were no significant differences in the occurrence of inflammatory diseases during the study between the groups.

Table 2.
Ingredients, chemical composition and nutritive value of diets fed to close up cows

	DMI	%		
Legume hay immature	4.17	32.61	Nel 3X (Mcal)	19.45
Corn silage immature	2.42	18.97	Nel 3X (Mcal/kg)	1.53
Wheat straw	0.46	3.63	RUP Kg	0.74
Corn gluten meal	0.13	1.01	RUP % of DM	5.84
Extrude linseed (flax)	0.41	3.21	RDP kg	0.74
Barley grain rolled	1.35	10.57	RDP % of DMI	1.12
Corn grain ground dry	1.50	11.71	CP Kg	1.86
Wheat bran	0.13	1.05	CP % DM	14.6
Canola meal	0.45	3.53	NDF %	37.31
Cotton seed meal (solvent extracted)	0.32	2.49	ADF %	23.01
Soybean meal, expellers	0.36	2.79	NFC %	39.81
Full-fat soy, roated	0.19	1.46	EE %	4.73
Fish meal, anchovy	0.18	1.44	Ca/P	3.48
Vegetable oil	0.15	1.17	Absorbable Ca (Kg/day)	0.16
Anionic supplement *	0.50	3.91	Dietary Ca %	1.24
Limestone	0.02	0.15	Absorbable P (Kg/day)	0.05
Toxin blinder	0.02	0.12	Dietary P %	0.36
Availa chrome	0.01	0.06	Mg %	0.57
Levucell	0.02	0.12	Cl%	0.87
Total	12.78	100.00	K%	1.44
Forage % of DMI	55.33		Na %	0.04
Concentrate % of DMI	43.67		S%	0.35
			DCAD (mEq/Kg Dm)	-81.46

*refer to Table 4

An *in vitro* study suggested that the effects of vitamin D on IL-6 expression in monocyte/macrophage could be slightly upregulated, depending on various factors, such as interleukin under consideration, the degree of maturation, and the stimulus. This study showed that vitamin D in monocyte/macrophage in the absence of TNF-α could markedly induce IL-6 gene expression [26]. Naghavi Gargari et al. showed that vitamin D supplementation up-regulated IL-6 and IL-17A gene expression in multiple sclerosis patients [27]. Vitamin D could affect IL-6 expression in multiple routes, including NFκB inhibition, p38 MAP kinase inhibition, and CoX-2 inhibition [28]. Both *in vivo* and *in vitro* studies showed that oxidative stress might cause underlying diseases in the transition period of dairy cattle [29, 30]. Oxidative stress causes a dysfunctional inflammatory reaction, raises IL-6 and TNF-α, and induces metabolic stress, which is important for the occurrence of underlying diseases, such as ketosis and fatty liver [31]. GPx represents a main intracellular anti-oxidant agent. The treatment effect tends to be significant for GPx value ($p < 0.06$) in the present study. A previous study in cattle showed that raising intracellular GPx indicated a proper condition against oxidative stress [30]. In addition, in the present study, hemolysate GPx was significantly higher in the treatment group. Thus, vitamin D administration could lead to lower inflammatory and oxidative stress due to better anti-oxidative performance. In our study IL-6, GPx in hemolysate, and probably GPx in serum amounts were significantly higher in vitamin D injected cows, although INF- γ, and TNF-α

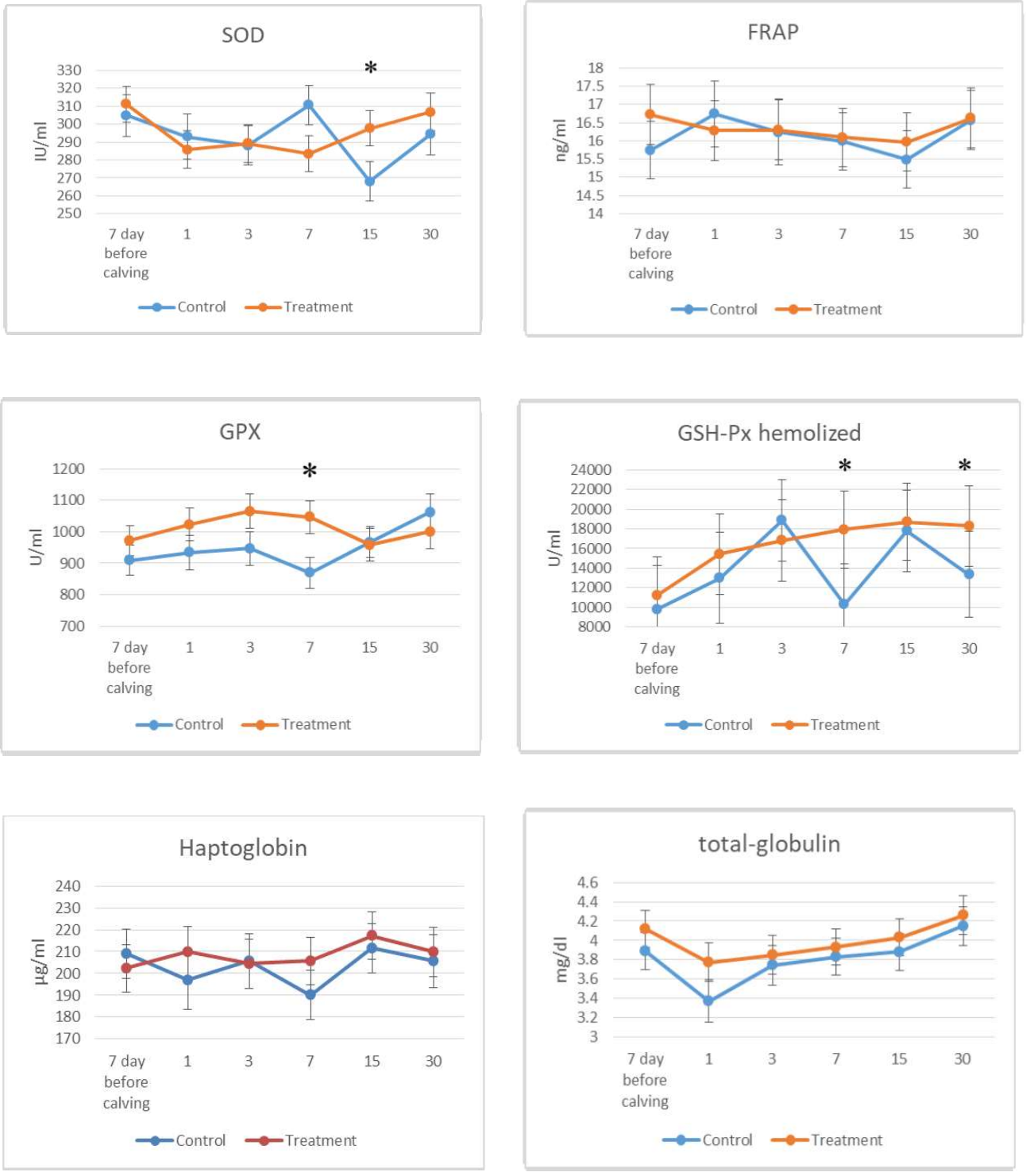

Figure 2.
Time related changes (LSM ± SE) and pairwise comparisons for the amounts of GPx, hemolysate GPX, FRAP, SOD, total globulin, and haptoglobin for trial groups. The asterisk shows significant difference between groups ($p \leq 0.05$).

amounts were not significantly different between trial groups. It seems that the increased amounts of GPx activity in serum and hemolysate similar to an increased amount of IL-6 were compensatory responses; however, its precise reason was not clear.

In conclusion, vitamin D injection increased the amounts of IL-6 and hemolysate GPx activity and tended to affect serum GPx activity. The number of animals used in this study was not large enough to show the exact differences between trial groups. Thus, it is necessary to conduct further studies with a larger number of animals to clearly understand the role of vitamin D as an immunomodulatory factor in transition dairy cows.

Materials & Methods

Cows, experimental design, and feed

The trial was conducted in a commercial dairy farm with about 1500 lactating Holstein cows in Tehran province, Iran. The rolling herd average for milk production was about 10,920 Kg. The herd used mix loose pens with adjacent outside yards and free-stall facilities with sand bedding. The animals had free access to water throughout the experiment. The predominant forages used in this farm were alfalfa hay, and corn silage, and the main concentrates consisted of corn, barley, soybean meal, canola meal, linseed meal, cottonseed meal, wheat bran, gluten feed, and sugarcane bagasse. Feed composition details for both close-up and early lactation cows are depicted in Tables 2 and 3. Total mixed ration (TMR) was offered to cattle twice a day and mixed by a mechanized feeder. Diet's ingredients included between 20,000 and 30,000 IU of Vitamin D3 per day. The farm held a policy of drying off the cattle 8 weeks before the expected calving day. All the cows in close up period were placed on a diet with anionic salts according to table-3 (500g/head/day) including ammonium sulphate, magnesium sulphate, and calcium chloride and a DCAD between -100 to -110 mEq/Kg of DM (Calculated as $(Na^+ \times 435mEq + K^+ \times 256mEq) - (Cl^- \times 282mEq + S^{2-} \times 624mEq)$). Cattle urine pH were measured twice weekly using a digital pH-meter (Jenway, Model 3040, England) from 2 days after the anionic salts were added until calving to make sure that the value was never less than 5.8 or more than 6.8.

Twenty-four Holstein cows were randomly selected and put in control and treatment groups. Blood sampling was conducted in the same season and time in the treatment and control groups. Both groups were approximately homogenous concerning body condition score (BCS) and number of parturition of cows. In the treatment group, 12 cows received a single dose of 8,000,000 IU vitamin D3 (cholecalciferol, Darou Pakhsh Co., Iran) intra muscularly before sampling at seven days before the expected calving time, and in the control group, 12 cows were injected placebo (injectable distilled water) at the same time. In our study, the dose of vitamin D was selected based on previously published studies and also for preventing of any probable toxicosis [32, 33]. Cows that did not calve within the expected time were eliminated from the study; the elimination criterion also included those who lost their health for any reason during the study. The parity of the cows ranged from 3 to 7 and its average for control and treatment groups was 4.42 and 3.83, respectively. The body conditions of all cows were scored in far-off period based on a 5-point scale and an increment of 0.25 (34). All scorings were performed by a single evaluator. All the cows in both groups were clinically healthy based on Duffield et al. and had a BCS between 3.25 and 4 initially in the far-off period [35]. The cows were also categorized into 2 groups based on BCS; BCS of ≥ 3.7 (control, n=5, and treatment, n=4) were enrolled as fat cow (FC), and a BCS of < 3.7 (control, n=7, and treatment, n=8) as nonfat cow (NFC) for statistical purposes.

Blood sampling and variables measurements

Blood samples were collected via jugular vein with disposable syringes between 8 to 10 A.M on 21 and 7 days before calving and 1,3,7,15, and 30 days after calving. We considered 21 days before calving as covariate for other sampling times. Blood samples were taken by disposable syringes on the plain tube and chilled immediately after collection, and sera were harvested immediately after centrifugation at 2200×g for 10 min. Serum was kept frozen at −20°C until transfer to the laboratory for further analysis. EDTA containing tube was also used for obtaining whole blood. Hemolysate was prepared as follows: After primary centrifugation of whole blood (1800 g for 15 min) plasma was removed and the remaining packed erythrocytes were washed three times with normal saline. Eventually four volumes of cold distilled water were added to one volume of the washed packed erythrocytes, shaken and after final centrifugation; supernatant hemolysate was aliquoted and stored at -20°C. 25(OH)Vitamin D, tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), haptoglobin, interleukin-6 (IL-6), and glutathione peroxidase (GPx) were measured in serum by cattle-specific enzyme-linked immunosorbent assay kits (Shanghai Crystal Day Biotech CO., LTD, Shanghai, China). Superoxide dismutase (SOD) and ferreic reducing agent of plasma (FRAP) in serum and GPx in hemolysate were also measured by commercial kits based on enzymatic reactions (Randox Laboratories Ltd., Ardmore,UK). Intra-assay and inter-assay coefficient of variation for all ELISA tests were less than 8% and 10%, respectively, and sensitivities are as following: 25(OH)Vitamin D: 0.53 ng/ml, TNF-α: 5.56ng/L, IL-6: 10.56 ng/L, INF-γ: 2.35 pg/ml, haptoglobin: 1.36 µg/ml, and GPx: 5.69 U/ml. The inter assay and intra assay of SOD measurement method were 7.07% and 3.58%, respectively, and the sensitivity was < 6.13 U/ml. The total assay and intra assay of GPx measurement method were 4.37% and 3.2%, respectively, and the sensitivity was 75 U/L. FRAP kit sensitivity was 2 µmol Fe^{2+}. All measurements were performed by biochemical auto-analyzer (Biotecnica, BT 1500, Rome, Italy). Control serum (Randox Laboratories Ltd., Ardmore, UK) was used for controlling measurement accuracy. For measuring of globulin amount, serum total protein, and albumin concentrations were measured by commercial kits (Pars azmoon, Tehran, Iran) and the amount of globulin was calculated by subtracting of albumin from total protein.

Data management and statistical analysis

Normality of variables was evaluated by PROC UNIVARIATE of SAS software, version 9.2 (SAS Inst. Inc., Cary, NC). Variables with Shapiro-Wilk values $p > 0.05$ were considered normal (25(OH)Vitamin D, TNF-α, INF-γ, IL-6, GPx, hemolysate GPx, SOD, total globulin) while all other variables were normalized using natural logarithm.

Data of serum profile and cytokines were then analyzed using repeated-measures ANOVA (Mixed procedure in SAS, version 9.2). The model for all serum metabolite contained the effects of time of sampling (-7, 1, 3,7,15, 30 days), BCS category, and parity group. Cows that had a BCS of ≥ 3.7 were enrolled as fat cow (FC), and a BCS of < 3.7 as nonfat cow (NFC). Parity was classified into 2 groups: cows with third and fourth parity as parity group 1 (control, n = 8, and treatment, n = 10) and cows with five and higher parity as parity group 2 (control, n = 4, and treatment, n= 2). Interactions between time of sampling and BCS were tested to see if BCS effect was significant. Pairwise comparisons for each

sampling time were conducted by *Tukey's* procedure. Differences with $p \leq 0.05$ were considered as significant, and $0.05 < p \leq 0.10$ were considered as a tendency. Least square means and standard errors are presented as LSM ± SE.

Animal Welfare Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee of Ferdowsi University of Mashhad approval has been received (3/43622). The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

Table 3.
Ingredients, chemical composition and nutritive value of diets fed to fresh cows

	DMI	%		
Alfalfa hay	3.704	17.02	NEL 3X (Mcal)	36.09
corn silage	2.83	12.99	NEL 3X (Mcal/kg DM)	1.85
Wheat straw	0.185	0.85	RUP (kg)	1.75
Corn gluten meal	0.518	2.38	RUP % of DMI	8.06
extruded linseed (full-fat flaxseed)1	0.446	2.05	RDP (kg)	1.83
Barley grain rolled	2.71	12.41	RDP % of DMI	8.39
Corn grain ground dry	2.64	12.13	CP kg	3.58
Wheat bran	0.21	0.94	CP % of DM	16.45
Canola meal	0.155	5.31	NDF % of DMI	33.73
Cotton seed W lint	0.531	2.44	ADF % of DMI	19.33
Soy Meal, expellers,	1.682	7.73	NFC % of DM	43.05
Soybean seeds, whole roasted	0.344	1.58	EE % of DM	4.68
Fish meal, Anchovy	0.165	0.76	Absorbable Ca (Kg/day)	0.18
vegetable oil	0.21	0.96	Dietary Ca % of DMI	0.82
beet Pulp, dried	3.532	16.23	absorbable P (Kg/day)	0.11
Calcium Carbonate	0.089	0.41	Dietary P % of DMI	0.51
DCP (Di-Calcium Hydrogen Phosphate)	0.048	0.22	Ca/P ratio	1.62
Sodium Bicarbonate	0.181	0.83	Mg % DMI	0.34
Salt	0.039	0.18	Cl % of DMI	0.36
Magnesium oxide	0.051	0.23	K % of DMI	1.64
Vitamin/Mineral supplement6	0.161	0.74	Na % of DMI	0.42
Bentonite	0.061	0.28	S % of DMI	0.25
Propylene glycol2	0.250	1.15	DCAD (mEq/kg DM)	+345.3688
Antimycotoxin3	0.015	0.07		
Yeast4	0.015	0.07		
Rumen protected methionine5	0.009	0.04		
total	20.78			
Forage% of DM	30.87	100		
Concentrate % of DM	66.75			

1Shayflax, Tehran, Iran

2Glycoline™, Vitalac Co, France

3mycosorb™, Alltech co, USA

4levucell™, (Saccharomyces cerevisiae CNCM I-1077), Nutritech co, USA

5mepron™, Evonik industries, Wien, Austria

6 vitamin/mineral supplement contain: Ca 13.4%, P 1.08%, Mg 3.4%, Na 10.35%, Cl 8.11%, Co 20 mg/kg, Cu 1500 mg/kg, I 70 mg/kg, Fe 3000mg/kg, Mn 4000 mg/kg, Se 370 mg/kg, Zn 5000 mg/kg, Vit A 700000 IU/kg, Vit D 200000 IU/kg, Vit E 2000 IU/kg

Table 4.
Ingredients of Anionic salt used in diet of cows in close-up period (ANIOMIX®)

Ingredient	Amount
Vitamin A	300000 IU
Vitamin D3	45000 IU
Vitamin E	3000 IU
Calcium (Ca)	150 gr
Chlorine (CL)	150 gr
Magnesium (Mg)	25 gr
Sulfur (S)	35 gr
Zinc (Zn)	1500 mg
Manganese (Mn)	1200 mg
Copper (CU)	500 mg
Selenium (Se)	8 mg
Cobalt (Co)	9 mg
Iodine (I)	12 mg
Chrome (Cr)	14 mg
Monensin	400 mg
Niacin (B3)	4 gr
Antioxidant	1000 gr
DCAD	-6473.21 mEq/kg
DCAB	-4285.714 mEq/kg

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Authors' Contributions

MH contributed to the main design of study, sample collection, laboratory tests, data analysis, and drafting the manuscript. MM contributed to main design of the study, data analysis, and reviewed and edited the manuscript. HAS contributed to the main design of the study and data analysis. All authors approved the final version of the manuscript for publication.

Conflict of interest

The authors declare they have no conflict of interest.

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Isolation and culturing myogenic satellite cells from ovine skeletal muscle

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ABSTRACT

Sheep satellite cells more than satellite cells of the rat and mouse are similar to human satellite cells. These cells are widely used in the modeling and treatment of diseases like heart insufficiency, neurological diseases, muscular dystrophy, cerebral cell transplantation for the treatment of migraines, screening, and the production of new drugs. This study was aimed to isolate and culture primary satellite cells (PSCs) obtained from sheep fetus, and perform clonal expansion of transfected PSCs. Skeletal muscle tissues of hind limbs were collected from sheep fetuses obtained from a local abattoir. After enzymatic digestion, flasks were replaced after 3 hours to isolate non-myogenic cells, such as fibroblasts. After six days, the cells were differentiated to myoblasts. Using a differentiation medium containing the horse serum, myotube cells were observed in the flask, indicating that the cultured cells were satellite cells. The mRNA expression of the PAX7 gene was used to confirm the presence of satellite cells. In addition, the results showed that satellite cells grow in a culture medium containing 5% FBS without differentiation, while 10% FBS initiates their differentiation.

Keywords

Myoblasts, PAX7, Satellite cells, Sheep

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Abbreviations

PSCs: Primary satellite cells
MDSCs: Muscle-derived satellite cells

Introduction

Skeletal muscle tissue is composed of different cell types. The growth and development of this tissue are controlled by several mechanisms [1]. Homeostatic and regenerative replacement of skeletal muscle fibers requires the activity of a dedicated pool of self-renewing muscle-derived satellite cells (MDSCs) [2, 3]. MDSCs are small mononucleotide fusiform cells, lying between the basal lamina and the sarcolemma of muscle fibers, making them difficult to isolate [4, 5]. The intermediate filament protein, desmin, and the striated muscle actin protein, a sarcomeric actinin that is abundantly expressed in skeletal muscle cells, are involved in the movement and can be used to identify skeletal muscle cells in other tissues. Furthermore, the myogenic regulatory factors, Myf5 and MyoD1, are markers of the proliferation and differentiation of satellite cells. PAX7 also has a vital role in the maintenance of satellite cells. In zebrafish, PAX7 expression marks muscle progenitor cells, and when muscle tissue is injured, PAX7+ cells migrate around the site of injury and enter the cell cycle, while adjacent fibers up-regulate the expression of myogenic regulatory factor [6]. Immunodetection assays have shown the presence of 93% PAX7 cells and 8% MyoD cells. PAX7 marks both quiescent and activated satellite cells, whereas MyoD marks activated satellite cells only[6, 7].

In addition to MDSC's role in the acute repair of damaged muscle tissue, satellite cells are of interest in some research fields such as aging, stem cell therapy, exercise, and neuromuscular diseases [8]. The first MDSCs were found in an electron microscopic study of the peripheral region of the frog skeletal muscle fiber [9], but later the viable satellite cells were isolated from adult rat skeletal muscles [10]. Satellite cells appear in the limbs at day 17 in the ovine embryo, after primary muscle fibers have formed [11]. A subpopulation of satellite cells may be derived from a more primitive stem cell [12]. Their origin is not known, but there is evidence that they are derived from the dorsal aorta [11].

Quiescent satellite cells are characterized by the expression of PAX7 and Myf5, but not by MyoD or Myogenin. Damage to the environment surrounding satellite cells results in the deterioration of the basal lamina and their exit from the quiescent state (satellite cell activation) [13]. During regeneration, activated satellite cells could return to quiescence to maintain the satellite cell pool. This ability is critical for long-term muscle integrity [13].

Skeletal muscle satellite cells have received a great deal of attention because they directly participate in skeletal muscle differentiation and repair of adult muscle tissue. Most MDSC studies have involved mice, rats, and humans, while few MDSC studies have focused on livestock, such as cows and sheep [14]. This study aimed to establish a protocol for isolating and culturing the primary satellite cells (PSCs) obtained from ovine fetal muscle tissue.

Results

At first, different types of cells with different morphologies were observed (Figure 1-A). By placing the cells inside the flask and replacing the medium after 1h, some of the fibroblast cells attached to the flask. After 10–14 h, other cell types began to attach to the plate; some of the adherent cells had round, spindle, or polygonal shapes (Figure 1-B). Most non-specific MDSCs remained in the initial flask by transferring the medium into other flasks. After the second round of selection, the remaining cells had a completely compressed appearance and were similar to cubic cells of the transplanted tissue. Other cells, such as the nerve and the fat, were removed after the third transfer (Figure 1-C). Cells obtained from the forth and the fifth transfer were muscle cell precursors. Although their size was small, but they required 4-5 days to grow larger (Figure 1-D and E). Five days after inoculation, half of the cells were fused, had long tubular shapes and significantly larger size than single MDSCs, and many nuclei could be seen in the swollen regions of the cytoplasm. These features indicate the formation of myotubes. In the first 48 hours fibroblast cells appeared, after 96 hours myoblasts, and on the sixth day, satellite cells were observed regularly with spherical shape (Figure 1-F).

RT-PCR reactions were performed with PAX7 (satellite cell-specific markers) primers. RT–PCR results showed that PAX7 is expressed, confirming that the isolated cells were MDSCs (Figure 2).

Bacterial contamination can easily be detected after a few days. No bacterial contamination was detected by PCR analysis (Figure 3).

Figure 4 depicts the cellular-growth curves for satellite cells determined by the trypan blue assay. In this figure, the "lag-phase" was present in all cellular lines during the first 24h of incubation. After this period, the "log-phase" started to indicate that the growth was more significant for 10%FBS than the rest of cellular lines. These cell showed the highest growth rate in this experiment; whereas the growth rates of the rest of other lines were as follows: 5% FBS > 0% FBS. The 5% and 10% contained FBS manifested three phases of cell growth, while the rest of cells only manifested the "lag and death-phases". In other words, cells in 0% FBS line never reached the "log-phase", as it remains practically on the "lag-phase", because the medium

did not have any FBS. In the 10% FBS, cells showed the highest max value at day 4; whereas, the 5%FBS cells has the lowest max value. It can be concluded that by increasing the FBS, the growth and differentiation of the satellite cells increased. Also, we might control the differentiation of satellite cells with media containing 5% FBS, consequently cell proliferation will speed up and we may have a chance to obtain more cells from limited primary cells.

Depending on the size indicated on the lam, the diameter of the satellite cells was measured. Each degree is equal to 0.01 mm. According to Figure 5, the diameter of the satellite cell is 1.5 degrees, which is equal to 0.015 mm.

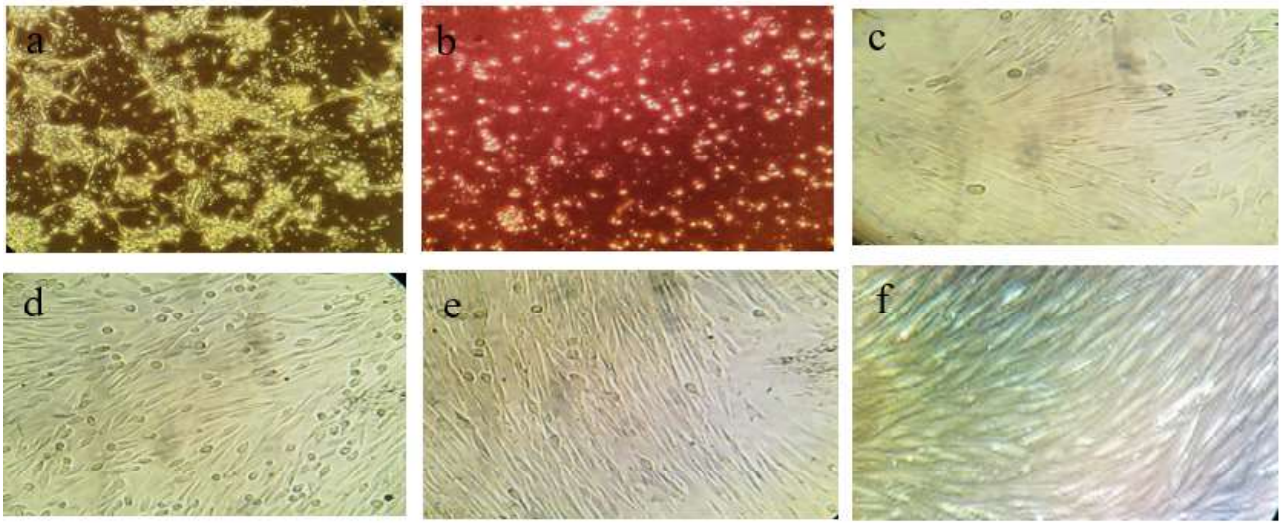

Figure1
Primary cells derived from hind limbs of sheep fetus from day one to six. a) The day-1 cultured cells including a combination of several different cell types. b) The day-1 non-specific muscle-derived satellite cells. c) The day-2 screening showing a variety of cell such as nerve and fat cells. d-e) Days 4-5, including primary skeletal muscle cells. f) The day-6 differentiated sheep skeletal muscle satellite cells.

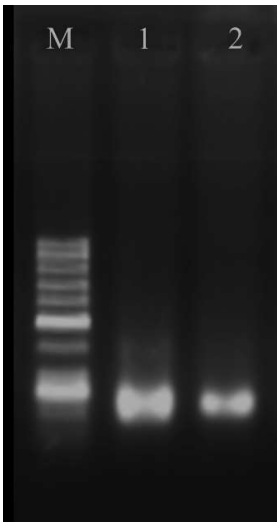

Figure 2
PAX7 was amplified with primers designed to amplify a 300-bp product (lanes 1 and 2) in the primary satellite cells. DNA size marker (M) is 1 Kbp DNA ladder.

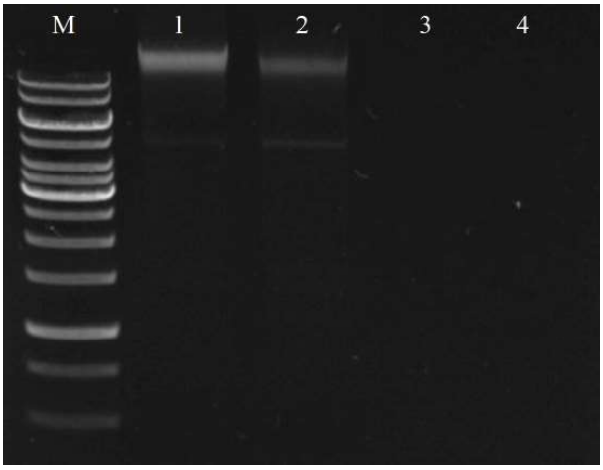

Figure 3
Agarose gel electrophoresis of PCR products for microorganism detection. Lanes 1 and 2: The genomic DNA was extracted from suspended cells in the medium, lane M: 1Kbp DNA size marker, and lanes 3 and 4 negative results of PCR reactions for Mycoplasma and bacterial contamination detection.

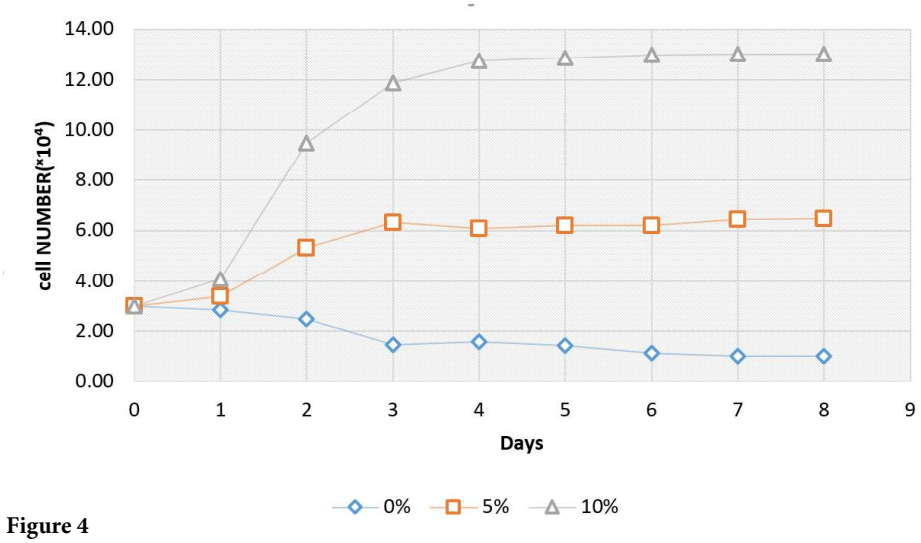

Figure 4
Cell growth curve of ovine MDSCs. The growth rate of the cells cultured in the medium containing 10% FBS was significantly higher than the cells cultured in medium containing 5% FBS.

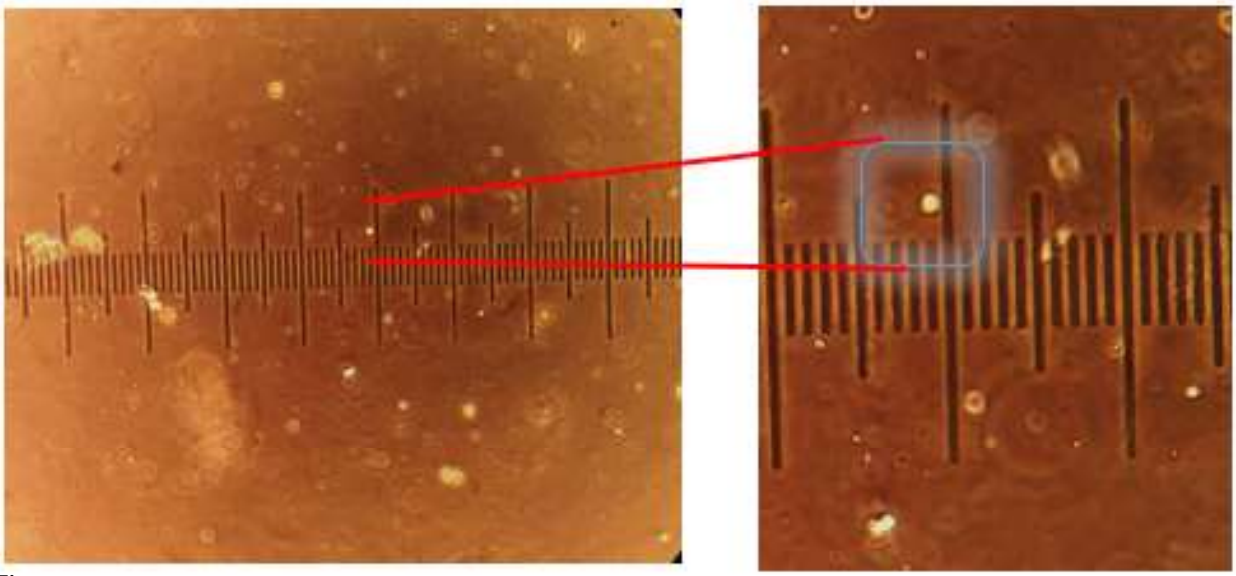

Figure 5
Morphological characteristics of isolated skeletal muscle satellite cells under the microscope. Each degree is equal to 0.01 mm.

Discussion

The purpose of this study was to establish a method for the in vitro isolation and purification of ovine MDSCs and find a more comprehensive identification method for these cells. The number of MDSCs decrease with age. We have successfully isolated sheep MDSCs, optimized their identification, and their cell proliferation and pluripotent differentiation capability assays.

Methods are being continuously updated with the developments in biotechnology [15]. The choice of methods depends mainly on the isolation scale and the subsequent experiments [17,18]. In the isolation process, 0.2% type I and IV collagenase and trypsin have been used to digest the skeletal muscle tissues. The cells have been grown in DMEM with 20% FBS and 1% penicillin/streptomycin [19, 14].

Skeletal muscle satellite cells are adult stem cells. Thus, the postnatal period is a suitable time for the isolation of skeletal muscle satellite cells. Mesires and Doumit (2002) indicated that the absolute number of these cells increased between 1 and 32 weeks of age [12]. However, the relative proportion of porcine skeletal muscle satellite cells gradually decreased from 1 to 64 weeks after birth. Satellite cells account for 30–35% of the sublaminal nuclei on myofibers in the early postnatal murine muscles, and this number declines to 2–7% in adult muscles [20]. Therefore, it is better to select newborn animals at no more than two weeks of age to obtain a high proportion of muscle satellite cells. In this study, 50-60 old-day sheep were used to isolate the skeletal muscle satellite cells and functional positive skeletal muscle satellite cells were obtained.

By adding DMEM containing 10% FBS and 2% HS, 90% of the cells in the flask differentiated to mononuclear myocytes or myotubes after four days. In adult skeletal muscle, all or most of satellite cells express the Pax3, Myf5, Barx2, M-cadherin, c-Met, α7-integrin, CD34, syndecan-3, syndecan-4, caveolin-1, Receptor Calcitonin, and Pax7. Some of the satellite cell markers (e.g. α7-integrin and CD34) are also expressed on other cell types within skeletal muscle, and thus should not be utilized alone to identify satellite cells. Pax7 is specifically expressed in satellite cells within skeletal muscle, in both quiescent and proliferating stages [13]. PAX7 is the best method to identify satellite cells. PAX7 can activate transcription in quiescent satellite cells and does not prevent the fusion of satellite-cell-derived myoblasts [21].

We successfully isolated and cultured sheep primary satellite cells via mechanical and enzymatic disaggregation. Two major approaches have been applied to isolate skeletal muscle satellite cells. The first approach was to break down the connective tissue network and myofibers to release the muscle satellite cells based on the mincing, enzymatic digestion, and repetitive trituration of the muscle mass. This was the classical and efficient method to obtain enough muscle satellite cells, although this method might obtain a heterogeneous population of precursor cells. The second approach was to isolate the muscle satellite cells from a single intact muscle fiber, which could result in relatively pure muscle satellite cells. This method has been successfully used in studies of muscle satellite cells in rats [22], mice [23], and humans [10]. Our findings provided an experimental basis for the research on ovine muscle-derived satellite cells and the methodology can be applied in other related research areas.

Materials & Methods

Reagents

The media and reagents used in this research included Dulbecco's Modified Eagle Medium, high glucose (DMEM-HG) (Gibco, Life Science, USA), fetal bovine serum (FBS) (Gibco, Life Science, USA), horse serum (HS) (Invitrogen, New Zealand), phosphate-buffered saline (PBS) (Sigma, USA), 0.25% Trypsin-ethylenediaminetetraacetic acid (Life Technologies, NY, Grand Island, USA), collagenase type I and IV (Sigma, St. Louis, MO, USA), 100x penicillin-streptomycin (10,000 U/mL) (Invitrogen, Carlsbad, CA, USA), 100% ethanol (Taghtir Khorasan, Iran), dimethyl sulfoxide (DMOS) (Sigma, USA), amphotericin (Cipla, India), and gelatin (Sigma-Aldrich, Louis, USA).

Sheep muscle tissues collection

Due to the critical stage of the skeletal muscle development at mid-gestation in sheep, muscle tissues were collected before mid-gestation from hind limbs of 50 to 60-day-old sheep fetuses [24]. Samples were kept in PBS supplemented with 10% penicillin-streptomycin and 10% amphotericin on ice before transfer to the cell culture laboratory.

Isolation and culture procedures of muscle satellite cells

The surface of the hind limbs was rinsed 3-4 times with PBS supplemented with 10% penicillin-streptomycin and 10% amphotericin. Then, the whole semitendinosus (ST) and semimembranosus (SM) muscles on the right and left legs were dissected. Visible adipose and connective tissues on the muscle mass were removed. The minced pieces of muscle were added to 6-well plates containing PBS, amphotericin, and penicillin-streptomycin. The muscle pieces were soaked in each well for 5 min to remove any surface contamination [19].

Each small tissue sample (~ 1mm^3 cubes) was transferred into a 50 ml tube containing 5 ml of collagenase solution (DMEM containing 10% FBS and 0.2% type I and IV collagenases), and incubated in a shaking incubator at 37 °C for 90 min. Then, trypsin was added (2 times the tissue volume) and the tube was incubated for 3 min in a 37 °C incubator [25]. To neutralize the effects of trypsin, the medium containing FBS was added and the tubes were centrifugated at 500g at 4 °C for 5 min. The supernatant containing the dissociated cells was transferred onto a cell strainer (40 µm) to collect cells. The collected cells were centrifugated at 500g at 4 °C for 5 min and were separated from the supernatant. The cells were resuspended with 5 ml of 2% FBS in DMEM and transferred into

T25 cell culture flasks coated with 0.2% gelatin [16], and incubated at 37 °C under 5% CO_2 for 1 h [19, 25].

Separation of non-myogenic cells

After 1 h, the fibroblasts quickly adhere to the bottom of the cell culture flask, while the skeletal muscle satellite cells remain in the supernatant. Thus, the supernatant containing the skeletal muscle satellite cells were collected from the supernatant in a 15 mL centrifuge tube after centrifugation for 5 min at 500g. The cell pellet was washed with 10 mL of PBS, was resuspended with 5 mL 37 °C preheated DMEM containing 10% FBS, and plated in T25 cell culture flask and incubated at 37 °C under 5% CO_2 [25]. The culture medium was replaced with fresh medium every 2 days. After 6 days, the cells were differentiated [14]. Sheep MDSCs were identified by a reverse transcript PCR (RT-PCR) reaction that amplified PAX7 transcript using forward, (5'-ATTGAGGACTACAAGAGGGAAAACC-3') and reverse (5'-CTGCTTACGCTTCAGAGGGAG-3') primers.

Total RNA was extracted by the total RNA extraction kit (Parstous, Iran). One µg of total RNA was used to make cDNA by the Easy cDNA synthesis kit (Parstous, Iran). The PCR reaction was conducted in a 25 µl reaction containing: 12.5 µl 2x mastermix (Parstous, Iran), 1 µl cDNA, 1 µl primer mix (10 µM) and 10.5 µl ddH_2O. PCR program consisted of 10 min at 94 °C as initial denaturation followed by 35 cycles of 94 °C (30 sec), 57 °C (15 sec) and 72 °C (30 sec), and the final extension at 72 °C for 5 min.

MDSCs growth curves

Once the cells reached 80-90% of confluence, the cells were counted (THOMA, Germany). Then the cells were seeded at a density of 30,000 cells per well in a corning 24-well plate. Different concentrations of FBS (0, 5 and 10%) were used to grow the cells before counting on day 8 [14].

Bacterial and Mycoplasma testing

Mycoplasma contamination of cells often goes unperceived. Unlike bacteria, mycoplasmas do not cause consistently observable alterations in cell culture, such as rapid pH change or culture turbidity [26]. Therefore, the cultured cells were assessed by a DNA PCR test for the presence of both mycoplasma and bacterial species. The sequence of primers for the detection of multiple mycoplasma (including: Mycoplasma Bovis, Mycoplasma pneumonia, Candidatus Phytoplasma aurantifolia, Candidatus Phytoplasma pruni, and etc.) and bacterial species is presented in Table 1.

Morphology of satellite cells

The neobar lam was used to measure cell diameter. Cells were separated from the T25 cell culture flask by using a trypsin-EDTA solution. They were incubated for 2 min, then neutralized with a DMEM containing FBS. 100 µl of it was cast on the lam. Finally, the diameter of the cells was measured under a microscope.

Authors' Contributions

ZR and AJ contributed to the sampling and in vitro experiments, as well as preparing the first draft of the manuscript. ND helped with the cell culture and editing the final proof. HD assisted with editing the manuscript and troubleshootings of cell culture., AJ designed the experiment, edited, and finalized the manuscript.

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Competing Interests

The authors declare that they have no conflict of interest.

Table 1.
Primer sequences of multiple mycoplasma and bacterial species detection

	Primer sequence
Mycoplasma	Forward 1: 5'CGC CTG AGT AGT ACG TTC GC3'
	Forward 2: 5'CGC CTG AGT AGT ACG TAC GC3'
	Forward 3: 5'TGC CTG GGT AGT ACA TTC GC3'
	Forward 4: 5'CGC CTG GGT AGT ACA TTC GC3'
	Forward 5: 5'TGC CTG AGT AGT ACA TTC GC3'
	Forward 6: 5'CGC CTG AGT AGT ATG CTC GC3'
	Forward 7: 5'CAC CTG AGT AGT ATG CTC GC3'
	Reverse 1: 5' GCG GTG TGT ACA AGA CCC GA3'
	Reverse 2: 5'GCG GTG TGT ACA AAA CCC GA3'
	Reverse 3: 5'GCG GTG TGT ACA AAC CCC GA3'
Bacteria	Forward: 5'ACG TCR TCC MCA CCT TCC TC 3'
	Reverse: 5'GTG STG CAY GGY TGT CGT CA3'

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Premedication for intrathecal anesthesia in dogs: xylazine versus propofol

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ABSTRACT

This study aimed to compare the effects of xylazine or propofol before intrathecal (IT) bupivacaine administration in dogs. The study was conducted in two groups of 10 dogs each. In group I (XG), intrathecal injection of 20 mg bupivacaine was administered into the subarachnoid space in the lumbosacral area after treatment with 1 mg/kg intravenous (iv) xylazine. In group II (PG), 4 mg/kg iv propofol was administered before IT bupivacaine administration. The onset, duration, and magnitude of sensory block (scale 0–3) were determined using the pin-prick test throughout the anesthesia. Duration of surgery (XG: 47.20 ± 5.01 min, PG: 50.85 ± 6.97 min) and duration of anesthesia (XG: 92.20 ± 7.02 min, PG: 94.50 ± 7.26 min) were not significantly different between the groups. This study concludes that propofol administration before IT anesthesia with bupivacaine maintains safe levels of IT anesthesia and can therefore be used as an alternative to xylazine treatment.

Keywords

Xylazine, Propofol, Intrathecal anesthesia, Bupivacaine, Dog

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Abbreviations

XG: Xylazine group
PG: Propofol group
SBP: Systolic blood pressure
DBP: Diastolic blood pressure
MBP: Mean arterial pressure
HR: Heart rate
RR: Respiratory rate
RT: Rectal temperature
AST: Aspartate aminotransferase
ALT: Alanine aminotransferase
BUN: Blood urea nitrogen

Introduction

Spinal or intrathecal (IT) anesthesia is performed by injecting local anesthetics into the subarachnoid space. This technique provides ideal anesthesia and muscle relaxation for pelvic and hind limb surgery. Administration of local anesthetics into the subarachnoid space and cerebrospinal fluid leads to sympathetic, sensory, and motor nerve block [1-3].

Bupivacaine is an amide-type long-acting local anesthetic. IT bupivacaine is safer than many other local anesthetics, such as lidocaine and levobupivacaine, which cause ventricular arrhythmia and cardiotoxicity. Therefore, the use of IT bupivacaine remains popular [2,4,5].

Spinal or IT anesthesia, known for its superiority over general anesthesia, is preferred for orthopedic surgery of the hind limbs [6-9]. However, IT anesthesia is associated with complications such as permanent nerve damage. These complications can be caused by local anesthetic-mediated neurotoxicity or pre-anesthetic intervention or injection manipulations within the theca or subarachnoid area [1]. Therefore, pre-medication is an equally important consideration as the choice of local anesthetic [2].

Humans should be conscious during IT anesthesia. However, such concerns are unwarranted during injections or IT anesthesia in animals. Therefore, in animals, IT anesthesia is often performed following sedation with xylazine. This study aimed to compare the effects of xylazine and propofol before IT bupivacaine administration in dogs.

Results

The study results were evaluated in terms of clinical, hemodynamic, and biochemical parameters. There was no statistically significant difference between the groups in terms of body weight (XG: 22.50 ± 4.93 kg, PG: 24.50 ± 4.77 kg), age (XG: 3.40 ± 1.57 years, PG: 3.70 ± 1.88 years), duration of surgery (XG: 47.20 ± 5.01 min, PG: 50.80 ± 6.97 min) and duration of IT anesthesia (XG: 92.20 ± 7.02 min, PG: 94.50 ± 7.26 min) as shown in Table 1. The IT injections were easy to perform and well tolerated by all dogs under sedation with xylazine (xylazine + desensitization of lumbosacral region and ligamentum flavum) or propofol (propofol alone). None of the dogs in either group experienced difficulty in the effort for reach to the sternal position or other abnormal conditions during the entire duration of IT anesthesia.

SBP, DBP, MBP, HR, RR, and RT were not significantly different at baseline and following recovery from anesthesia ($p > 0.05$), although statistically significant differences in baseline values were observed between IT anesthesia with xylazine versus propofol application ($p < 0.05$). DBP and HR were statistically significant at 60 min ($p < 0.05$). MBP was statistically significant at 15, 30, and 60 min post-IT injection ($p < 0.05$). RR and RT were not significantly different between XG and PG ($p > 0.05$). These values and differences are summarised in Table 2.

Serum glucose, ALT, AST, and BUN values were not significantly different between the groups. Although statistically significant differences were observed within groups for these parameters, they were all within the reference range of values (Table 3).

Discussion

This study aimed to compare the use of xylazine and propofol as pre-medication during IT anesthesia in dogs undergoing orthopedic surgery. We used bupivacaine in our study because of its proven reliability and wide use as an IT anesthetic [1,9,10]. Similarly, xylazine is widely used for pre-medication and its effects are well-known [11,12]. Therefore, it was used in the control group in this study. However, the use of propofol has not been evaluated in this context before.

Despite its weak analgesic effect, propofol is often administered with many different combinations before anesthesia to immobilize the patient, maintain safe and comfortable anesthesia and aid easy recovery

Table 1.
Distribution of body weight, age, duration of surgery, and IT anaesthesia in different groups.

Groups	Body weight (kg)	Age (year)	Duration of surgery (min)	Duration of IT anaesthesia (min)
XG (n=10)	22.50 ± 4.93	3.40 ± 1.57	47.20 ± 5.01	92.20 ± 7.02
PG (n=10)	24.50 ± 4.77	3.70 ± 1.88	50.80 ± 6.97	94.50 ± 7.26
p	0.418	0.691	0.079	0.342

Table 2.
Mean ± SD of heart rate (HR), systolic arterial blood pressure (SBP), diastolic arterial blood pressure (DBP), mean arterial blood pressure (MBP), respiratory rate (RR), and rectal temperature (RT) in different groups.

Values	Groups	Time (min)								*p*
		0	Premedication	5	15	30	60	90	120	
SBP	XG	138.20 ± 7.48^{a}	124.60 ± 7.68^{bc}	117.10 ± 7.56^{bc}	112.60 ± 9.51^{c}	124.00 ± 6.51^{bc}	139.70 ± 7.06^{a}	131.60 ± 9.78^{ab}	126.60 ± 9.43^{b}	0.000
	PG	135.90 ± 7.58^{a}	118.00 ± 9.92^{bd}	110.70 ± 7.97^{bc}	109.60 ± 7.17^{c}	121.80 ± 3.55^{d}	136.00 ± 4.32^{a}	131.20 ± 3.88^{a}	128.30 ± 4.22^{ad}	0.000
p		0.198	0.082	0.124	0.440	0.406	0.113	0.446	0.644	
DBP	XG	96.70 ± 6.13^{a}	92.10 ± 5.86^{ab}	87.80 ± 5.20^{b}	96.60 ± 4.33^{b}	88.80 ± 4.34^{b}	96.10 ± 5.13^{a}	94.20 ± 5.20^{ab}	92.10 ± 3.84^{ab}	0.000
	PG	95.40 ± 4.81^{a}	91.20 ± 4.83^{ab}	86.40 ± 3.50^{bc}	84.10 ± 3.67^{c}	84.30 ± 4.19^{c}	94.40 ± 5.48^{a}	91.10 ± 5.17^{ab}	87.30 ± 3.12 b^{c*}	0.000
p		0.509	0.618	0.322	0.057	0.013	0.234	0.133	0.001	
MBP	XG	111.70 ± 4.42^{a}	106.80 ± 3.99^{ab}	101.80 ± 3.49^{b}	101.10 ± 3.45^{b}	105.00 ± 4.67^{b}	111.60 ± 3.50^{a}	110.00 ± 5.31^{a}	107.80 ± 5.01^{ab}	0.000
	PG	111.20 ± 2.70^{a}	106.10 ± 3.45^{b}	99.20±2.70^{c}	94.90 ± 2.68^{c*}	94.20 ± 3.19^{c*}	110.60 ± 3.13^{a}	108.60 ± 5.25^{ab}	100.20 ± 5.12^{c*}	0.000
p		0.551	0.466	0.012	0.000	0.000	0.138	0.196	0.007	
HR	XG	85.50 ± 1.84 ^{a}	81.60 ± 1.57^{b}	78.40 ± 1.26^{c}	78.20 ± 1.47^{c}	76.70 ± 2.40^{c}	83.90 ± 2.60^{ab}	82.60 ± 2.11^{b}	78.10 ± 2.02^{c}	0.000
	PG	85.60 ± 2.22 ^{a}	81.40 ± 1.89^{b}	77.80 ± 1.75^{c}	77.90 ± 0.99^{c}	78.90 ± 2.02^{c}	84.20 ± 1.98^{ab}	82.80 ± 1.68^{b}	81.50 ± 1.26^{b*}	0.000
p		0.931	0.823	0.468	0.541	0.082	0.769	0.853	0.003	
RR	XG	26.80 ± 3.01^{a}	18.30 ± 1.88^{bc}	17.10 ± 2.07^{b}	17.10 ± 2.02^{b}	19.30 ± 1.88^{b}	26.00 ± 3.27^{a}	25.40 ± 2.71^{a}	21.40 ± 1.71^{c}	0.000
	PG	27.80 ± 2.89^{a}	16.70 ± 3.53^{b}	17.40 ± 2.63^{b}	17.60 ± 2.50^{b}	19.70 ± 2.11^{b}	27.20 ± 2.57^{a}	25.90 ± 2.80^{ac}	23.20 ± 2.34^{c}	0.000
p		0.596	0.269	0.799	0.563	0.637	0.484	0.747	0.091	
RT	XG	38.59 ± 0.11^{a}	38.45 ± 0.15^{ab}	38.10 ± 0.10^{c}	37.96 ± 0.12^{c}	37.98 ± 0.04^{c}	38.42 ± 0.19^{abc}	38.19 ± 0.53^{bc}	38.21 ± 0.53^{bc}	0.000
	PG	38.60 ± 0.13^{a}	38.30 ± 0.23^{b}	38.09 ± 0.17^{bc}	37.96 ± 0.11^{c}	37.96 ± 0.08^{c}	38.56 ± 0.06^{a}	38.38 ± 0.21^{ab}	38.16 ± 0.15^{bc}	0.000
p		0.832	0.110	0.872	1.000	0.555	0.106	0.290	0.766	

a-c: different letters (a-b) on the same line ($p < 0.05$) in group, *Significantly different between groups ($p < 0.05$)

Table 3.
Distrubition of serum glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and blood urea nitrogen (BUN) values between groups

Values	Groups	Time (min)					*p*
		0	5	15	60	**120**	
Glucose mg/dl	XG	71.64 ± 5.10^{a}	77.04 ± 4.88^{ab}	78.28 ± 3.73^{b}	80.08 ± 4.33b^{c}	84.80 ± 4.01^{c}	0.000
	PG	70.20 ± 4.62^{a}	76.55 ± 5.74^{ab}	77.08 ± 6.39^{ab}	81.01 ± 8.43^{b}	81.94 ± 12.11^{b}	0.016
p		0.420	0.861	0.657	0.827	0.374	
AST U/I	XG	49.61 ± 4.01^{a}	42.94 ± 2.78^{b}	40.42 ± 2.55^{bc}	36.26 ± 3.33^{c}	36.53 ± 3.19^{c}	0.000
	PG	48.45 ± 2.37^{a}	43.19 ± 2.23^{b}	37.82 ± 2.92^{c}	35.31 ± 3.84^{c}	38.24 ± 4.36^{c}	0.000
p		0.899	0.859	0.058	0.336	0.316	
ALT U/I	XG	65.81 ± 8.79^{a}	44.16 ± 4.36^{b}	39.45 ± 6.28^{bc}	31.49 ± 6.27^{cd}	26.54 ± 3.13^{d}	0.000
	PG	54.47 ± 8.39^{a}	43.81 ± 4.34^{b}	38.14 ± 5.11^{b}	29.45 ± 3.97^{c}	27.73 ± 2.93^{c}	0.000
p		0.791	0.870	0.602	0.257	0.458	
BUN mg/dL	XG	7.18 ± 1.86^{a}	9.48 ± 3.36^{ab}	10.44 ± 2.61^{ab}	15.35 ± 5.59^{bc}	17.61 ± 6.74^{c}	0.000
	PG	7.29 ± 2.30^{a}	8.60 ± 1.57^{a}	11.10 ± 3.46^{ab}	15.37 ± 5.71^{b}	17.28 ± 6.11^{b}	0.000
p		0.928	0.518	0.556	0.993	0.835	

a-c: different letters (a-b) in a row denote significant differences ($p < 0.05$)

from anesthesia with minimal side effects [9,13]. The dogs in the PG group in our study were prepared for anesthesia using propofol before IT anesthesia. Effective induction was achieved in a short time in all cases in this group. The subarachnoid injection was easily performed and induction enabled manipulations. In the XG group, ligamentum flavum desensitization was required for performing spinal injections. Manipulations during subarachnoid injections and local anesthesia of the ligamentum flavum may be areas of concern despite the animal being sedated. Therefore, we believe that propofol provides the advantage of the comfortable application of subarachnoid manipulations.

The use of xylazine or propofol as pre-medication did not affect the duration or depth of anesthesia. The duration and depth of anesthesia were statistically similar in both groups (Table 1).

Slowing of venous circulation during intrathecal anesthesia is known to induce hypotension [9]. Accordingly, HR, SBP, DBP, and MBP values in our study were depressed during IT anesthesia but were not significantly different at baseline or following recovery from anesthesia. However, DBP and HR were significantly different at 60 min and MBP was significantly different at 15, 30, and 60 min after administration of IT anesthesia (Table 2). These decreases were found to be statistically significant between the groups and were clinically acceptable. Depression in both the circulatory and respiratory systems was higher in the PG group. This may be considered a disadvantage compared with xylazine use. Apnoea and/or dose-dependent respiratory depression with propofol use was previously reported (9,13). Therefore, it is recommended that propofol should be administered at the lowest possible dose and i.v. injections should not be performed very quickly.

There were no significant differences between the groups in terms of biochemical parameters, but significant changes were observed within the groups during IT anesthesia (Table 3). However, glucose, ALT, AST, and BUN values were following the reference values [14].

Cranial migration of the local anesthetic into the cerebrospinal fluid within the medullar canal is undesirable. Several factors, such as the anatomical structure of the animal, density of the local anesthetic used and speed of injection affect cranial migration [1,15]. To avoid this migration, the head of the animal should be kept elevated above the rest of the body by tilting the operating table. This restricts the local anesthetic to a limited region due to the effect of gravity [9]. In our study, no anesthesia-associated complications were observed.

Despite its respiratory depressant effect, propofol is widely used for induction in gas anesthesia owing

to advantages such as comfortable and safe recovery from anesthesia, minimum side effects and short-term general anesthesia. However, the analgesic effect of propofol is weak [9,13].

In this study, no comparison was made between the groups in terms of postoperative analgesia because IT anesthesia provided postoperative analgesia and good muscle relaxation. Furthermore, it has been previously reported that a 20% increase in HR, SBP, and RR values during surgery may be indicative of intraoperative pain [9,10,16]. In our study, pulse and respiration values were depressed during anesthesia but were the same as baseline values after recovery from anesthesia. Additionally, there was no evidence of intraoperative pain or any other complication.

In dogs, the use of xylazine for pre-medication has advantages such as good sedation and muscle relaxation. However, the animal may be unduly stressed or complications may occur during the manipulation of spinal injections. Additionally, desensitization of ligamentum flavum is required during xylazine injection. Propofol is primarily used for the induction of gas anesthesia or in combination with other anesthetics. Our study found propofol to be more beneficial than xylazine for pre-medication in spinal anesthesia, particularly during intrathecal injections. Nonetheless, propofol-mediated respiratory depression should also be considered.

Materials & Methods

The study was conducted on 20 dogs following approval by the Animal Research Local Ethics Committee of Kafkas University (KAU-HADYEK, 2015/040). Twenty client-owned dogs scheduled for orthopedic surgery were enrolled in a blunt randomized, prospective, clinical study after obtaining written consent from the owners.

The animals were categorized into two groups: one group receiving xylazine (XG, n = 10) and the other receiving propofol (PG, n = 10). In the XG group, the dogs were sedated with xylazine (2% Rompun®, Bayer, Turkey, 1 mg/kg i.v.). Dogs were placed in the prone position and the lumbosacral region was shaved and disinfected. Next, local infiltration anesthesia (Adokaine®, Sanovel, Turkey, 4 ml) was performed on the area from the skin to the intrathecal space. An 18 G spinal needle (Exelint Spinal Needles) was inserted into the lumbosacral space as previously described [6-9] and 20 mg (4 mL total dose) of bupivacaine (Marcaine®, 5 mg/mL AstraZeneca, Turkey) was injected under aseptic condition. In the PG group, 4 mg/kg propofol (Propofol® 1%, 20 ml inj., Fresenious Kabi, Germany) was administered as a single dose i.v. five minutes later, IT anesthesia was performed using 20 mg bupivacaine, similar to that performed in the XG subjects.

All dogs in the XG and PG groups were placed in the lateral position on the operating table at an approximately 30° angle to prevent cranial migration of the local anesthetic. Before surgery, a 22 G polyurethane catheter was aseptically placed into the ramus dorsalis of the saphenous vein and an electrolyte solution (0.9% saline) was administered i.v. at 10 ml/kg/h for the duration of IT anesthesia.

Each dog was monitored with Veterinary Monitor®, MMED-6000DP S6-V (Germany). Systolic blood pressure (SBP), diastolic blood pressure (DBP) mean arterial pressure (MBP), heart rate (HR), respiratory rate (RR), and rectal temperature (RT) were recorded pre-medication (time 0) and at 5, 15, 30, 60, 90 and 120 min after IT injection.

Serum glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and blood urea nitrogen (BUN) values were determined colorimetrically before injection and at 15, 30, 60, and 120 min after IT injection.

The onset, duration, and magnitude of sensory block were assessed by pinpricks using a 23 G needle. A superficial (needle used to prick the skin) and deep (needle inserted up to the muscle layer) pinpricks were performed. Furthermore, painful stimuli were created to assess superficial and deep pain ventral to the caudal abdomen, tail, perineum, and hind limbs. The needle prick was evaluated on a scale of 0 –3 as previously reported [1,9,10]: 0 = no analgesia and normal strong reaction to a stimulus, 1 = mild analgesia and depressed reaction to a stimulus, 2 = moderate analgesia, and no response to superficial needle-prick stimulation of the skin and 3 = complete analgesia and no response to insertion of the needle deep into muscle tissue.

The needle prick procedure was continued until the end of anesthesia even if the operation ended early. Additionally, the depth of anesthesia was checked by compressing the paw and tail end with forceps during anesthesia.

Routine nursing procedures such as suitable postoperative analgesics (Carprofen, 4 mg/kg, intramuscularly, Rimadyl®, Pfizer, Turkey) were provided daily.

Statistical analyses of the data were performed using the Minitab-16 packet program. The Anderson–Darling test was used to determine the normality of data distribution, the Kruskal–Wallis test was used for non-parametric data, and ANOVA (One-way Analysis of Variance-Tukey's pairwise comparisons) with $p < 0.05$ accepted as significant.

Authors' Contributions

SY, EK, CSE, UA, and IO performed the experiments. MO analyzed the data. EC provided research space and equipment. SY and EC wrote the paper.

Conflict of interest

The authors declare that they have no conflicts of interest.

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The impact of aerobic training intensity on skeletal muscle PGC-1α, interferon regulatory factor 4, and atherogenic index in obese male Wistar rats

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ABSTRACT

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is the main regulator in energy metabolism. Training stimulates many processes like mitochondrial biogenesis, glucose metabolism, and fatty acids metabolism. It also increases the capacity of fat oxidation. The purpose of this study was to investigate the effects of eight-week aerobic training of different intensities on PGC-1α, interferon regulatory factor 4 (IRF4), and atherogenic index in obese male Wistar rats. Twenty-four obese male rats induced by a high-fat diet (weight: 250 to 300 gr, BMI >30g/cm^2) were divided into three groups: aerobic training of moderate intensity (MI), aerobic training of high intensity (HI), and the control group (C). The MI and HI training groups carried out exercise training by eight weeks of walking on a treadmill for five sessions/week, 60 min per session, and at a speed of 28 m/min and 34 m/min, respectively. The levels of PGC-1a in the MI and HI groups significantly increased compared to the C group ($p < 0.05$). Moreover, there was no significant differences between IRF4 levels of MI and HI groups ($p > 0.05$). The serum HDL-C levels increased only in the MI group compared to the C group ($p < 0.05$). The LDL-C, TG, TC, and atherogenic index levels reduced more significantly in MI and HI groups than in the C group ($p < 0.05$). The results show that eight-week aerobic training of two moderate and high intensities may be the signaling pathways to the activation of the PGC-1α protein (i.e., a key regulator of energy metabolism and mitochondrial biogenesis) in skeletal muscle.

Keywords

Aerobic training, PGC-1α, IRF4, atherogenic index

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Abbreviations

PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
IRF4: Interferon regulatory factor 4
FNDC5: Fibronectin type III domain-containing protein 5
AMPK: AMP-activated protein kinase
MI: Moderate intensity aerobic exercise
HI: High-intensity aerobic exercise
C: Control
HDL-C: High lipoprotein lipase-C
LDL-C: Lipoprotein lipase-C
TG: Triglyceride
TC: Total cholesterol
BMI: Body mass index
M ± SD: Mean ± Standard Deviation
PPAR-γ: Peroxisome proliferator-activated receptor gamma
UCP1: Uncoupling protein 1

Introduction

Obesity is one of the most common health problems worldwide [1]. It happens when excess adipose tissue is accumulated in the body [2]. The body contains two types of white and brown adipose tissue that can easily be distinguished through their color. Although white adipose tissue is used to store extra calories in the body, the brown adipose tissue burns adipose tissue in the body and produces heat [3]. In recent studies, researchers have shown that the interferon regulatory factor 4 (IRF4) plays a key role in the process of producing heat in the brown adipose tissue and regulating energy consumption and cold resistance. In this process, some specific hormones, including epinephrine, are activated with a reduction in temperature, and brown adipose tissue produces heat by the activities of a group of genes, collectively referred to as the heat-generating gene expression program. One of the most famous of these genes encodes the uncoupling protein 1 (UCP1) [4, 5].

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is one of the well-known UCP1 encoding genes, the expression of which in the muscular tissue could be effective on UCP1 gene expression and the resultant thermogenesis of brown adipose tissue outside the muscle tissue [6]. The PGC-1a protein is a transcriptional cofactor with a molecular weight of 90 kD with an SR region and a specific RNA adhesion, which can lead to many thermogenesis adaptations [7, 8]. Besides, it is capable of indirect transcriptional stimulation of genes such as UCP1. PGC-1α is a PPAR-γ activating factor that exerts many of its biological effects on energy metabolism [6]. In addition, it has been shown that this factor is expressed as a result of exercise, and it stimulates many processes, such as mitochondrial biogenesis, angiogenesis, alteration of the type of the fiber, and prevention of muscle atrophy [9]. This cofactor is not capable of binding to DNA [5]. According to studies, IRF4 has been identified as a transmitter of the effects of PGC-1α on DNA, which has led to an increase in heat production [5]. Therefore, IRF4 is a key factor in the formation of adipose tissue, the management of fats, and the production of IRF4 with reduced fat intake. On the other hand, animals with adipose tissue lacking IRF4 become obese and are resistant to insulin, and cannot withstand cold [5].

One of the best treatments for obesity is one that simultaneously involves modifying the diet, changing physical activity, and behavioral therapy. The results of the studies have shown that regular exercises could lead to a type of adaptation in antioxidant systems and increased resistance to oxidative stress by increasing the function and the level of PGC1-α & IRF4 and decreasing excess body fat [5, 10, 11]. In this regard, Suwa et al. investigated the effect of low-intensity aerobic exercise (20 meters per minutes per day for 90 days) and high-intensity (30 meters per minutes, for 60 days); they concluded that after 14 days, in both test groups, the levels of SIRT1 and PGC-1α, hexokinase, mitochondrial proteins, and glucose 4 (GLUT4) are increased in the soleus muscle of male rats [12]. Norheim et al. studied the effects of 12 weeks of combined exercise (aerobic-resistance) each week with four sessions of exercise on 26 inactive men aged 40 to 65 years old. However, before the 12 weeks of exercise, the participants experienced an acute endurance activity for 45 minutes with an intensity equal to 70% of the maximum oxygen consumption. They concluded that there is a significant relationship between the increases of muscle mRNA PGC-1α and FNDC5 mRNA after 12 weeks of exercise. Also, despite the reduction of irisin levels after 12 weeks of exercise, its levels increased after severe activity [13]. Gurd et al. reported that six-week intense periodic exercise could lead to an increase in skeletal muscle enzymes by 36% and in PGC-1α protein by 16% after four days of exercise [14].

Participating in regular physical activities, especially aerobic exercises, relying on muscle glycogen fuels, together with the evacuation of these reservoirs, improves the metabolism of lipids in the body, and by using them, it can increase the energy supply [15]. The beneficial effects of regular exercise in preventing obesity, diabetes, and its complications, and improving health have already been proven [16], but the molecular mechanisms of these beneficial effects remain elusive. The results are not precise about administering the most effective intervention program and their mechanisms for changing the adipose tissue phenotype. Therefore, the purpose of this study is to answer the question that which of the aerobic exercise intensities can stimulate the expression of muscle protein PGC-1α, IRF4, adipose tissue, and atherogenic index in obese male Wistar rats.

Results

At the end of the eight-week aerobic training, the body weight of rats was reduced. Moreover, there was a significant difference between the effects of moderate and high-intensity aerobic exercises on body weight ($p < 0.05$) (Table 1).

According to Table 2, there was no difference between the effects of the eight-week moderate and high aerobic exercise on the concentration of PGC-1α in the muscle tissue of male Wistar rats ($p > 0.05$). Based on the results of *Tukey's* post hoc test, there

was a significant difference between mean concentrations of PGC-1α in muscle tissue of the moderate and high-intensity groups and the control group ($p < 0.05$); while there was no significant difference in IRF4 protein concentration between the moderate and high-intensity groups ($p > 0.05$).

The serum HDL-C level significantly increased in the moderate intensity aerobic exercise group compared to the control group ($p < 0.05$); however, serum HDL-C levels in the high-intensity group increased compared to the control group, but these changes were not statistically significant ($p > 0.05$). There was a significant difference in mean concentrations of LDL-C ($p < 0.05$), serum TG concentration ($p < 0.05$), serum TC concentration ($p < 0.05$), and atherogenic index ($p < 0.05$) between the moderate and high-intensity groups and the control group. There was no difference between the eight-week moderate and high aerobic exercise on the atherogenic index in obese male Wistar rats.

Table 1.
The variation of PGC-1α, IRF4 proteins, lipid profile, and atherogenic index in the experimental and control groups after eight weeks of aerobic exercise.

Variable	C [a] (Mean ± SD)*	MI [b] (Mean ± SD)*	HI [c] (Mean ± SD)*
PGC-1α (ng/ml)	0.27 ± 0.01	0.31 ± 0.01‡	0.30 ± 0.00‡
IRF4 (ng/l)	41.70 ± 1.85	44.04 ± 4.03	43.35 ± 5.54
HDL-C (mg/dl)	25.37 ± 3.01	28.72 ± 3.37‡	27.58 ± 2.52
LDL-C (mg/dl)	10.42 ± 1.08	9.33 ± 0.98‡	9.24 ± 0.63‡
TG (mg/dl)	68.33 ± 2.16	48.35 ± 9.32‡	48.50 ± 9.73‡
TC (mg/dl)	85.62 ± 8.35	74.11 ± 5.37‡	72.63 ± 9.40‡
Atherogenic index (Unit)	2.41 ± 0.52	1.73 ± 0.32‡	1.68 ± 0.52‡

*: Data are presented as Mean ± Standard Deviation; ‡: The mean difference is significant at 0.05. C [a]: control, MI [b]: moderate exercise, HI [c]: high-intensity aerobic exercise

Table 2.
The content of high-fat and standard food

Contents	Protein	Fat	Carbohydrate	Fiber	Ash	Calcium	Phosphorus	Salt	Humidity	Lysine	Methionine	Methionine + cysteine	Reunin + Tryptophan	Other materials	Kcal energy in grams
High fat (%)	18	39	20	2	1	1	0.7	0.5	5	1.15	0.33	0.63	0.95	10	4.8
Standard (%)	20	3.5	25	14.5	10	1	0.7	0.5	10	1.15	0.33	0.63	0.95	11	3.9

Discussion

The purpose of this study was to compare the effect of eight weeks of aerobic exercise with two different intensities on IRF4 and PGC-1α levels and lipid profile of obese male Wistar rats. Based on the study results, the moderate-intensity aerobic exercise program and high-intensity aerobic exercise resulted in a significant increase in the concentration of PGC-1α in the muscular tissue of obese male rats compared with the control group. This finding is consistent with the results of Jung et al., Oliveira et al., and Norheim et al. [13, 22-23], but this result is inconsistent with Pekkala et al. [24]. Jung et al. conducted an eight-week ladder-climbing exercise, performed three sessions a week on male rats and found that PGC-1α, AMPK, and mitochondrial biogenesis significantly increased at the end of the period [23].

Oliveira et al. examined the effect of running on a treadmill at speeds of 0.8 to 1.2 km/h, 50 min/day five days a week for eight weeks, on obese male rats. Their results showed that the values SIRT1, AMPK, PGC-1α, and metabolic enzymes increased significantly [22]. Norheim et al. assessed the effect of one session of acute endurance activity for 45 minutes with an intensity of 70% of maximal oxygen consumption and then 12 weeks of combined exercise (aerobic-resistance), four sessions a week, on 26 inactive men aged 40 to 65 years, and concluded that there was a significant relationship between the increase of muscle mRNA PGC-1α and FNDC5 mRNA after 12 weeks of combined exercise. Irisin levels decreased after 12 weeks of combined exercise and increased after an acute activity session, and there was no relationship between uncoupling protein 1 mRNA and expression of FNDC5 in subcutaneous adipose tissue or skeletal muscle or plasma irisin levels [13]. On the other hand, Pekkala et al. examined the effects of four types of exercises, one hour of low-intensity aerobic exercise, high-intensity exercise, 21 weeks of long-term aerobic exercise, and a combination of exercises (long-term exercise + resistance exercise) on irisin levels and FNDC5 mRNA expression. They concluded that there was no significant change in PGC-1α, FNDC5 skeletal muscle, and serum irisin in low-intensity aerobic exercise groups, 21 weeks of long-term aerobic exercise, and combined exercise [long-term exercise + resistance exercise] [24].

The difference in findings may be attributed to different subjects in research that were diabetic or obese. These differences can also be related to the different exercise protocols, including volume and the intensity and different types of exercise programs, measurement methods of the indicators, or differences in the level of plasma or serum levels. The PGC-1α protein encoded by the PPARGC1A gene is a transcriptional activator and acts as an activator of PPARγ, and regulates the expression of the UCP1 gene and thermogenesis in the brown adipose tissue. Also, in some cases, it was observed that this protein controlled mitochondrial biogenesis and oxidative metabolism in many cells. This protein can cause mitochondrial biogenesis, angiogenesis, and changes in the type of muscle fibers in the skeletal muscle. It also has resistance to dystrophy and muscle atrophy [25].

It acts as a mediator in many of the biological mechanisms involved in energy metabolism. PGC-1α was named upon identifying its role in PPARγ activation. Studies have reported an increase in the gene expression of this protein in a cold environment. This activator controls the mitochondrial biogenesis and respiration by the UCP1 proteins and the respiratory factors of the nucleus [26]. Although this protein is essential for the thermogenetic reaction of brown adipose tissue, it does not affect the browning process, and in various experiments that increased PGC-1α expression in the subcutaneous tissue resulted in a change in the phenotype of the adipose tissue in brown adipose tissue; this change was associated with increasing the expression of the UCP1 gene, the respiratory chain proteins, and the oxidative enzymes of fatty acids [27]. In rats whose body fat is resistant to PGC-1α, the expression of mitochondrial genes and exothermic in the subcutaneous fat is slower. However, in rats lacking the PGC-1α gene, the expression of biogenic mitochondrial genes in white adipose tissue was not dependent on PGC-1α. However, its presence was indispensable for inducing the expression of UCP1 and other brown adipose-specific genes. This protein may also be involved in controlling blood pressure, regulating cellular cholesterol homeostasis, and developing obesity [28]. Despite the important roles that have been reported for PGC-1α in the browning process, data on PGC-1β is still not widely available, and researchers do not consider a significant role for that. The mechanisms by which PGC-1α activates the expression of the gene is not well known. Besides, C-terminals include the SR motif and the RNA binding area. This area needs to stimulate the expression of specific endogenous genes and interact with the proteins involved in the processing of RNA and the transport protein particle (TRAPP) complex involved in the initiation of transcription [29]. Moreover, PGC-1α is related to two other transcriptional complexes with acetyl transfer activity, including GCN5 and TIP60. GCN5 directly acetylated the PGC-1α in the lysine residue, thereby reducing the PGC-1α transcription activity. In contrast, SIRT1 increases the function of this cofactor by distilling the PGC-1α. By SIRT1 activation through activating PGC-1α, mitochondrial biogenesis, free radical production, and oxidation of fatty acids are controlled [30]. It is believed that two factors of nutritional and hormonal messages affect the levels of PGC-1α [31]. Researchers believe that reducing glucose and increasing glucagon, catecholamine, and glucocorticoid hormones triggers SIRT1 activation and PGC-1α deacetylation and increases the oxidation of mitochondrial fatty acids by activating PPAR [32]. Regular long-term exercises seem to positively regulate SIRT1 and PGC-1α indices [33-34].

According to the study results, the HDL-C serum levels in the moderate-intensity aerobic exercise group were significantly higher than the control group, while serum HDL-C levels in the high-intensity group increased compared to the control group, but these changes were not statistically significant. Differences in the mean concentrations of LDL-C,

TG, TC serum, and atherogenic index were significantly different between the two moderate intensity exercise groups and the high-intensity exercise group compared to the control group. These findings are consistent with the results of Adrien et al. [35], but this result is inconsistent with the findings of Freitas et al. and Romero et al. [36-37]. Adrien et al. found that the concentration of low-density lipoprotein, triglyceride, and total cholesterol serum decreased significantly at the end of the training period by examining the effect of eight weeks of aerobic exercise four sessions a week, along with a diet on lipid profiles [35]. Freitas et al. studied the effects of resistance exercise and aerobic exercise on the lipid profile of inactive young men; they concluded that 14-week exercise in the resistance group only led to a significant decrease in total cholesterol, and it has a significant effect only on triglyceride levels in the aerobic exercise group; it did not lead to significant changes in other variables [36]. Romero et al. reported that 24 weeks of aerobic exercise on treadmill resulted in no significant changes in the serum level of high density lipoprotein [37].

Given the nature of the intervention in this study, which is aerobic, it can be stated that this type of exercise can play a significant role in reducing the level of fatty acids. However, the interference of different variables, such as different laboratory methods, for estimating the results, exercise type, the number of sessions, the duration of the exercise session, and the intensity of exercise, can be other reasons for the difference in our research results [38-39]. But there is still debate about whether the intensity or duration of exercise is effective in reducing body fat [40]. It has been reported that intense exercise reduces appetite and increases resting metabolism, ultimately leading to an increase in the negative energy balance [39]. Therefore, by controlling the volume of exercise, we can examine the effect of exercise intensity, which is another aspect of exercise. But in a low-intensity exercise causes a more considerable decrease in body fat compared to high-intensity exercise. It has been shown that lipolytic activity is heterogeneous in various fat stores [subcutaneous or intra-abdominal]. Intraperitoneal adipose tissue is the most active site for lipolytic activity [41]. Despite the high rate of lipolytic activity of the intra-abdominal fat, it is unlikely to have an important role in providing fatty acids for muscle oxidation during exercise. Therefore, most fatty acids entering the bloodstream are extracted from subcutaneous adipose tissue [42].

During moderate aerobic exercise, approximately half of the needed fatty acids are supplied by subcutaneous adipose tissue, where the fatty acid share of trunk and upper limbs adipose tissue is greater than the lower limbs [43]. The relative share of plasma fatty acid and intracellular triglycerides to the total amount of fat oxidation during exercise with varying intensity has been studied more in athletic individuals than those non-athletic. During moderate-intensity exercise, most of the oxidized fatty acids result from plasma fatty acids, which, with increasing exercise intensity, the relative share of muscular triacylglycerols increases and can account for half of all fat oxidation. The amount of energy consumed during the intensive exercises (more than 70% of maximum oxygen consumption) is relatively high, and the total fatty acid oxidation is suppressed over its levels during the moderate-intensity exercise [44].

In summary, it can be said that eight-week aerobic exercise with two moderate and high levels of intensity by increasing the amount of muscular PGC-1α protein and improving lipid profile, including high-density lipoprotein increase and a significant decrease in low-density lipoprotein, total cholesterol, triglyceride, and the atherogenic index, can play an important role in changing the phenotype of adipose tissue from white to brown and be effective in preventing obesity and insulin resistance. Therefore, for more accurate results, further research has to be conducted on the effects of exercise on levels of myokine.

Materials & Methods

Animals

The present research was an experimental study on Wistar rats. Twenty-four healthy Wistar male rats (14 weeks, weighing 250 to 300 g, BMI higher than 30 g/cm^2) were used. The BMI was determined using the formula: BMI = Body weight (g) /body length ^{2} (cm^2). The rats were fed with a standard diet (Behparvar Factory, Mashhad, Iran) for a week to adapt to the laboratory conditions. In order to induce obesity, the obese group had a high-fat diet from week 6 for nine weeks; the high-fat diet contained higher calories and fat than the standard food (energy of 4.8 vs. 3.9 kcal/g and fat of 39 vs. 3.5%). All rats were fed this way for 14 weeks and had free access to water and food throughout this study phase (Table 3). At the end of week 14, the approximate weight of rats reached 250 to 300 grams. All rats had free access to water and food. Animals were randomly divided into three groups: 1) moderate aerobic exercise (n = 8), 2) high-intensity aerobic exercise (n = 8), and the control group (n = 8). It should be noted that the principles of the Helsinki Declaration and the opinions of the Ethics Committee were respected in all research phases. The ethics approval was also obtained from the Ethics Committee for the Research of the Ferdowsi University of Mashhad (Ethic code: IR.MUM. FUM. REC.1396. 131).

Familiarization Stage and exercise protocol

According to Table 4, a moderate and high-intensity aerobic exercise program was designed for eight weeks, five days a week, and one session per day for 60 minutes between 08:00 and noon at different speeds on a rodent treadmill (Mobin Bionic Research Group, Mashhad, Iran; with the ability to adjust the slope of the device from -15 degrees to + 15 degrees, set several successive programs, adjust speed, slope, shock, and acceleration for each individual program). After completing the adaptation level, the

animals (to learn how to adapt to the aerobic exercise protocol) were placed on the treadmill during the first week and walked at a speed of 10 m/min with a zero-degree gradient for 15 minutes. During the second and third weeks, the speed and duration of the training gradually increased so that the average speed rate on the treadmill was 28 m/min, 70-75% of the maximum use of oxygen, and the speed of the high-intensity group on the treadmill was at 34 m/min, equal to 80-85% of the maximal oxygen consumption. In total, the exercise volume in the moderate group was 8.4 km/week, and the high-intensity exercise group was 10.2 km/week [17-19]. After completing the training program, to cool down, the speed of the device was reduced inversely so that the machine's speed reaches zero.

Biopsy and measuring the variables

After 48 hours of the last exercise session and 12 hours fasting, rats of all groups were transferred to the Pharmacology Faculty of the Ferdowsi University of Mashhad. In the first place, the animals were anesthetized in a special sampling space (sterile environment) by a combination of Ketamine (30-50 mg/kg) and Xylazine (3-5 mg/kg). After the confirmation of anesthesia, 5 ml of blood was taken by syringe from the right ventricle of each rat and immediately poured into non-anticoagulant test tubes (20). The adipose and soleus tissue were then quickly removed and transferred to a microtube of 1.5 ml and immediately stored in liquid nitrogen and kept at 80 °C.

The tissue samples were poured into the homogenizer tube and combined with RIPA buffer solution and then mixed with a homogenizer (Potter Elvehjem, Mashhad, Iran) for 15 seconds. All the steps were taken in an ice container. At the last step, the sample was drawn from the tube by the sampler and poured into a 1.5 ml microtube. The microtube was centrifugated for 20 minutes at a rate of 20,000 RPM at 4 °C. After the completion of the centrifuge, the microtube was removed from the device and supernatants were removed again and transferred to the new microtube, and then the samples were kept in a freezer at -80 °C. The PGC-1α protein level of muscle tissue and IRF4 level of adipose tissue was measured with a rat-specific ELISA kit (EASTBIOPHARM, Hangzhou, China) with Cat. No. CK-E91412 and Intra-Assay CV of <10%. Serum lipid profile level was measured with Pars Azmoon kits (Tehran, Iran). Equation (1) was used to measure the atherogenic index [21]:

Equation (1) = Atherogenic index: High density lipoprotein/(high density lipoprotein - total cholesterol)

Statistical analysis

The data were analyzed by SPSS 16 at a significant level of $p < 0.05$. After ensuring the normal distribution of the data, using the Shapiro-Wilk statistical test and the homogeneity of variances by Levene's test, one-way analysis of variance (ANOVA) with *Tukey's* post hoc test was used to examine the differences between groups. Furthermore, the criteria for accepting or rejecting hypotheses were considered at the level of $p < 0.05$.

Table 3.

Program exercise with moderate and high intensities

weeks	Moderate intensity	High intensity
First	15 min/speed 10 m.min	15 min/speed 10 m.min
Second	27 min/speed 15 m.min	27 min/speed 15 m.min
Third	34 min/speed 20 m.min	35 min/speed 20 m.min
Fourth	40 min/speed 21 m.min	45 min/speed 22 m.min
Fifth	46 min/speed 23 m.min	54 min/speed 24 m.min
Sixth	52 min/speed 24 m.min	59 min/speed 27 m.min
Seventh	58 min/speed 26 m.min	60 min/speed 31 m.min
Eighth	60 min/speed 28 m.min	60 min/speed 34 m.min

Table 4.

The variation of weights in experimental and control groups before and after eight weeks of aerobic exercise.

Variable	Stages	C [a] (Mean ± SD)*	MI [b] (Mean ± SD)*	HI [c] (Mean ± SD)*
Weight (gr)	Pre test	295.75 ± 1.81	295.16 ± 1.40	295.41 ± 2.15
	Post test	302.58 ± 1.16**€	287.41 ± 4.25**€	287.08 ± 4.23**€

*: Data are presented as Mean ± Standard Deviation
‡: The mean difference is significant at 0.05
**: The mean difference is significant in compareison with pre-test values
€: The mean difference is significant in comparison with control group
C [a]: control, MI [b]: moderate exercise, HI [c]: high-intensity aerobic exercise

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Authors' Contributions

KH: Conception of the study, designed research, conducted research, analyzed data, edited the final version of the paper, had responsibility for the final content of the paper. SRAH: Conception of the study, designed research, conducted research, analyzed data, edited the final version of the paper, had responsibility for the final content of the paper, had primary responsibility for the content of the paper. MF: Designed research, data interpretation, edited the final version of the paper. MMZ: Conception of the study, designed research, conducted research, analyzed data, edited the final version of the paper, had responsibility for the final content of the paper.

Competing Interests

The authors declare that they have no competing interests.

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Purification and biological analysis of specific antigens (ESAT6/CFP10) from Mycobacterium tuberculosis

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ABSTRACT

The pathogenesis of *Mycobacterium tuberculosis* (Mtb) is related to its low molecular weight proteins mainly ESAT6 and CFP10 that are highly specific and potentially useful for the diagnosis of tuberculosis. This research focused on isolation, purification, and characterization of low molecular weight proteins from Mtb. Cultures of Mtb were inactivated by heating at 68 °C for 90 min and 100 °C for 3 hrs, respectively. Inactivated cultures were filtered and the proteins in the supernatant fluid precipitated with two rounds of ammonium sulfate, at 4 °C. The collected precipitates were dialyzed and subjected to gel chromatography (G-50) and the obtained fractions were analyzed for protein concentrations and molecular weight. ESAT6 and CFP10 protein complex in the purified fraction was confirmed by Western blotting. Guinea pig sensitization assay was used for estimating the potency of the purified fraction compared to the standard PPD. The maximum amount of low molecular weight proteins were precipitated by 20% ammonium sulfate. SDS-PAGE analysis revealed protein bands of approximately 10-15 kDa. The purity of the proteins was ≥95%, as confirmed by SDS-PAGE. The presence of the ESAT-6/CFP10 complex was confirmed by Western blot analysis. The purified fractions showed no cross-reaction with BCG or *M. avium* strain. ESAT-6/CFP-10 purified by the ammonium sulfate method appeared to be suitable for the development of a diagnostic kit for the detection of Mtb.

Keywords

Mycobacterium tuberculosis, ESAT-6/CFP10, Ammonium sulphate, Chromatography, Western blotting

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Abbreviations

AA: The first step of precipitation with ammonium sulfate
AT: The first step of precipitation with TCA
BA: The second step of precipitation with ammonium sulfate
BCG: Bacille Calmette-Guérin
BT: The second step of precipitation with TCA
CFP10: Culture filtrate protein
ECL: Enhanced chemiluminescence
ESAT6: Early secretory antigenic target
GMP: Good manufacturing practices
HIF: heat-inactivated filtrate
HRP: horseradish peroxidase
IgG: Immunoglobulin G
Mtb: Mycobacterium tuberculosis
PBST: Phosphate buffer saline with tween
PPD: purified protein derivative
RD1: Region of difference 1
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TB: Tuberculosis
TST: Tuberculin skin test
TCA: Trichloroacetic acid
WHO: World health organization

Introduction

Tuberculosis (TB) is an infectious bacterial disease caused by *Mycobacterium tuberculosis* (Mtb). TB is a major public health problem. Each year about eight million new cases of TB are notified, of which 2 to 3 million prove fatal [1]. To control the disease, one of the most important requirements is the early and specific diagnosis of TB.

Mantoux test or the tuberculin skin test (TST) is one of the most widely used diagnostic procedures that is based on the detection of the cell-mediated immune response [2]. Tuberculin is the crude protein extracts of thermally treated cultures of Mtb. These proteins "referred to as purified protein derivative (PPD)" is used for *in vivo* skin diagnostic tests for detecting sensitization to mycobacterial antigens that might have resulted from infection, vaccination, or environmental exposure [3].

The primary concern with TST diagnostic procedure is the high number of false-positive results, as the test is unable to distinguish between Mtb infection and either exposure to non-tuberculosis mycobacteria or vaccination with *M. bovis* Bacille Calmette-Guérin (BCG) [4, 5]. False-negative results are also reported especially in children and immune-compromised individuals [6, 7].

The low specificity of the TST is attributed to the undefined nature of PPD antigens. Therefore, the presence of cross-reactive antigenic epitopes existing in Mtb and other mycobacterial and non-mycobacterial strains has hampered the development of specific immunodiagnostic test methods [8, 9].

Efforts have been made to identify and characterize immunologically active antigens from Mtb that could react specifically with antibodies from TB patient's sera. Several secretory antigens that are secreted into the extracellular environment by Mtb are known as the target of the immune response in the infected host. Up to date a number of these antigens have been purified, characterized, and evaluated. Among these antigens, the low molecular weight proteins called early secretory antigenic target (ESAT6) and culture filtrate protein (CFP10) are known to be involved in the virulence of Mtb [10]. These complex protein antigens are known to be specific targets for the diagnosis of TB infections. The genes for culture filtrate protein (CFP10) and early secretory antigenic target (ESAT6) are located within the 9455 bp region of difference 1 (RD1 region). This region is known to be highly specific for Mtb complex and is absent in M. bovis BCG strain [11]. Both ESAT6 and CFP10 mediate a specific Th-1 host immune response and have a strong diagnostic potential for both the virulent and latent form of Mtb [12-15].

Different purification procedures have been used for the purification of secretory antigens from the culture filtrate of Mtb. Seiber et al. (1934) used trichloroacetic acid (TCA) for the precipitation of proteins from heat-killed Mtb cultures [26]. The obtained product designated as PPD is a complex mixture of several protein antigens and their aggregated and degraded products. Hence, poor sensitivity and low specificity of the PPD in a diagnostic test might be due to the mixture of protein antigens present in the TCA purified fractions [14].

Thus, the development of new improved test procedures that could specifically diagnose Mtb and have higher sensitivity and specificity might be an effective tool for accurate diagnosis of TB and the control of the disease. In this study, we precipitated and purified low molecular weight secretory proteins (ESAT6 and CFP10 complex) from heat-inactivated Mtb. The purified proteins were then analyzed by Western blotting and finally, animal studies were performed.

Results

Protein/antigen purification

The Mtb C strain was grown on Lowenstein-Jensen solid medium and Dorset Henley liquid medium. As seen in Figure 1, 20% w/v of the mentioned salt allowed maximum precipitation of low molecular proteins, compared to other methods. Figure 1 clearly shows the protein bands more pronounced in $(NH_4)_2SO_4$ precipitated filtrates inactivated at 68 °C, for 90 min. (NH4)2SO4 precipitation yielded proteins with a molecular weight of about 10-50 kDa. While by the TCA method, higher molecular weight proteins ranging from 18 to 90 KDa was apparent. Fraction BT and the standard PPD appeared as a smear on the gel showing no precise protein bands that might be due to the presence of complex protein mixtures.

The results showed that the precipitation of tuberculin solution with 40% TCA at 100°C (BT) had the highest protein concentration. However, the precipitation with 20% ammonium sulfate at 68 °C (AA) had the lowest protein concentration (Table 1).

The amount of protein in fraction AA increased from 0.58 to 1.48 mg/ml after PEG 6000 concentrations. Figure 2 indicates the protein profile of concentrated AA fractions. After subjecting the fraction AA to Sephadex G-50 column chromatography four peaks (F1 to F4) were obtained (Figure 3). Fractions 1 to 3 showed the presence of protein while F4 showed no protein.

All fractions were further analyzed by SDS-PAGE to estimate their protein profile. Fraction 3 showed a single protein band of approximately 12 KDa which corresponds to the ESAT6/CFP10 complex (Figure 4). Fractions 1 and 2 (F1 and F2) had several pro-

tein bands, while no protein bands were observed in fractions 4 (F4). Table 2 represents the purification steps with its recovery during this study. So that by performing chromatography in the last stage, the recovery was about 3.25% and 38.65 mg/ml yield was observed at the F3.

The presence of purified ESAT-6/CFP10 complex protein in F3 fraction in the range of approximately 12 kDa was confirmed, as anti ESAT6/CFP10 monoclonal antibodies blotted with the protein in the mentioned fraction (Figure 5).

Animal study

Thirty-six male guinea pigs were divided equally into four groups and sensitized with Mtb, BCG vaccine, *M. avium*, and PBS, respectively. The animals were later injected with the PPD produced at Razi Institute of Iran as the positive control, purified ESAT6/CFP10 complex (F3 fraction), and PBS as the negative control, respectively (Figure 6).

The results were read after 24 hours and indurations occurring on the skin of the animals were measured (Table 3). The animals sensitized with *M. avium* reacted to PPD fractions, however, the level was approximately 5 mm, while this reaction was enhanced in BCG sensitized animals. The purified ESAT6/CFP10 complex protein showed positive results in Mtb sensitized animals only, while the non-sensitized animals (PBS control) showed no skin reaction to any of the tested treatments.

Table 1.
The concentrations of protein in the four fractions as determined by the Lowry method

Precipitation Method	TCA precipitation		$(NH_4)_2SO_4$ precipitation	
	AT	BT	AA	BA
Inactivation method	68 °C 90 min	100 °C 3 h	68 °C 90 min	100 °C 3 h
Protein concentration (mg/ml)	1.6 ± 0.22 to 2.2 ± 0.31	3.3 ± 0.45 to 5.1 ± 0.28	0.3 ± 0.02 to 0.6 ± 0.08	1.1 ± 0.2 to 1.8 ± 0.33

The results are expressed as mean (± SD) of three individual experiments

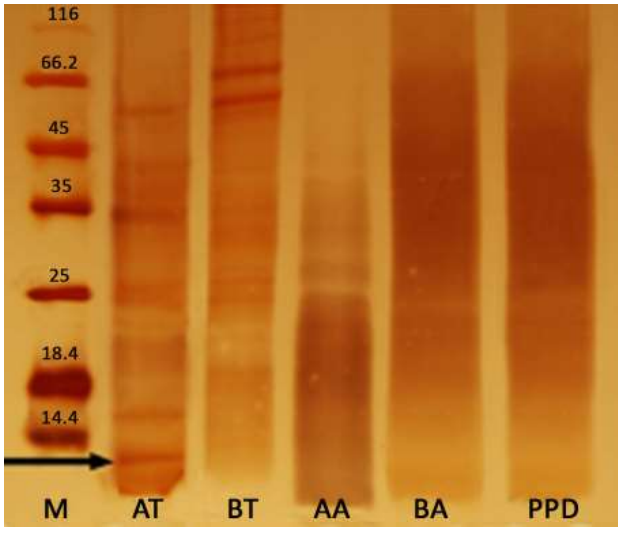

Figure 1.
The protein profile of the ammonium sulphate and TCA precipitated fractions on 15% SDS-PAGE. AT: Cell free filtrate of cultures inactivated at 68 °C for 90 min and precipitated with 40% TCA; BT: Cell free filtrate of cultures inactivated at 100 °C for 3 hrs and precipitated with 40% TCA; AA: Cell free filtrate of cultures inactivated at 68 °C for 90 min and precipitated with 20% ammonium sulphate; BA: Cell free filtrate of cultures inactivated at 100 °C for 3 hrs and precipitated with 20% ammonium sulphate ; PPD: Standard tuberculin produced at Razi Vaccine and Serum research Institute, Iran; M: Standard protein molecular weight marker 10-120 KDa.

Discussion

The worldwide burden of TB occurs in low- and middle-income countries where the diagnosis of this disease still relies on sputum smear microscopy and clinical findings including chest radiography. There is a great need for rapid diagnostic tests at all levels of the health system especially in developing counties [22].

Secreted proteins of Mtb have been reported as a rich source of immunogens. In the last decades, these proteins have gained specific attention as virulence factors, vaccine, and diagnostic candidates [22]. ESAT-6 and CFP-10 have been described as dominant antigens recognized by T-cells and considered as virulence factors in Mtb. These two major proteins (as a complex 1:1) facilitate translocation of Mtb from the phagosome into the host cell cytoplasm at later stages of infection. They do induce a strong T-cell response and, presumably, are involved in the lysis of the host cell membrane or the overall host cell. [35]. In this regard, ESAT6/CFP10 complex proteins are recognized by over 70% of TB patients and are considered promising candidates for the accurate diagnosis of TB in man and animal [15, 23]. The ESAT6/CFP10 has been demonstrated recently to be one of the major targets for memory effector cells during the recall of memory

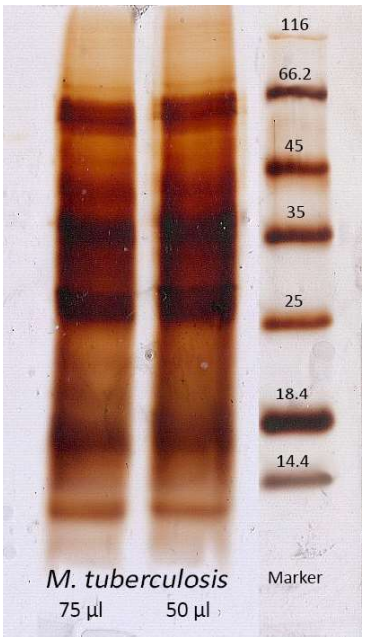

Figure 2.
SDS-PAGE analysis of two different concentrations of fraction AA after concentrating it one-to-tenth volume by PEG.

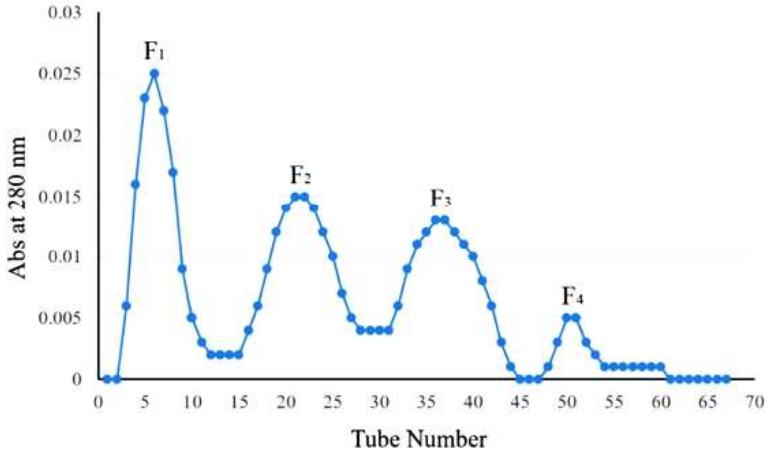

Figure 3.
Sephadex G-50 chromatography of fraction AA of *Mycobacterium tuberculosis* C strain

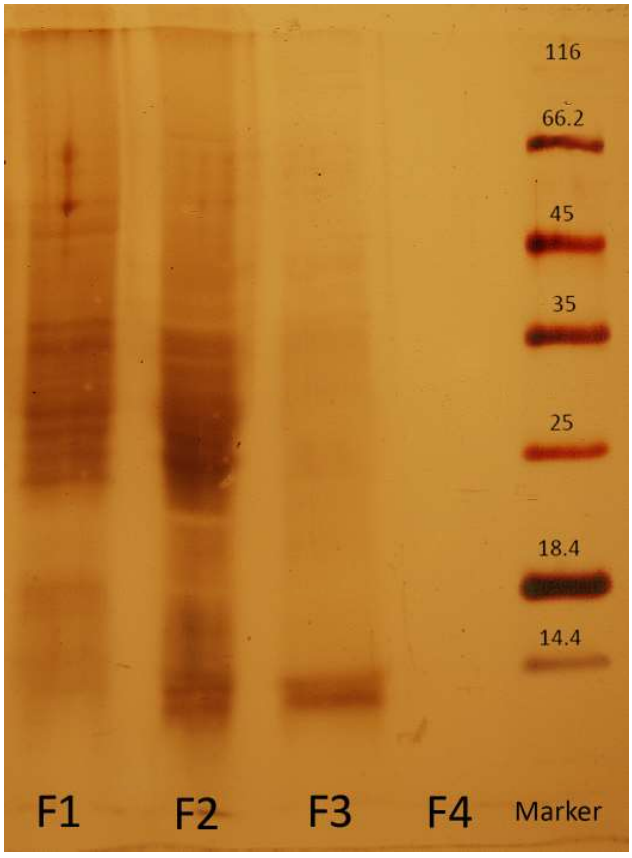

Figure 4.
SDS-PAGE analysis of four fractions obtained after gel filtration in Sephadex G-50 columns; F1, F2, F3 and F4 are the fractions collected. Lane M is molecular weight marker (10-180 kD).

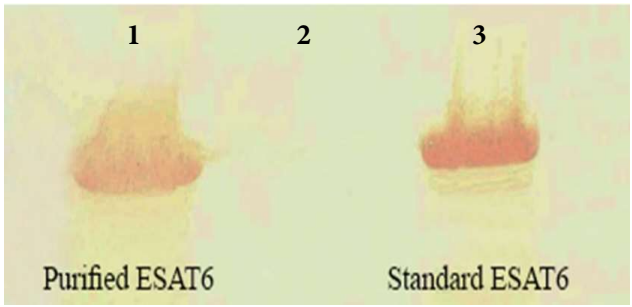

Figure 5.
Western blotting of ammonium sulfate-purified low molecular weight protein fractions using ESAT6 specific antibody. lane 1, purified ESAT6 protein; lane 2, negative control; lane 3, positive control.

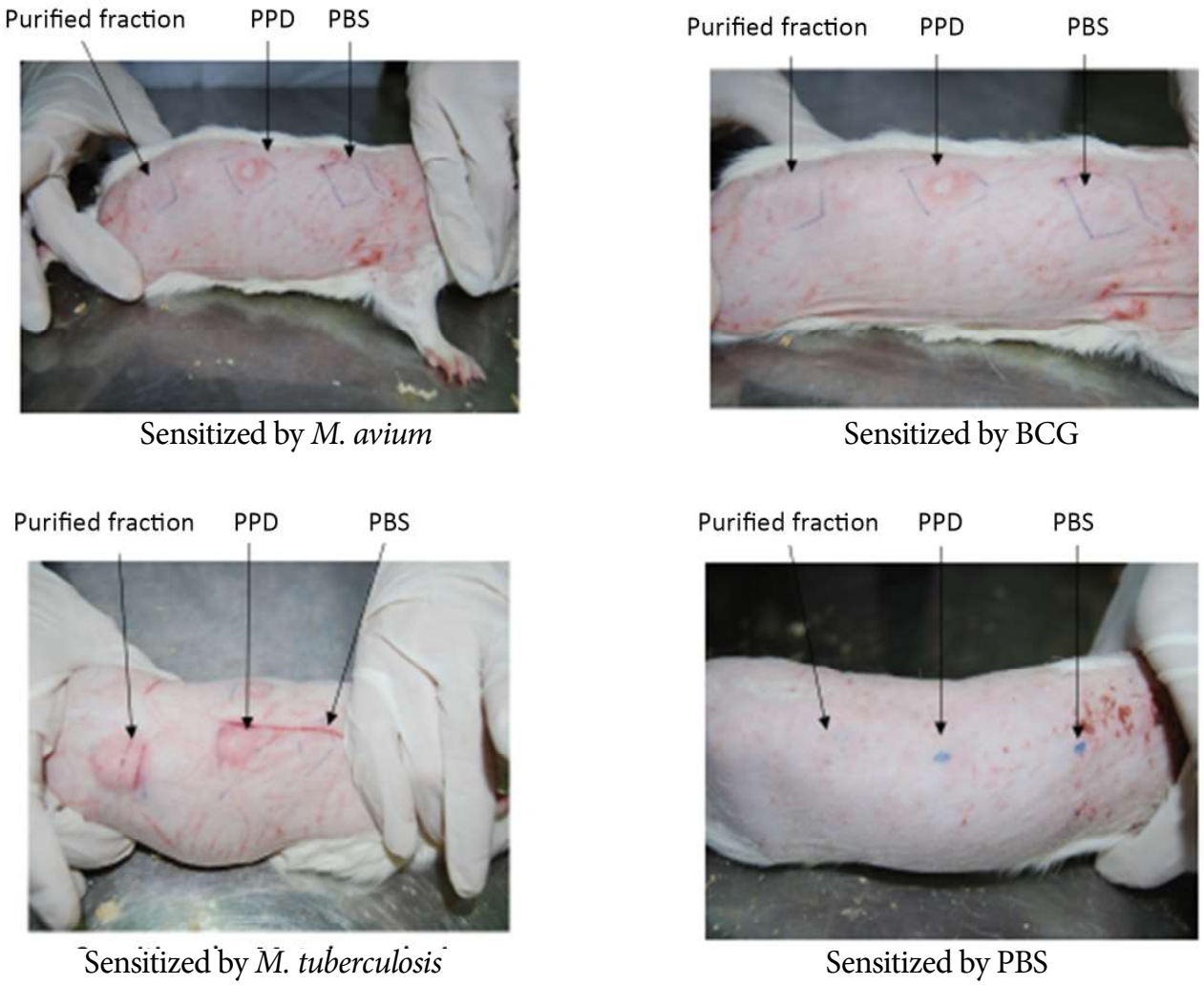

Figure 6.
Skin reactivity after injecting PPD, purified ESAT6/CFP10, and PBS in guinea pig models sensitized with *M. avium*, *M. tuberculosis*, BCG, and PBS.

Table 2.
Purification stages adopted for purification of low molecular weight secretory proteins form Mtb.

No.	Purification stages	Initial Volume (ml)	Final volume (ml)	Protein (mg)	Total Protein mg/ml	pH	Recovery %	Yield mg
1	Heat inactivated (HI)	350	-	12.68	4438.0	6.7	100	-
2	HI filtered (0.2 µm)	350	170	12.63	2147.1	6.6	48	-
3	NH_4SO_4 ppt (20%)	170	70	-	-	-	-	-
4	NH_4SO_4 2nd (20%)	70	38	0.28	10.64	6.2	0.64	533
5	Dialysis	30	58	0.24	13.92	-	-	-
6	PEG concentration	58	35	1.31	45.85	6.9	2.13	578
7	Gel Chromatography	F1	21	1.42	29.82	-	1.38	3.25	386
		F2	18	0.45	8.1		0.18	
		F3	75	0.47	35.25		1.64	
		F4	15	0.067	1.005		0.05	

Table 3.
Skin indurations measured after 24 h in sensitized guinea pigs injected intradermally with PPD, purified ESAT6/CFP10, and PBS

Treatments	Dose	Skin reaction (SD ± mean diameter in mm)			
		M. tuberculosis	BCG	*M. avium*	PBS
PPD	5 IU	12.64 ± 0.92	6.19 ± 1.14	4.21 ± 0.61	0
F3	0.001 mg/ml	16.91 ± 2.76	0	0	0
PBS	0.1 ml	0	0	0	0

PPD: purified protein derivative
F3: purified ESAT6/CFP10 protein obtained during gel chromatography
PBS: Phosphate buffer saline

immunity in a mouse model of TB [24].

Previous studies have studied the specificity of recombinant ESAT6, CFP10 antigens for the diagnosis [31-33], but in this study, we chose to improve our existing PPD product by replacing TCA with ammonium sulfate and autoclaving of culture filtrate with heat inactivation at lower temperatures (68 °C, 90 min).

Several impurities in the tuberculin PPD has been reported, including polysaccharides, nucleic acis, ash, and metals such as iron, sodium, zinc, copper, etc [25, 26]. The aim was to purify low molecular weight proteins mainly ESAT6 and CFP 10 that could have enhanced specificity and sensitivity for skin testing. In contrast to TCA, ammonium sulfate is predicted to precipitate tubercle proteins with lower nucleic acid contaminations and additionally maintain the protein integrity in its natural state [3, 27].

As mentioned earlier, these proteins are usually obtained by cloning methods and gene expression as they are expressed in small quantities under *in vitro* conditions [22]. However, some methods such as cloning might not have desirable outcomes as the recombinant or synthetic proteins might not possess the same biological functions as the natural proteins [14].

In this study, we used the ammonium sulfate precipitation technique to isolate and purify the target proteins while maintaining their natural integrity. The method appeared to be especially suitable for the purification of low molecular weight proteins from the culture filtrate of the bacterium. Although we observed lower yields for the ammonium sulfate method compared with the TCA method, the homogeneous protein precipitates achieved during two rounds of 20% ammonium sulfate constituted mainly of low molecular weight proteins including ESAT6 and CFP10. Hence, it was predicted that this purified fraction would be significantly more specific for the diagnosis of TB compared to the currently available Razi institute PPD.

A skin test was performed in guinea pig models that were sensitized previously with Mtb, BCG vaccine, and *M. avium*. Guinea pig models are considered the most suitable animal models for studying vaccine response and additionally for the development and standardization of PPD [28].

Previously, a group of researchers approved the use of guinea pig models for evaluating the specificity and sensitivity of combinations of ESAT6/CFP10 antigens. According to their reports, a skin test reagent containing these two molecules together could be considered highly specific and sensitive reagent for the detection of TB infection [23, 29].

Similarly, our purified fraction which had both ESAT6/CFP10 protein complex demonstrated greater specificity compared to the standard PPD in these animal models. The purified fraction injected in sensitized guinea pigs was able to distinguish between Mtb from BCG and *M. avium*. While PPD showed cross-reactive immunity to BCG and *M. avium* strain. Hence we could differentiate the exact agent responsible for TB infections. These results are consistent with previously reported studies and showed that ESAT6 protein can elicit skin test reaction in guinea pigs immunized with Mtb but not in the BCG vaccinated guinea pigs [30-33].

Since PPD is a mixture of a large number of proteins, its production in uniformity and compliance with good manufacturing practices (GMP) is very difficult, as a batch to batch variations are often recorded. We here were able to show that our purified fraction had greater specificity to TB than our manufactured PPD at the Razi Institute. This also offers a greater advantage to develop a skin test that utilizes the lower molecular weight proteins particularly ESAT6 and CFP10 rather than a whole mixture of proteins. The use of these antigens is likely to produce consistent and easily interpretable responses when used. However, as might be expected in a genetically diverse population, the sensitivity of a diagnosis based on a single antigen is lower than that with a complex

antigen mixture [34].

In summary, our results confirm that the ESAT6/CFP10 antigen is more specific than PPD to Mtb infection and could be used in more specific skin tests for detection of Mtb in animals and humans. However, further studies need to be performed to identify additional low molecular weight proteins of Mtb strain C. Additionally, alternate separation and detection methods must be employed to resolve and identify extremely basic proteins. Based on the immune response to these proteins in suspected cases, it seems that these proteins might be a suitable candidate for effective vaccination against TB.

Materials & Methods

Bacterial strain and Cultural conditions

The Mtb strain C (ATCC 35808) employed in this study was obtained from the Tuberculosis Reference Laboratory at Razi Vaccine and Serum Research Institute, Karaj, Iran. The bacterial strain was cultivated on Lowenstein-Jensen medium at 37 °C for 40 days and later inoculated into 15 Liters of Dorset Henley liquid medium (with a final pH of 6.8). Confluent growth of Mtb was observed after 6 weeks on the liquid medium.

Inactivation of Mtb strain C

To inactivate the tuberculosis bacterial cells, the freshly grown cultures (15 L) were divided into two equal parts and placed in a hot water bath set at 68 °C for approximately 90 min (Fraction A), and 100 °C for 3 h (Fraction B), respectively. To assure complete inactivation of the bacterial cells, drops of heat-treated cultures were placed on Lowenstein-Jensen solid medium and incubated at 37 °C for 40 days. No growth after 40 days indicated complete inactivation of the bacterium.

Filtration and collection of cell free extracts

The inactivated cultures (Fraction A and B) were passed through Buchner funnel to strain the cell debris and then filtered under pressure using K7 and 0.22 µm filters (Millipore, USA). The collected cell-free extracts were stored at 4 °C for further use. To assure complete inactivation, few drops of the filtered liquid were transferred to Lowenstein-Jensen medium and incubated for approximately 8 weeks. The work proceeded after no growth was observed after the incubation period (at 37 °C for 40 days). The obtained heat-inactivated filtrate (HIF) were further assessed for sterility according to in-house protocols.

Protein Precipitation and concentration

The HIF (fractions A & B) were further divided into two parts (AT, BT and AA, BA). AT and AA fractions were TCA and ammonium sulfate precipitated fractions of the filtrates inactivated at 68 °C for 90 min, respectively. While BT and BA were the culture filtrates inactivated at 100 °C for 3 h and inactivated by TCA and ammonium sulfate, respectively. For $(NH_4)_2SO_4$ precipitation, solid ammonium sulfate was added with stirring, in a 40-min period to reach 70% salt saturation at 4 °C. Based on gradient analysis, two rounds of 20% $(NH_4)_2SO_4$ was selected for further studies. The required amount of the salt was dissolved slowly in the appropriate fractions and kept on a stirrer for 4-8 h. Later, the precipitates were collected by centrifugation at 7000 rpm for 30 min. After dissolving the precipitates in phosphate buffer (pH 6.8), the trace amount of the salts were removed by dialysis against sterile distilled water, overnight at 4 °C. Finally, the dialyzed fractions were concentrated to one-tenth of the original volume by polyethylene glycol 6000. For the TCA method, the fractions (A and B) were subjected to the acid at 40% final concentrations. All purified fractions were stored at refrigerated temperatures until use.
Determination of protein concentrations and Molecular Weight. The protein concentrations were determined by the Lowry method using BSA as standard [17]. The approximate molecular weight of the obtained protein fractions was determined by 15% SDS-PAGE [16] and subsequent staining with Silver staining [18].

Gel Filtration Chromatography

The obtained concentrated filtrate was subjected to size exclusion chromatography with G-50 Sephadex (Sigma, USA) columns. The fractions were filter-sterilized (0.45 µm) and applied to Sephadex G-50 packed column (2×150 cm), at 4 °C. The column was equilibrated with 50 mM phosphate buffer (pH 6.8) and eluted with the same buffer. Fractions of 4 ml were collected at a flow rate of 6 mL/hour. Fractions were pooled and monitored at 280 nm [19].

Western blot analysis

The SDS-PAGE-fractionated antigen mixture was transferred to a nitrocellulose membrane, as described by Franco et al. [20]. Transfer efficiency was monitored by checking for the presence of pre-stained marker bands on the membrane. After transfer, the membrane was blocked with a blocking reagent (PBS/Tween-20 0.05%; PBST) containing 5% dried skim milk, with gentle agitation at room temperature. The membranes were incubated for 2 h at room temperature in 1:2000 diluted anti-ESAT6, anti-CFP10 (Abcam, UK) antibody, washed repeatedly and incubated with the secondary antibody i.e. horseradish peroxidase (HRP) conjugated antibody (goat anti-mouse IgG, Abcam). After thorough washes, the blots were developed by ECL (Enhanced chemiluminescence, Promega, USA) kit and visualized for immune-reactive bands.

Sensitization and Skin test in Guinea pigs

The purified fractions were tested for their potency based on a six-point assay, by sensitizing guinea pig models according to WHO recommendations [21]. 36 male outbred guinea pigs weighing 400-450 g, obtained from Razi Vaccine and Serum Research Institute, Animal care unit were divided into four groups of nine. Potency test was performed after 30 days of guinea pig sensitization with Mtb strain C (ATCC 35808), *M. aviam* strain D4 (ATCC 35713), BCG vaccine, and PBS, respectively. The purified fraction containing 2 mg/ml of the protein was diluted to obtain 0.001 mg/ml concentrations. The animals were injected intradermally with 5 IU PPD (positive control), 0.001 mg/ml of the purified fraction, and 0.1 ml of PBS (negative control) in the flank. The test results were read based on the size of erythema and edema at the injection site of the animals after 24 hrs. The diameters above 8 mm were regarded as a positive reaction.

Ethics committee approval

The present study was approved by the Ethics Committee of Razi vaccine and serum research institute, Karaj, Iran.

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Authors' Contributions

The experiment was designed by N Mosavari. The experiments were conducted by MB, NS, MMT, and AG. The data were interpreted by N Mojgani and N Mosavari. The figures were prepared by MB. The manuscript was written by MB. The manuscript was reviewed by N. Mosavari, N. Mojgani, and KSB. The final manuscript was read and approved by all authors.

Conflict of interest

The authors declare no conflict of interest.

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Short Communication

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Identification of ectoparasites of ornamental birds in the north of Sistan and Baluchestan (southeast Iran)

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ABSTRACT

Several species of ectoparasites infect birds. These parasites that are considered arthropods include: mites, ticks, lice, bugs, fleas, mosquitoes, and flies. This study aimed to identify the ectoparasites species on ornamental birds and determine their prevalence in Zabol and Zahedan in the northern part of Sistan and Baluchestan. A total of 318 birds were examined and inspected for ectoparasites. Parasites were collected by forceps and stored in 70% ethanol. In parallel to the identification of their species, the samples were cleared in 10% KOH following which light microscopy was used to identify the parasites according to their morphological characteristics and the descriptive keys proposed for each species. The overall prevalence of ectoparasites in birds was 21.7%. The ectoparasites were identified as *Menopon gallinae*, *Menacanthus stramineus*, *Columbicola columbae*, *Goniodes pavonis*, *Myrsidea fasciata*, an unknown species from *philopetrus* genus *Argas reflexus*, *Pseudolinchya,* and *Culicoides*. So far few studies have been performed on parasites in birds in Sistan and Baluchestan. Identification of parasites (such as lice in birds) in any region of the country helps us to improve our knowledge about parasitic fauna in this area.

Keywords

ectoparasites, ornamental birds, Sistan and Baluchestan

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Abbreviations

CI: confidence interval
M. fasciata: *Myrsidea fasciata*
C. heterografus: *Cuclotogaster heterografus*

Short Communication

The birds housed and bred for an exclusively ornamental use are called pet birds. This category includes mainly Passeriformes like canaries, finches, mynah, and sparrows. They are also called songbirds and Psittaciformes includes parrots, parakeets, budgerigars (*Melopsittacus undulates*), and love birds (*Agapornis roseicollis*), quail (*Coturnix coturnix*), pheasant (*Phasianus colchicus*), Partridge, peacock, and guinea fowl [1]. Because this avian group is in a close relationship with humans, it is of great importance to pay attention to their health and hygiene. Ectoparasites that infect birds are considered mites, ticks, lice, bugs, fleas, mosquitos, and flies. One of which that causes damage in birds is lice. Lice are divided into two types of chewing (*Ischnocera, Amblycera*) and sucking parasite which are permanent obligate ectoparasites. Chewing lice are mostly parasitic on bird species and feed on feathers and skin scales. Sucking lice can cause skin irritation, and suck blood. Lice can have very harmful effects that lead to anemia and low production in the birds [2]. The first study conducted on the identification of avian lice from domestic birds in Iran was by Rafyi et al. in 1968 [3]. Eslami et al. performed research in 2009 on native birds from Golestan Province in which six lice species were reported, including *Menopon gallinae, Menacanthus stramineus, Lipeurus caponis, Goniodes dissimilis, Coclotogaster heterografus* and *Dermanyssus gallinae* that is a type of mite [4]. In another study, 106 birds were examined for lice infestation in the east of Iran which 52 (49.05%) were infested and 11 lice species were identified [1]. Dik and Halajian have examined 79 wild birds in northern Iran and have demonstrated 15.2% of them infested with 11 lice species [5]. In another study that was conducted in 2013 by Azizi et al. on 26 eagles taken to Shahr-e-Kurd's veterinarian clinic, *Laemobothrion maximum* louse was identified [6]. Despite the existence of a rich fauna of birds in Sistan and Baluchestan, few works have been done for the identification of avian ectoparasites. This study aimed to determine the prevalence of ornamental bird infestation with ectoparasites in the northern side of Sistan and Baluchestan province and to identify the parasites' species to increase the knowledge of parasites fauna in the region.

This study was performed in Zabol and Zahedan (cities in the north of Sistan and Baluchestan province in the southeast of Iran) from March to December 2016. A total of 318 birds belonging to four orders of Psittacines, Columbiformes, Passeriformes, and poultry were examined for ectoparasites. Some of them were randomly selected from the zoo and some from the bird shops in Zabol and Zahedan. The feathers of a different part of the body including head, neck, under the wings, legs, and anus were raised and thoroughly examined for ectoparasites. The attached ectoparasites such as louse, fly, mosquitos, and ticks were removed and transferred in labeled tubes to the parasitology lab of the Faculty of Veterinary Medicine, University of Zabol. The lice were cleared for one day in 10% KOH and another day they were stored in distilled water. After 24 hours, dehydration was carried out in graded series of ethanol (70%, 80%, and 90% and 99%) each for 24 hours, respectively, and then the specimens were mounted [7]. The collected specimens were identified using diagnostic keys [8, 9, 10, 11, 12, and 13]. To confirm the diagnosis, some specimens were sent to the Department of Parasitology, University of Salamanca, Spain. The Pearson *chi*-square test was used for statistical analyses. The population prevalence of ectoparasites with a 95% CI was calculated using a binomial distribution.

Out of 318 birds, 69 (21.70%) (95% CI: 17.3% - 26.6%) of them were infested with ectoparasites. The prevalence of ectoparasites in Psittacines, Passeriformes, Columbiforms, and poultry were 18.6%, 18.2%, 34.4%, and 9.8%, respectively. The difference in the prevalence of ectoparasites between the four groups of birds was statistically significant ($p = 0.003$).

The isolated ectoparasites were identified as follows: *M. gallinae*, *M. stramineus*, *M. fasciata*, *C. columbae*, an unknown species from *Philopterus* and *G. pavonis* genera. The soft tick found in this study was *A. reflexus*, which is mostly found in bloodsucking state on hosts.

Infestation with *Pseudolynchia* and their larvae was seen in adult pigeons and their chicks and a species of *Culicoides* mosquito was identified in an investigation of a cage resided by several rose-ringed parakeets.

According to Figure 1a, the highest infestation rate (40%) in the Psittacine group belongs to cockatiel and the lowest prevalence rate was scored for budgerigar in which no infestation was diagnosed. In Passeriforms, finch and canary were found with no infestation and a high rate of prevalence (44%) was related to myna (Figure 1b). In Columbiforms, collared dove (*Streptopelia*) showed no infestation (Figure 1c) and the highest rate of prevalence was documented for peacock as a member of the poultry group (Figure 1d).

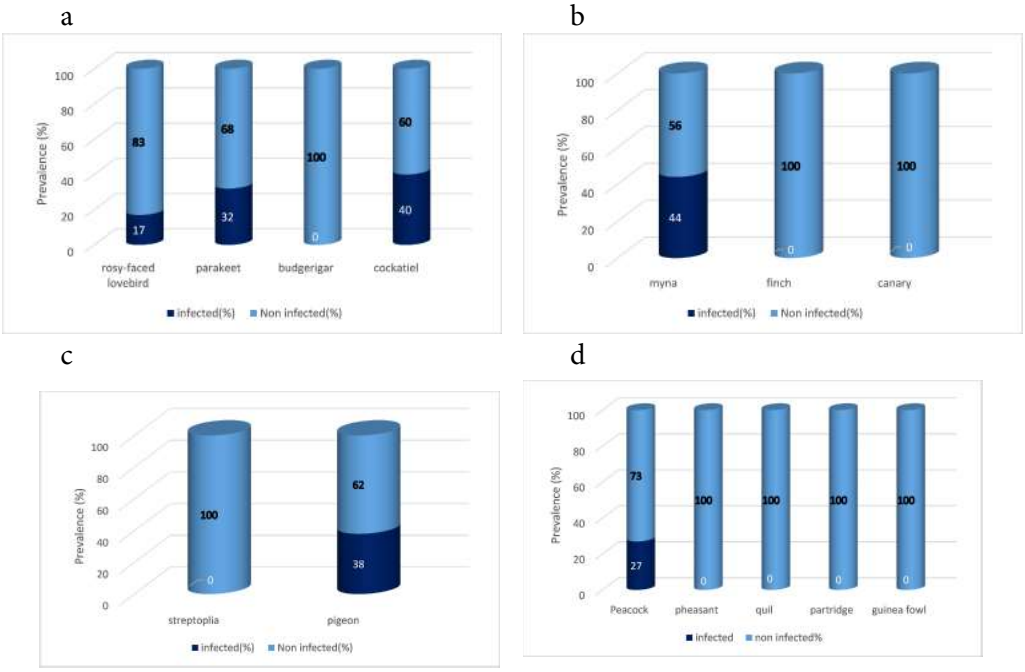

Figure 1.
a) The prevalence of lice infestation (%) in Psittacines. b) The prevalence of lice infestation (%) in Passeriformes.
c) The prevalence of lice infestation (%) in Columbiforms. d) The prevalence of lice infestation (%) in poultry.

The results showed the highest prevalence of ectoparasites in myna with 44%, while other birds in this study include budgerigar, rosy-faced lovebird, finch, canary; Eurasian collared dove, pheasant, quail, partridge and guinea fowl were found with no infestation with ectoparasites.

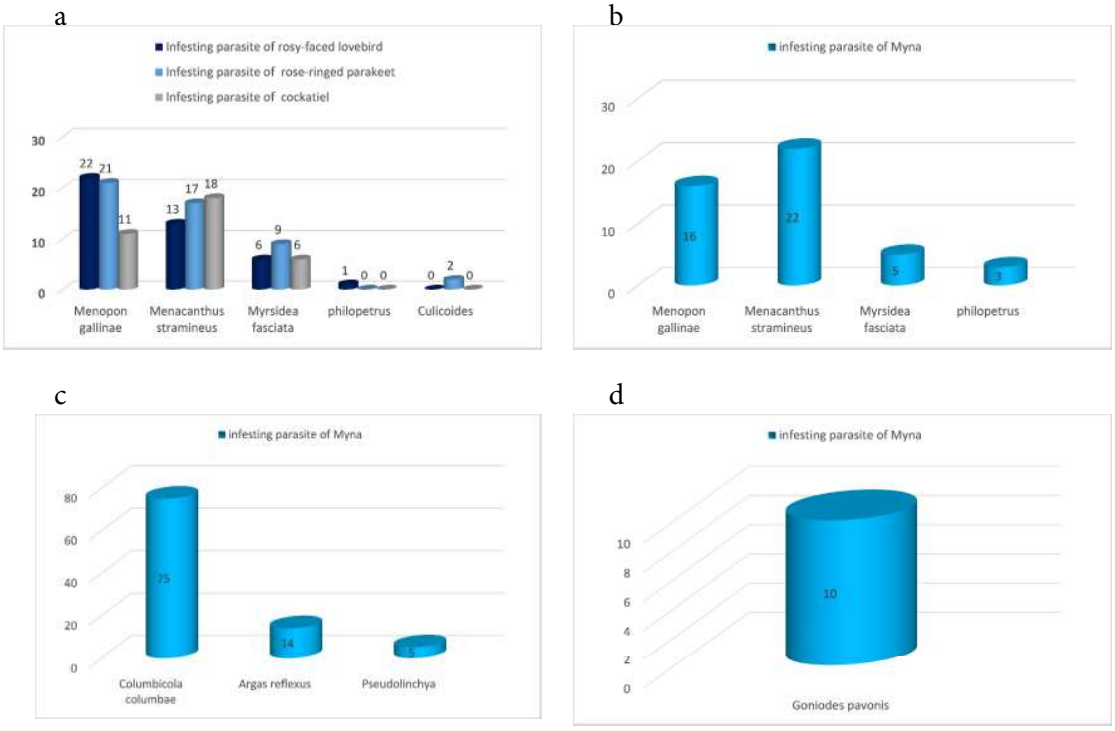

Figure 2.
a) The species and number of infesting parasites of each of Psittacine bird. b) The species and number of infesting Passeriformes.
c) The species and number of infesting parasite of each of Columbiform bird. d) The species and number of infesting parasite of each of poultry.

The investigation of the ectoparasites in birds is of great importance because they can lead to feather reduction, injury and skin damages, and nervous stress and reduction of production. Furthermore, they can be also considered as a vector of pathogens. On the other hand, the people that are in contact with infected birds are exposed to infection, and although the infection is usually for a short time it can cause discomfort. The studies have shown that fleas and lice cause the birds' body growth and egg production to be reduced from 2% to 25% and even more [2]. In Iran, bird's ectoparasites fauna is almost unknown, but recently few studies have been carried out to identify that, especially the recognition of the lice in birds. By identifying several ectoparasites infestations in ornamental birds in the southeast of the country, the present study took a step towards perfecting the fauna of ectoparasites in Iran.

It was assumed that the ornamental birds from Sistan and Baluchestan have been infested with special species of ectoparasites due to the adjacency of the region to the country's eastern borders and illegal and uncontrolled entry of the birds to the region. According to the results, myna has shown infestation with a new species of *Philopterus spp.* featuring morphological properties from the previously recognized features. This is the first time that *Myrsidea fasciata* and *Philopetrus* have been reported in this region. In the meanwhile, infestation with *M. fasciata* lice has been reported for the first time in Iran's ornamental birds.

In a study that was carried out by Hashemzadeh et al. in 2008 on 50 domestic fowls in Tabriz, 44 cases (88%) of the poultry were infected with ectoparasites. In their study, louse species as *C. heterografus* in 86%, *M. gallinae* in 80%, *G. dissimilis* in 62%, and *l. caponis* was found in 26% of the cases. *K. mutans* was found responsible for 8% of the infections and *A. persicus* tick accounted for 18% [14]. In another study that was conducted by Eslami et al. in 2009 on native birds from the Golestan province, infection with lice species such as *M. gallinae*, *Me. stramineus*, *L. caponis*, *G. dissimilis, C. heterografus,* and *D. gallinae* were 40%, 40%, 32%, 38% , 8%, and 20%, respectively [4]. Nazarbeigy et al. in an investigation of parasitic infestation in 60 domestic fowls native to the city of Ilam have reported that 56 of the studied fowls (93.3%) were infested with ectoparasites. These parasites were *M. gallinae* (55%), an unknown goniodes (18.3%), *L.caponis* (53.3%), *Me. stramineus* (58.3%), *G. gallinae* (81.6%), *K. mutans* (23.3%) and an anonymous geralobia (33.3%) [15].

In 2013, Moodi et al. performed a study on 106 passerine birds. In this study, 52 (49.05%) out of 106 birds were infested with louse. In overall, 456 lice belonging to 11 species were identified, all of which had been reported for the first time in Iran [1].

Authors' Contributions

VMF: Performed the experiments. FSS: Designed the experiments, supervised the dissertation, identified the parasites, analysed the data, and wrote the manuscript. MG: supervised the dissertation, identified the parasites, and wrote the manuscript. MJ: supervised the dissertation. JL-A: Identified the parasites.

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Conflict of Interests

The authors declare that there is no conflict of interest.

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Morphological aspects of the brain in the Indian grey mongoose (*Herpestes Edwardsii*)

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ABSTRACT

Mongoose is a common name for 29 to 34 species in 14 genera of the family *Herpestidae* which are found in vast areas of southwestern Asia, especially southern Iran. Anatomical and morphological studies of the brain have always been of interest to the researchers in the field of anatomy, due to its high importance in various fields of veterinary and zoology. Because of the lack of information about the brain structure in wild carnivores, the present study was conducted to better understand the morphological features in Indian grey mongoose. For this purpose, 4 carcasses of adult mongooses were used. They were found in different areas of Fars province. The mongooses had died due to natural causes. The brain was carefully separated from the skull and the measurements and observations were made on different parts of it. In this study, it wa found that the brain's structure has an ovoid appearance. Also, distinguished olfactory bulbs, deep transverse and longitudinal fissures, and relatively large cerebellar vermis were observed. According to the current study, it can be concluded that the anatomical features of the brain in the mongoose are similar to those of other carnivores and are in perfect harmony with the sensory and motor capabilities of the animal.

Keywords

anatomy, brain, mongoose, morphology

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Number of References: 13
Pages: 73-76

Different varieties of mongoose are relatively widespread geographically and are found in many areas in Iran [1, 2]. Due to a lack of information about the anatomy of the brain in mongooses, the present study was designed and conducted with the aim of an overview of the morphological aspects in the mature mongooses. To conduct the present study, 4 Indian grey mongooses were used for two years. The samples were cases that had recently died of natural causes and were abandoned in the wild, prepared from different areas of Fars province.The skull was opened completely and after separating meningeal layers, the morphological examinations were performed and the required images were taken.

From the dorsal view, in the caudal position of the transverse fissure, there was a relatively large cerebellum included a protrusive and developed vermis. The vermis area was greater than the cerebellar hemispheres and was separated from right and left cerebellar hemispheres by two shallow grooves. The vermis seemed stripped by numerous and arranged transverse grooves. The number of grooves in the samples were found to be 10 to 11. The grooves make the cerebellum seems like cauliflower despite its spherical shape. Moreover, the cerebellar hemispheres also include many irregular bulges and concavities in different directions that povide a stripped appearance (Figure 1). The morphological investigation of the cerebellum structure showed that it is similar to that in other carnivores and different from rodents [3,4,5]. The vermis is a bit larger than each cerebellum hemisphere, which shows the considerable balance and coordination of this animal in motion [6].

The two cerebral hemispheres in mongoose had relatively big and developed olfactory bulbs that could be observed on the frontal pole. The olfactory bulb on the right side seemed bigger in all samples. In the caudal side of the bulbs, there were frontal lobes and seemed relatively smaller. The frontal lobe was separated from the parietal lobe by a specific cruciate groove. The longitudinal and transverse fissures were clear and deep and the transverse fissure between the cerebrum and cerebellum was deeper compared to the longitudinal one. Since the frontal pole of the cerebral hemispheres was extremely pressed from the sides and the back pole is wider, the whole cerebrum and cerebellum hemispheres seem oval from the dorsal view. There were clear sulci and gyri on the outer surface (Figure 1).

The hemispheres look square and bulgy on frontal pole from the dorsal view in dogs. Nonetheless, the elongation of the brain structure is distinct in different dog breeds and follows the shape of the skull [7]. The general structure of cerebral hemispheres in cats looks spherical with no angles. Also, the olfactory bulbs in mongooses are big compared to the total size of the brain and this is consistent with the studies conducted on other carnivores [7,8]. No difference was found between mongoose and other carnivores in terms of the number and pattern of the gyri and sulci on the cerebral cortex (Figure 1). [9,10]. Although, due

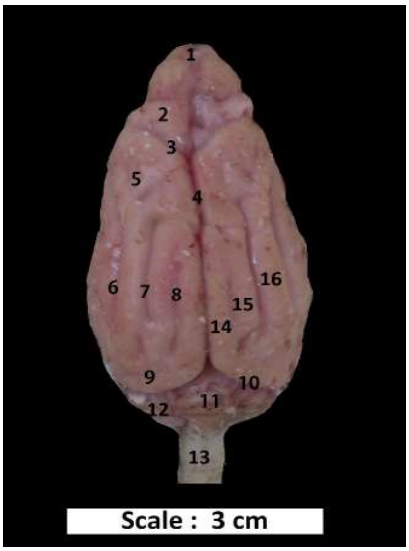

Figure 1.
Dorsal view of the brain in male mongoose.
1.Olfactory bulb, 2.Frontal lobe 3.Cruciate sulcus 4.Longitudinal fissure 5.Parietal lobe 6.Suprasylvian sulcus 7.Ectomarginal sulcus, 8.Marginal sulcus, 9.Occipital lobe, 10.Transverse Fissure,11. Vermis of cerebellum 12. Cerebellar hemisphere, 13.Spinal cord, 14.Mrginal gyrus, 15.Ectomarginal gyrus, 16.Ectosylvian gyrus.

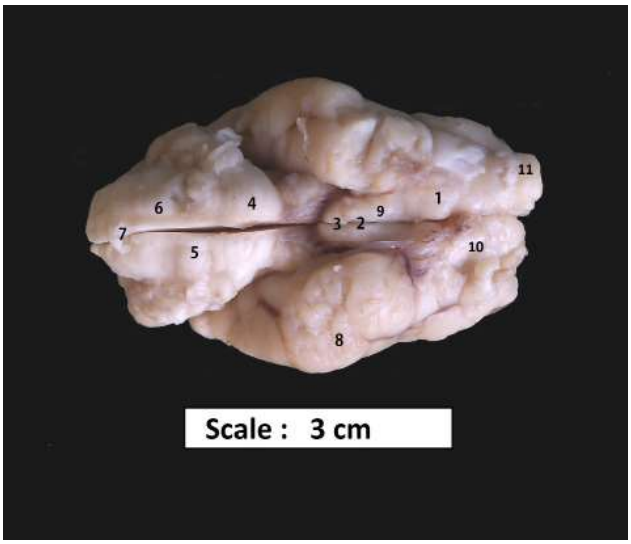

Figure 2.
Ventral view of the brain in male mongoose.
1.Olfactory tract, 2.Infundibulum, 3.Hypophysis, 4.Pons, 5.Trapezoid body, 6.Pyramid band, 7.Medulla oblongata, 8.Piriform lobe, 9.Optic chiasma, 10.Olfactory bulb, 11.Olfactory peduncle.

to the smaller brain size in mongoose, no sylvian sulcus was seen from the lateral view of the brain.

The olfactory bulbs were in the most anterior area of the brain and the olfactory peduncle, as the holder and base of the olfactory bulb, was in the ventral position in the piriform lobe. From this view, the cerebral hemispheres seemed tangled and in triangle form. Moreover, the transverse fissure was the deepest at this view. Also, the occipital lobe that forms the occipital pole of the cerebral hemispheres covered the frontal half of the cerebral hemispheres dorsally. The corpus callosum was broad and sickle-shaped; it was the main identifiable interhemispherical structure on the seriate cuts and clearly distinguished the lining cortex from the internal cerebral segments. The hypothalamus and pituitary gland were also large and developed, as in other carnivores [8]. The medial surface of the thalamus formed the lateral boundary of the third ventricle and the caudal border of the thalamus was wider than the rostral border.

From the ventral view, the olfactory bulb and optic tract could not be observed clearly. There was an infundibulum pore at the caudal of these structures and a mammillary body was observed at the anterior position. The mammillary body was in the form of two bulges separated by a shallow groove. The structure was relatively big and reached the optic chiasma cranially. Following the brainstem, the pons and medulla oblongata appeared as bulges with their borders very clear as a transverse groove. The presence of numerous transverse fibers on the pons helped to identify its area from the medulla oblongata (Figure 2).

The medulla oblongata was wider in comparison to the brain dimensions and it was convex on the surface. The pyramidal bands that form the origin of medulla oblongata were not very clear. However, a trapezoid body was seen as a bulge of transverse fibers between the pons and the medulla oblongata. The longitudinal groove was on the ventral surface of these structures and continued to the beginning of the spinal cord. In fact, it was the rest of the ventral longitudinal groove of the spinal cord (Figure 2).

By looking at the brain from the ventral view, it was found that, like other Felines, the piriform lobe was large. The studies conducted on other mammals such as mice and horses show that the piriform lobe is small [3, 9,11]. Pons was in the shape of a bulge with numerous transverse fibers. The studies conducted on humans, mice and cats are consistent in this regard. However, the pons in the dog is flat and its bulge is not very clear [12,13].

The trapezoid body was observed as a bulge similar to other carnivores between the pons and medulla oblongata [7, 8]. Since it is the origin of the acoustic nerve, the size of this area can be related to the highly developed auditory system in carnivores. Moreover, consistent with this property, a complicated auditory connection with various and continuous frequencies were reported in mongooses [2].

According to this study, it can be inferred that the morphological properties of mongoose brain is generally very similar to other carnivores and is consistent with the behavioral and social habits of the animal. The large olfactory bulb, trapezoidal body, and the vermis area in the brain is justifiable according to the smelling and hearing senses, and the animal's high balance in quick movements and grabbing the baits.

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Authors' Contributions

BR and YK performed the experiments. SG designed the research project and drafted the manuscript.

Competing Interests

The authors declare that they have no conflicts of interest.

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SHORT COMMUNICATION

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The effect of Iranian capripoxvirus vaccine strains on neutralizing antibody titer in cattle

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ABSTRACT

Lumpy skin disease (LSD) virus, Goat-poxvirus (GPV), and Sheep-poxvirus (SPV) are members of genus capripoxvirus (CaPV) and have close genetic similarity. The use of CaPV-vaccine strains would be useful to protect the cattle against LSD. This study aimed to compare the neutralizing antibody titer of Iranian heterologous sheep pox and goat pox vaccines against LSD in cattle. A total of 100 calves was vaccinated with Gorgan-GPV and Ramyar-SPV vaccines on separate farms. Neutralizing antibody titer and side effects of vaccines were evaluated at days 14, 28, 45, 90, and 180 post-vaccination. The mean of rectal temperature in SPV was higher than GPV and persisted for up to 3 days. Also during the onset time of fever, ocular and nasal discharge were observed, whereas in the GPV and control group no clinical signs were observed. In each vaccinated group, the first detectable antibody titer was after 14 days and rose to peak at 28-45 days post-vaccination, then it decreased in the following days. Although, the mean of the neutralizing index (NI) titer between GPV and SPV was relatively similar and there was no statistically significant difference ($p > 0.05$) at all days of the experiment, but in GPV the titer appeared slightly higher than SPV and reached to protective level (NI ≥ 1.5) on day 45 post-vaccination. There was a high antibody titer ($Log10^{1.07}$) in the day 180 post-vaccination. The results showed that GPV vaccine because of the induction of the protective level of antibody titer, and persisting within a long period for up to 180 day post-vaccination, has a good immunogenic response, so is considered a suitable vaccine to control LSD.

Keywords

sheep pox virus, goat pox virus, capripox vaccine, Neutralizing antibody

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Number of References: 30
Pages: 77-82

Abbreviations

CaPV: Capripoxvirus
DPV: Day's post vaccination
GGPV: Gorgan-goat pox virus
GPV: Goat pox virus
GVC: GGPV-vaccinated calves
LSD: Lumpy skin disease
NI: Neutralizing index
OIE: The world organization for animal health
RSPV: Ramyar-sheep pox virus
RVC: RSPV-vaccinated calves
RVSRI: Razi vaccine and serum research institute
SNT: Serum neutralization test
SPV: Sheep pox virus

Lumpy skin disease (LSD) is an infectious disease of cattle caused by a double-stranded DNA virus of the capripoxvirus genus of the *Poxviridea* family [1, 2]. Lumpy skin disease due to the rapid spread and economic importance in cattle has been considered in "list A" of bovine diseases by OIE [3, 4]. Several capripoxvirus (CaPV) vaccine strains are used for the prevention and control of LSD [5-7]. According to many studies, it has been proven that CaPV strains share a major neutralizing site, so that animals are infected with one strain of CaPV family and survived from it, will be resistant to infection with any other strains. Therefore, the use of vaccine strains of CaPV derived from sheep and goats would be useful to protect cattle against LSD [5, 8-10]. In Iran, two live attenuated strains of CaPV are used as vaccines for the control of LSD [11]. These are strains of goat and sheep pox virus that are produced by the Razi Vaccine and Serum Research Institute (RVSRI). After a recent outbreak of LSD in Iran in 2014, an emergency vaccination program with heterologous existing vaccines including GPV and SPV vaccines was carried out in the bovine population of the country [10]. Inadequate protective immunity can cause the outbreak of the disease in cattle populations after exposure to the LSD virus [12, 13]. Therefore, evaluation of immune response characteristics of vaccines against LSDV in field trials is very important to assess the status of the existing vaccine strains and to select the best vaccine strain that effectively protects the cattle population against LSDV.

The study aimed to compare the effect of Iranian capripoxvirus vaccine strains on neutralizing antibody titer in cattle. Accordingly, to the evaluation of humoral immunity, the neutralizing antibody levels of vaccinated calves were monitored weekly, to indicate the levels of protection expected.

Monitoring of adverse reactions

The individual rectal temperature value of vaccinated calves was recorded daily and rectal temperatures higher than 40 °C were considered febrile. The fever in GPV-vaccinated calves (GVC), and SPV-vaccinated calves (SVC) was first observed 24 h post-vaccination, continued for up to 72 h post-vaccination, then remained within normal range until the end of the experiment. In general, the mean of fever in SVC was higher than GVC, and persisted for up to 3 days. Also, in 32% of SVC (16 calves), ocular and nasal discharge was observed, whereas in GVC and control group any clinical signs were not observed.

Neutralizing antibody titers

The serum neutralizing antibody titer of all treated calves were negative before vaccination, but after the vaccination, the neutralizing antibodies were detected. In each vaccinated group, the first detectable neutralizing antibody titer was after 14 days and rose to peak at 28-45 days post-vaccination (dpv). Then it decreased in the following days, while in the control group there was no antibody response. Although, the mean of the neutralizing antibody titer between vaccinated groups at days 14, 28, 45, 90, and 180 post-vaccination were relatively similar, but in GVC was slightly higher than SVC, and in the 90 and 180 dpv it had a higher antibody titer ($Log10^{1.07}$) than SVC. As shown in Fig. 1, the mean neutralization index was higher for SVC on days 14 and 28 post-vaccination and for GVC on days 45, 90, and 180 post-vaccination.

Comparison of the seroconversion status of vaccinated calves

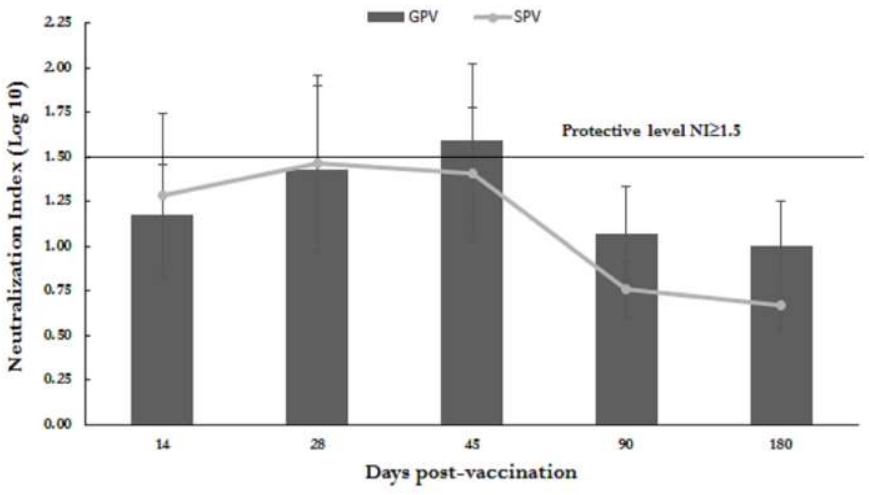

Figure 1.
Comparison of the neutralizing antibody titer of vaccinated calves in response to sheep pox virus (SPV) and goat pox virus (GPV) vaccines on different days post-vaccination.

Table 1.
Generalized estimating equations test results in evaluating the effect of time and vaccine type and their interactions on altering the antibody titer

Variables	Parameter	B	95% confidence interval for B	*p*-value
Vaccine	SPV	-0.26	-056 to 0.04	0.085
	GPV (reference)	.	.	.
Time	Day 180	0.04	-0.21 to 0.29	0.75
	Day 90	0.06	-0.12 to 0.24	0.51
	Day 45	0.52	0.29 to 0.75	<0.001
	Day 28	0.30	0.11 to 0.49	0.002
	Day 14 (reference)	.	.	.
Vaccine*Time	SPV*Day 180	-0.56	-0.89 to -0.24	0.001
	SPV*Day 90	-0.50	-0.82 to -0.18	0.002
	SPV*Day 45	-0.40	-0.72 to -0.08	0.015
	SPV*Day 28	-0.18	-0.44 to 0.08	0.17
	SPV*Day 14	.	.	.

According to the obtained results, the effect of "vaccines" (two Iranian capripoxvirus vaccines) in immune response stimulation and antibody production was not significantly different and the mean of antibody titer in each vaccinated group was similar (p = 0.59, DF = 1, Wald's *Chi*-Square = 0.29). But, the effect of "time" in antibody production was significant, so that the mean antibody titer in each vaccinated group were statistically significant different in five measurement periods (p < 0.001, DF = 4, Wald's *Chi*-Square = 65.16).

Also, the interactive effects of "time" and "vaccines" type on immune response were significant, and the mean antibody titer between the two types of the vaccines did not remain constant over time (p = 0.018, DF = 4, Wald's *Chi*-Square = 11.94). Table 1 shows the details of the generalized estimating equations statistical parameters. In this table, the B value shows the difference of mean antibody titer of each vaccinated group relative to the reference group.

In evaluating the effect of the vaccine type on antibody production, the B = -0.26 value was obtained for SPV, and indicating that the mean antibody titer in SPV was about 0.26 units less than GPV, and this difference was not significant. In interpreting the effect of time on antibody production, the 14th-day post-vaccination was considered as reference day, and the mean antibody titer of the other days was compared to the 14th day.

The mean antibody titer on days 180 (p = 0.75, B = 0.04) and 90 (p = 0.51, B = 0.06) in comparison to day 14 (reference day) were not significantly different, but on day 45 (p <0.001, B = 0.52), and 28 (p = 0.002, B = 0.30) this difference was significant. In evaluating the interaction between the two variables; time and vaccine type, the mean antibody titer in SPV was 0.56 units less than of GPV on day 180 (compared to day 14), and this difference was significant (B = -0.56, p <0.001). Also, on days 90 and 45, these differences were -0.50 and -0.40 significant. But, on day 28, the mean antibody titer of the two vaccines was not significantly different (B = -0.18, p = 0.17).

Because of reasonable prices, vaccination has been considered as a practical and effective method to control LSD [4-6]. To prevent and control lumpy skin disease, several capripoxvirus vaccine strains are currently used [14], and despite regular LSD vaccination of cattle, the cases of vaccine failure and re-occurrence of the disease have been reported [15]. However, little information about the immune dynamics and vaccine-cytokines response to LSD is available. In this study, Gorgan-GPV and Ramyar-SPV vaccine-induced immune responses were measured by neutralization antibody titer, because it is a critical parameter in immune response and can be related to the durability of protection.

Clinical examination of both SVC and GVC was performed daily, and in SVC, mild local reactions

and mild swelling were appeared in the site of vaccine injection, as in the previous studies has been shown [3, 10, 16-18]. While in GVC the local reaction at the vaccination site was lower than SVC [7, 10, 19, 20]. According to the incubation period of the disease, as well as reports of disease in this area, it is likely that SVC was in the incubation period and then showed symptoms following the vaccine injection [7, 21]. In many studies, the effective and protective immune system against LSD and other capripoxviruses is the cellular immune response, although both cellular and humoral immunity play a role in regulating of immune responses [22]. For example, Norian et al [10] and Barman et al [23] showed that not only the cellular immunity but the humoral immunity actively protect the cattle against the capripoxvirus disease.

In our study, the neutralizing antibody titer was detected in both groups of vaccinated calves. The vaccinated calves were able to produce antibodies before day 14 post-vaccination. Similarly, in many research studies, it has been indicated that the vaccinated cattle produce neutralizing antibodies before day 7 post-vaccination [20, 21].

The mean of NI antibody titer of two vaccine strains was increased on each day of follow-up and reached the high value at day 45 and decreased until the follow-up ends at day 180. There is accumulated evidence that the capripoxviruses (LSD, goat, and sheep pox viruses) are genetically related as well as their induced antibodies cross-react one another [4, 24]. Accordingly, though our viral strain vaccines were derived from sheep pox (Ramyar strain) and goat pox (Gorgan strain), a significant difference was not observed in antibody induction among the two viral strains used for our experiment across the study time. Between the two vaccinated groups, the seroconversion rates were relatively higher in GPV compare to SPV. The probable and known variables which influence the immune response to capripoxvirus vaccines include differences in virus strain, the dosage of the vaccine, or a difference in the percentages of exotic germplasm composition. The result of this study showed that all calves were able to produce antibodies in response to vaccine strains and reached to protective level at 28 and 45 day post-vaccination [4, 10, 24-26].

Executive Limitations

There were two executive limitations in this study: First, due to the inability to provide the necessary biosecurity, as well as the lack of funding, we were unable to perform the challenge of LSD virus in the vaccinated cattle by the capripoxvirus. While for approval of the protective effect of CaPV vaccines against LSD virus, we need to expose the calves with LSD virus. But since the LSD virus was on the list A of OIE protocol, this exposure was impossible. Second, the stimulation of immune systems and increases of neutralizing antibody titer may be due to vaccine antigens or the presence of other antigens in the animal's environment. So, the experiment was not performed in the antigen-free area.

Based on the above study we concluded that the Gorgan-GPV and Ramyar-SPV heterologous vaccines stimulated the humoral immune response against LSDV, and induced comparable SNT antibody response after vaccination. Both vaccines induced neutralization antibodies before 14 days, increased regularly thereafter, reached the peak at day 45, and then decreased with a gentle slope until the follow-up ends at day 180 post-vaccination. It seems that the GPV vaccine due to inducing a high level of neutralizing antibody titer against LSD virus, and longer shelf life, can be a suitable vaccine to control the disease in the field.

Study Area and Study Population

This study was conducted in the Zanjan province of Iran. 100 Holstein breed calves with approximately 5-6 months of age were selected from several dairy farms. The calves on each farm were divided into two groups; vaccinated calves as a treated group and unvaccinated calves as a vaccine control group.

Types of Vaccine

The two types of vaccine strains used were Ramyar-Sheep pox virus (SPV) and Gorgan-goat pox virus (GPV), produced by RVSRI of Iran. These vaccines were live attenuated, lyophilized and one dose of each vaccine for goat and sheep contained $10^{3.2}$ TCID50/ml of the virus. A ten-fold dose of vaccines was prepared according to the manufacturer's instructions for emergency use against LSD in cattle [10, 27].

Vaccination

In the vaccination trials, treated groups in each farm were vaccinated with GPV or SPV vaccines separately, and the control groups received PBS alone. The calves were received 5 ml volume of vaccine through subcutaneous, according to the manufacturer's instructions. All immunized calves were daily examined to the appearance of adverse reactions and clinical signs for 180 days following vaccination.

Sample collection

Blood samples were collected regularly at days 0 (before vaccinations), 14, 28, 45, 90, and 180 post-vaccinations. The collected samples were transported to the animal viral vaccine department of RVSRI institute and allowed to clot for 16-24 hrs at room temperature and then the sera sam-

ples were transferred to cryovials under a laminar airflow hood in ordered to avoid contamination and the serum was kept at -20°C temperature until processed.

Virus preparation

The GPV- and SPV-vaccine strains and LSDV were obtained from RVSRI and were used to make a master stock of the virus, and also, to eliminate a potential source of variability, a single batch of the virus was used. Virus cultivation was carried out according to the standard protocol of the department of animal viral vaccines of RVSRI following OIE manual [4, 27], and the titer of stock prepared virus, calculated by Reed & munch method [28].

Serum Neutralizing Test

In OIE, the serum neutralizing test (SNT) is the gold standard method to detect neutralizing antibodies against capripoxviruses [4, 29, 30]. Therefore the evaluation of the humoral immune responses of all samples was done by this method. The level of neutralizing antibodies index was measured in a micro-neutralization assay in duplicate, according to the standard protocol of RVSRI institute following the OIE manual [4]. Results were read on day seven for the neutralization index. The neutralization titer was recorded as the reciprocal of the initial dilution of serum which caused suppression of cytopathic effect. The neutralization index (NI) was calculated following the method of Reed and Munch [20].

Statistical analysis

For evaluation of the effects of vaccine type and time (as independent variables) on antibody titer changes (as dependent variable), and because of abnormal distribution of antibody titer and time interval differences, the generalized estimating equations method (GEE) was used. A *p*-value of less than 0.05 was considered significant.

Authors' Contributions

Conceived and designed the experiments: MS and HRV. Performed the experiments: HI, HRV, and MS. Analyzed the data: HI and HRV. Research space and equipment, reagents/materials/analysis tools: HI and HRV.

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Competing Interests

The authors declare no conflict of interest.

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Case Report

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Long-term outcome after surgical treatment of a congenital flexor tendon deformity in a pony

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ABSTRACT

Equine congenital or acquired flexor tendon deformity can occur immediately after birth or at any stage in the first 24 months of life. The long term prognosis after treating a severe flexor tendon deformity in horses may be poor. Although unfavorable prognosis of flexion deformities is a concept, but results of this presented case reveals that performing an appropriate treatment without any complications, will result in a functional improvement even in older patients, such as in this very case. The aim of this report is to present the long-term outcomes after the surgical treatment and postoperative supports of a congenital flexor tendon deformity in a pony.

Keywords

Limb Deformities, Congenital, Tenotomy, Pony

Number of Figures: 2
Number of Tables: 0
Number of References: 13

Abbreviations

DIPJ: distal interphalangeal joint
AL-DDFT: Accessor ligament of the deep digital flexor tendon (inferior check ligament)
DDFT: deep digital flexor tendon
iv: intravenously

Introduction

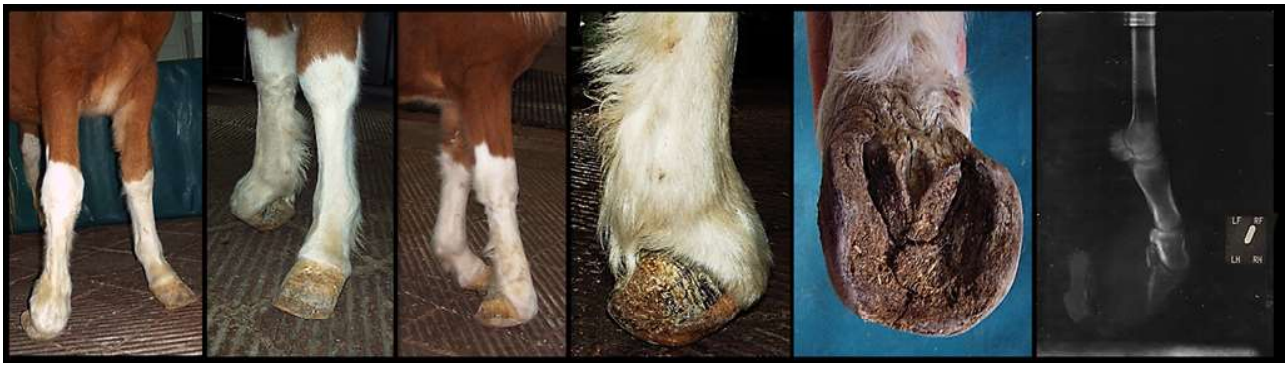
Flexural limb deformities are common orthopedic problems in horses [1-3]. They can occur immediately after birth or in the first 24 months of life [1] and identified as congenital or acquired [1-10], or unclear [3]. No single treatment regime is always successful for the flexural deformity [3,4,7,8,11]. The prognosis is unfavorable in younger foals with severe deformities of the distal interphalangeal joint (DIPJ) that result in weight-bearing on the dorsal surface of the hoof wall [4]. In the presented case, it was aimed to emphasize that the prognosis can be changed positively with an appropriate treatment protocol despite advanced age and severity of the deformity.

Case presentation

A 2 years old, 120 kg male pony was brought to the Large Animal Clinic of the Surgery Department, Faculty of Veterinary Medicine, Uludag University with a congenital postural defect of the right front limb. According to history, the former owner of the pony said this anomaly came from birth. And the current owner bought the pony with this deformity one year ago. A splinting bandage was previously applied to the affected limb by a private veterinarian when the current owner bought it, but there wasn't any improvement.

A tendon deformity that had caused a Grade II hyperflexion of the right DIPJ (Figure 1a-d) was observed with an irregular shaped elongation on the sole (Figure 1e). The right hoof sole did not touch the ground during stepping and the dorsal hoof ground angle was >115°. There was a hyperextension on the left hoof with an irregular shaped elongation on the sole (Figure 1a-c). The general health status and hematological analyses of the pony were normal. In the radiological examination, the right third phalanx toe was in the vertical position and the right DIPJ was hyperflexed. No osteophyte proliferation was determined in any of the phalanges (Figure 1f).

A desmotomy on the right inferior-check-ligament (AL-DDFT) was performed using a standard technique [5] under dissociative anesthesia. Preanesthesia was carried out with xylazine HCl (Alfazyne® 2%; Ege Vet, Turkey), 0.5 mg/kg, intravenously (iv). Anesthesia was induced with ketamine HCl (Alfamine® 10%, Ege Vet, Turkey) 2.2 mg/kg, IV, and midazolam (Dormicum® 15 mg/3 ml, Deva, Turkey) 0.1 mg/kg, IV. Anesthesia was maintained with a combination of low dose ketamine HCl with midazolam. Penicillin (Clemipen-Strep®; Topkim, Turkey), 22 000 UI/kg body weight and flunixin meglumine (Finadyne®, Sanofi DIF, Turkey) 1.1 mg/kg were administered iv before surgery. The pony was positioned in lateral recumbency with the affected limb down. The right metacarpal area was prepared for aseptic surgery. A longitudinal skin incision (4-6 cm long) was made at the point where the inferior check ligament becomes hidden by metacarpal IV. The subcutaneous tissues were incised parallel to the skin incision. A curved hemostatic forceps was used to separate the DDFT and the AL-DDFT. The AL-DDFT was transected with a surgical blade. The foot was extended to see the gap between the cut ends of the AL-DDFT (Figure 2a, b). The subcutaneous tissues were closed with a simple continuous pattern and skin was closed with simply separated sutures using 2-0 absorbable sutures in separate layers. Then, trimming on the hooves of the front feet (Figure 2c) and a bandage was applied on the right pastern. The hyperextension on the left DIPJ was resolved after trimming.



a b c d e f

Figure 1. The photos of the case which had a grade II hyperflexion of the right DIPJ.
a-d) A hyperflexion in the distal interphalangial joint (DIPJ) on the right distal front limb and a hyperextension on the left distal front limb. e) An irregular shaped elongation on the right hoof sole. f) The right DIPJ hyperflexion and no osteophyte proliferation were seen on the preoperative radiograph.

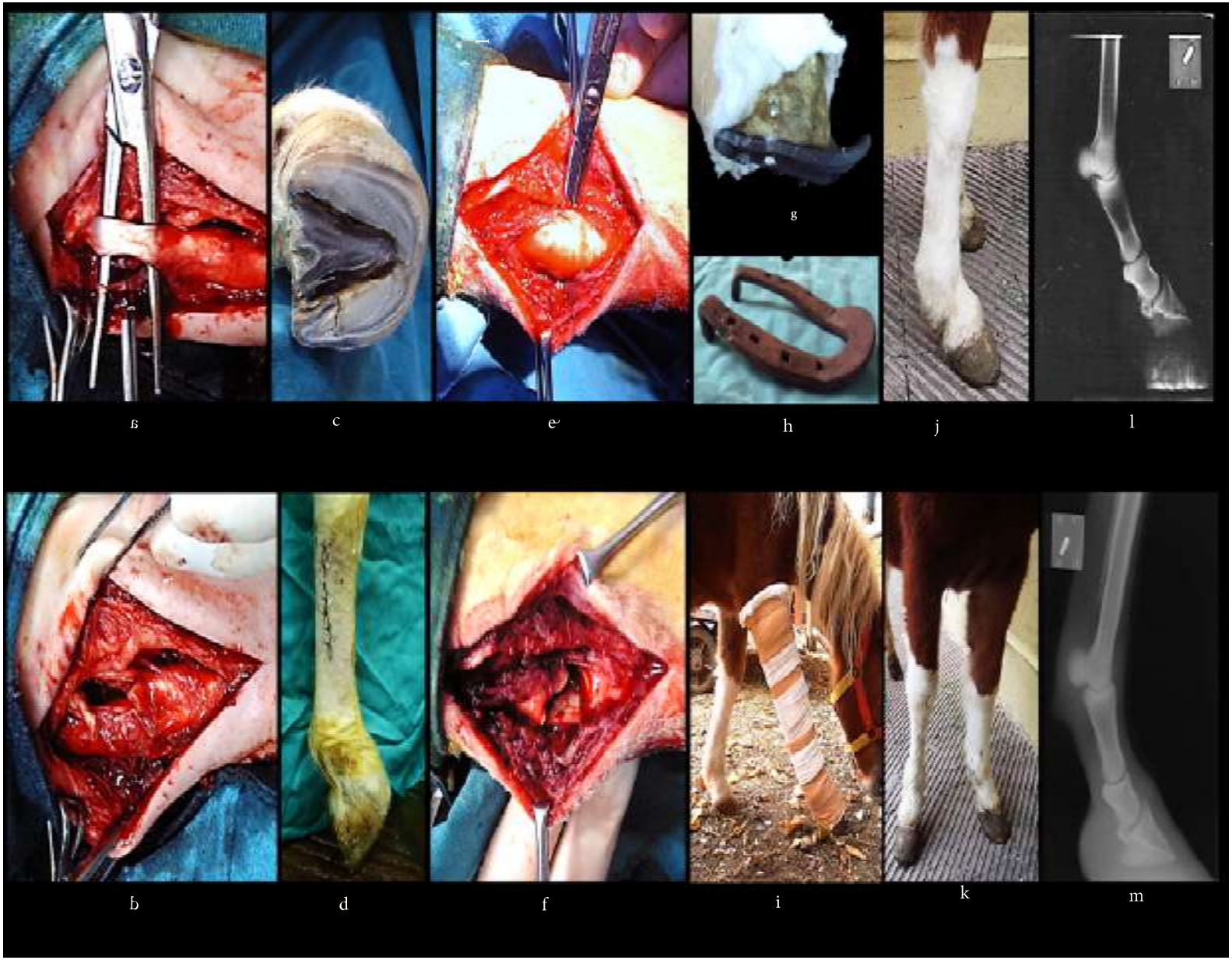


Figure 2. The intraoperative and postoperative photos of the case which had a grade II hyperflexion of the right DIPJ. a & b. Desmotomy of the right AL-DDFT. c) The right front sole after trimming. d) No recovery was observed on the flexion severity one week after desmotomy. e & f). Tenotomy of the right DDFT. g) An orthopedic-shoe with a toe extension. h) A splinted bandage on the right front leg. j-l) Clinically and radiologically functional improvement on the right front-limb at day 49. m) Radiological follow-up examination seven years after the tenotomy.

Results & Discussion

After one week, no improvement was observed relating to the flexion severity (Figure 2d). A tenotomy on the deep digital flexor tendon (DDFT) was performed using a standard technique [5] under the same anesthesia and medication protocol. The pony was positioned in lateral recumbency with the affected limb down. The right metacarpal area was prepared for aseptic surgery. A skin incision (10 cm long) was made on the lateral side of the mid metacarpal region. The digital synovial sheath was opened. The DDF tendon was elevated and incised transversely (Figure 2e-f). Then the ability to extend the foot was controlled. The subcutaneous tissues and skin was closed with suitable suture patterns using 2-0 absorbable sutures in separate layers.

Then, an orthopedic-shoe with a toe extension was applied (Figure 2g) with a splinted bandage on the posterior side of the right front leg (Figure 2i). Resting of the animal was recommended to the owner. Seven days after tenotomy, the stance hyperflexion on the right DIPJ had remarkably disappeared, although tendon callus on the DDFT tenotomy region had not been seen ultrasonographically. The orthopedic-shoe on the right hoof was changed with another horseshoe which has 1 1.5 cm spurs (Figure 2h). Resting was advised to the owner for another three weeks. At the end of the 3 weeks, the right-foot was observed in the normal situation during both standing and walking. Forty-nine days after DDFT tenotomy and application of the orthopedic shoe, functional recovery was observed on the right front limb (Figure 2j,k,l). The owner was advised to increase the speed and time of walking, gradually and to avoid hard exercises, during the next 5 months. Seven years after the tenotomy, no recurrence of flexor tendon deformity was observed based on clinical and radiological follow-up examinations (Figure 2m). The pony has been living a healthy life as a riding horse.

Flexural deformities seen in horses occur more frequently in the forelimbs [6-9, 12], one side more severely affected [7,9,12]. Its etiology is obscure, although the in-utero fetal position may be involved for congenital deformities [7]. Genetic factors [6, 10], nutrition (excessive carbohydrates 'energy' and unbalanced minerals) [6, 10, 12], and excessive exercise [10] thought to play roles as the causes of acquired flexural deformities. In the presented case (2 years old), the etiology was thought should be related to the intrauterine fetal position, because the right forefoot had a severe hyperflexion from birth due to history. Carlier et al. (2016) have reported the success rate as 56% for the surgical correction of the abnormal hoof conformation [2]. Jansson and SØnnichsen (1995) emphasized that the normal function and appearance of the hoof and toe can be achieved by surgery and reported a higher success rate for a desmotomy as 90% (26/29) [8]. A desmotomy was reported as successful for Grade I cases with a hoof ground angle between 90°-115°, but a DDFT tenotomy was advised for Grade II cases (>115°) [2,4,6]. Potential complications such as incision swelling dehiscence, postoperative scarring, an infection with serious complications like carpal tenosynovitis or desmitis could be seen after surgery [13]. In this case, no adhesion or other complications were observed during the recovery period. Corrective shoes with a toe extension [2,8,13] should be used for 2-3 months after surgery [13]. If the heels could be elevated, it can reduce tension in the DDFT [8, 11] and relieve the pain. In this case, the horseshoe applications made the pony move more comfortably. Exercise is important [2,6,7] but controlled exercise should be done to prevent over-tension in the tendon in the treatment process [6]. Controlled exercise can elongate palmar soft tissues while protecting limbs from overuse [4]. In this case, the owner advised to increase the speed and time of walking, gradually and to avoid hard exercises, during the next 5 months, since the pony was not a racehorse. The prognosis varies according to the chronicity and severity of the deformity [4]. A better prognosis can be achieved in younger patients than six-months-old [2] and also in patients younger than 24 months old [2, 13]. In this case, the age of the pony and the severity of the flexion deformity may have been obstacles to improvement at the desired level after desmotomy. Recurrence of the deformity can be seen in operated cases [1]. The presented case, although was 2 years old and suffering from a severe DIPJ deformity, treated successfully without any recurrence. Nevertheless, it was thought, the treatment success was increased because there was no osteophyte on the foot.

In conclusion, clinical outcomes of this case presentation reveal that appropriate treatment protocols, concurrent with thorough post-operation care, lead to a better prognosis, and provide functional improvement without any complication, even in older patients.

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This case report was presented as a poster at The 14th National Veterinary Surgery Congress in 2014 in Antalya, Turkey.

Authors' Contributions

Performed clinical evaluations and the anesthesia of the case, and wrote the manuscript: ÜGÇ and GÇA; Performed the surgeries and control examinations of the case: GÇA; Performed the surgeries of the case: HS; Analyzed the radiographic and ultrasonographic evaluations of the case: NÇ.

Coflict of Interests

The authors declare that they have no competing interests.

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Case Report

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A rare case with the absence of a distinct common brachiocephalic trunk in an adult mixed-breed horse

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ABSTRACT

The only branch that detaches from the aortic arch of adult horses is the common brachiocephalic trunk. This report describes a rare case of the unusual arterial arrangement of the aortic arch in a female adult horse. The aortic arch was found to lack a common brachiocephalic trunk and had instead two branches, the first being a brachiocephalic trunk and the second being a left subclavian artery. The direct origin of the left subclavian artery from the aortic arch is very rare in horses and a similar case has not been reported previously. The potential embryologic and phylogenetic aspects of this variation are discussed.

Keywords

Common brachiocephalic trunk, horse, left subclavian artery

Number of Figures: 2
Number of Tables: 0
Number of References: 16
Pages: 88-92

Abbreviations

BCT: Brachiocephalic trunk
LSA: Left subclavian artery
RSA: Right subclavian artery

Introduction

During the growth of the embryo and fetus, the architecture of the great vessels cranial to the heart derived from the aortic arches, ventral aortae, and cranial parts of the dorsal aortae is altered by the cephalic folding and elongation of the neck. As a result, the aortic arch descends from the cervical region to its definitive position in the thorax. Several species-specific changes also take place that ultimately determine the final branching pattern of the aortic arch [1]. Therefore, depending on the species of animal, the number of the branches that arise directly from the aortic arch differs from three or four to one [2]. However, in domestic animals, two or three of these branches merge at their origins, reducing them to one or two [3]. The only branch that leaves the arch of the aorta in ungulates including equines and ruminants is the common brachiocephalic trunk (BCT) (Figure 1A) [4]. It in turn gives rise to the left subclavian artery (LSA) and the right subclavian artery (RSA) at the level of the second intercostal space or the third rib and the first rib, respectively. Then, it continues as the bicarotid trunk. In pigs, dogs, and cats, the arch of the aorta gives off two separate branches, namely the brachiocephalic trunk and the left subclavian artery [3].

In addition to clinical significance, the anatomic variations of the great vessels at the base of the heart of equines may be of great interest for the clarification of the embryonic primordium. Hence, we here describe a rare case of an adult horse with the absence of the common brachiocephalic trunk and discuss the potential embryologic and phylogenetic aspects of this variation as well.

Case Presentation

During routine anatomical dissection of the large vessels within the mediastinum to teach veterinary gross anatomy, an unusual branching pattern of the aortic arch was observed in an adult mixed-breed mare, four years old. The case had a history of trauma and showed a severe cervical deviation (scoliosis) concaved to the left and lameness in the right forelimb. Due to unresponsiveness to treatment, it had been donated by its owner to the Anatomy Unit of Shiraz University. Following an injection of IV sodium pentobarbital (88 mg/kg), the horse was humanely euthanized by exsanguination and embalmed with standard formaldehyde solution. After opening the thoracic cavity, the lungs were removed and the pericardium covering the heart, aorta, and great vessels were carefully dissected. To test the arrangement of the aortic arch branches, an incision was made along the concave aspect of the aortic arch to see inside its lumen and determine whether the branches have a common or separate exit from the aorta [5].

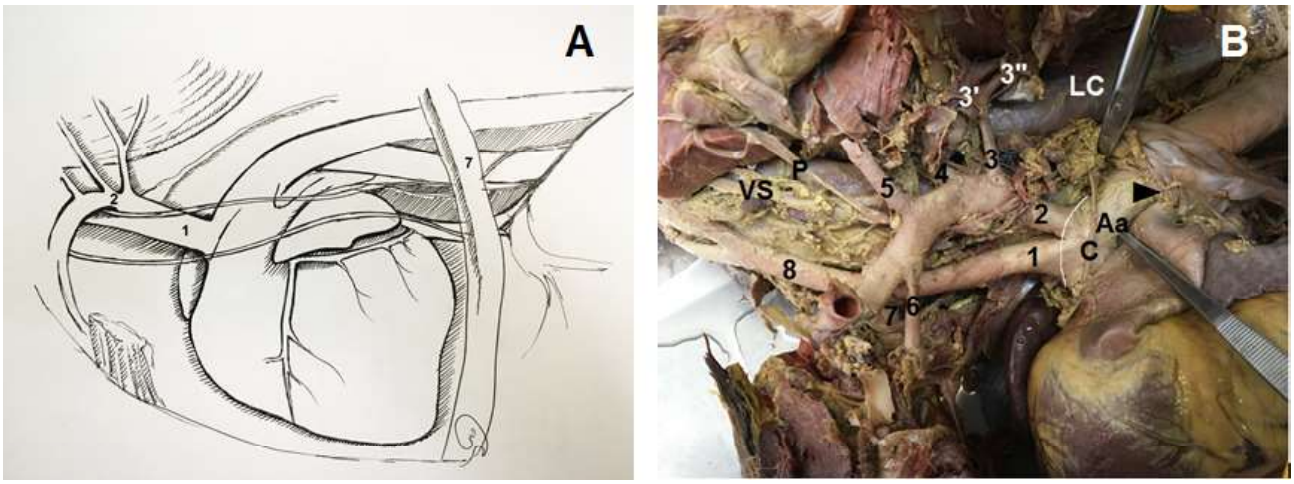

Figure 1.
A: Schematic drawing of the left equine thorax showing the brachiocephalic trunk (1) and left subclavian artery (2). B: The left lateral view of the thorax. The left lung has been removed and the left side of the pericardial sac has been reflected caudally to expose the aortic arch and the great vessels. The brachiocephalic trunk (1); the left subclavian artery (2); the costocervical trunk (3); the dorsal scapular artery (3'); the supreme intercostal artery (3"); the deep cervical artery (4); the vertebral artery (5); the internal thoracic artery (6); the right subclavian artery (7); the bicarotid trunk (8); the cardiac nerve (C); the phrenic nerve (P); the vagosympathetic trunk (VS); the distal end of the ligamentum arteriosum (the arrowhead) and the longus colli muscle (LC). Note the cranial curvature of the aortic arch (Aa) which is demarcated with the curved white line.

Results and Discussion

The curvature of the arch of the aorta was located opposite the second intercostal space. It was observed that the aortic arch gave off two great branches rather than the usual one in the cranial direction. The BCT with a length of 7.3 cm and a diameter of 1.64 cm was the first branch to leave the aortic arch giving origin to the RSA. Then, it continued at the level of the first rib as the bicarotid trunk. The second branch was the LSA (with a diameter of 1.18 cm) which separately arose from the similar surface of the aortic arch immediately distal to the BCT origin (Figure 1B). Each branch had a separate exit from the aorta with a ridge projecting between them (Figure 2A), comparable to that of dogs (Figure 2B).

The distal end of the ligamentum arteriosum (LA) joined at the lateral aspect of the aortic arch at the level of the third intercostal space, 3.2 cm caudal to the origin of the LSA.

No other arteries branched off from the BCT in its course toward the first rib. The branching patterns of both subclavian arteries were different on each side. The LSA branched into the costocervical trunk, deep cervical artery, vertebral artery, and internal thoracic artery in order as separate branches. The right costocervical trunk and the deep cervical artery were separated from the RSA by a long common stem, while the vertebral artery independently originated from the RSA (Figure not shown). The bicarotid trunk 6.7 cm cranial to the root of the RSA was bifurcated into two common carotid arteries along the ventral aspect of the trachea.

Encountering anatomic variations during veterinary dissection classes, necropsy or surgical interventions drives us to attempt to clarify their embryological basis. Disposition in various great vessels arising from the aortic arch is attributed to the unusual modes in the development and transformation of aortic arches during the embryonic period [6].

According to Vitums (1969), the definitive aortic arch and the common brachiocephalic trunk in the equine embryos are formed in three distinct phases of development. The typical events that take place in the third phase of development include the elongation of the brachiocephalic trunk and the fusion of the proximal origins of the left and right common carotid arteries into a bicarotid trunk. In this phase, the origin of the LSA along the aortic arch is more cranial in position and lies close to that of the brachiocephalic artery, between the latter and the distal end of the ductus arteriosus (DA). Finally, the aortic arch reaches its definitive position opposite the third to fifth intercostal space and a single great vessel (the common brachiocephalic trunk) develops from the fusion of the BCT and LSA [2]. In the case described here, although the arch of the aorta had completely migrated and reached its final position at the level of the third thoracic vertebra, the lack of formation of the common brachiocephalic trunk may be due to the impaired fusion of the BCT and LSA in the final phase of development. Furthermore, the aortic arch had taken its final form because the distal end of the DA on it was displaced from a more ventral position in the early third phase to a lateral position in the final phase of development. On the other hand, as mentioned earlier, the formation of the bicarotid trunk precedes that of the common brachiocephalic trunk and occurs early in the third phase of the development of equine embryos. Hence, the presence of a bicarotid trunk and

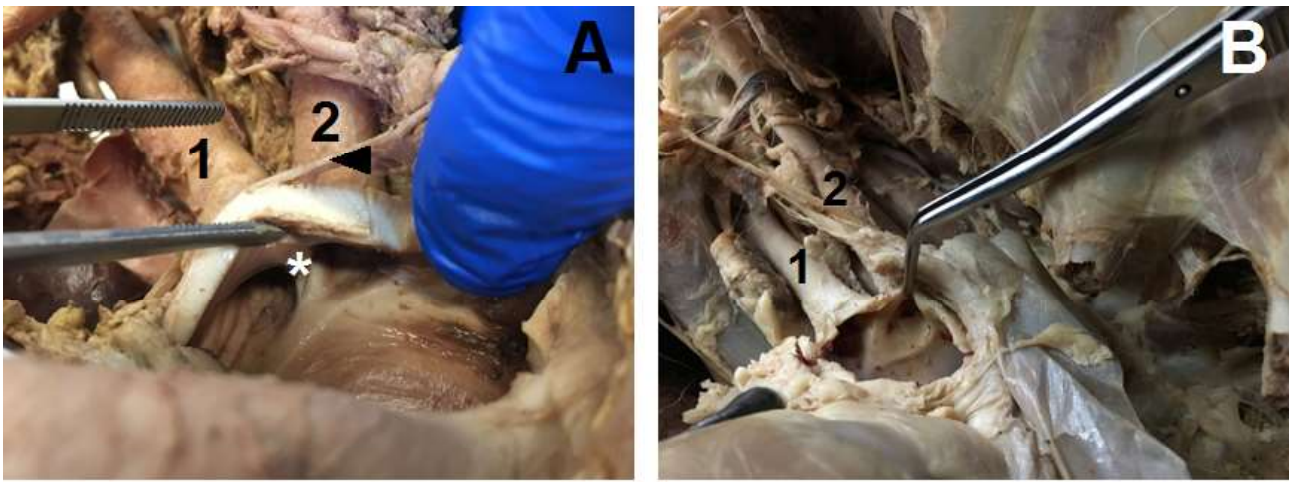

Figure 2.
The aortic arch was opened to show the separate outlets for the brachiocephalic trunk (1) and left subclavian artery (2) and a ridge between them (the asterisk) in the present case (A). The aortic arch of a dog (B) was also opened for comparison with (A). The arrowhead shows the cardiac nerve.

the direct origin of the LSA cranial to the distal end of the LA in this case may indicate an arrest in the late third phase of development.

A review of comparative morphology associated with the different branching arrangements of the aortic arch can provide a clear understanding of the abnormal conditions in horses and other domestic mammals [7]. A gradually increasing fusion of two or more of the branches forms a common trunk leaving the aorta. Normally, a fusion of the RSA and the right carotid artery produces an innominate artery that has been described in humans [8], monkeys [5], and some other mammals [9-11]. In dogs, pigs, and cats, condensation becomes more complete. however, the LSA remains separate so that the aorta gives the two branches of BCT and the LSA [3].

According to Parsons (1902) the animals with laterally compressed thoraces possibly show the least degree of separation. In ungulates, such as horses and ruminants, the thorax inlet is often twice as deep as it is broad. Furthermore, in these animals, the fusion of great vessels reaches its extremity and only one vessel (the common BCT) arises from the aorta [12]. The direct origin of the LSA from the convex aspect of the aortic arch in horses has not been previously reported in the veterinary literature. This peculiar disposition resembles that normally described in pigs, dogs, and cats [3], as well as other exotic animals such as the white-eared opossum [13], and chinchilla lanigera [14, 15]. In horses, only one large outlet from the aorta belongs to the common BCT is identifiable. But in the present case, two separate outlets for the BCT and LSA visible from inside the aorta confirmed the absence of a common brachiocephalic trunk. This indicates a reversion toward a more primitive condition in which the BCT and LSA take their origins independently from the aorta.

Regarding the branches of the subclavian arteries, the right costocervical trunk and the deep cervical artery arose from the RSA by a common trunk, whereas the vertebral artery arose independently from the RSA. This arrangement on the right side is similar to that observed in the donkey [16]. Because in horses, the right deep cervical artery usually arises from the RSA after the costocervical trunk [4].

To sum up, the present case would potentially be of great interest because the aortic arch had achieved its definitive development before the common BCT was formed.

Authors' Contributions

YK detected the anatomic variation, and wrote the primary draft. ZK revised and reviewed the final draft.

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Competing Interests

There was no conflict of interests.

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Abstracts (in Persian)

بررسی هیستوپاتولوژی، فراساختاری و مولکولی گونه های سارکوسیستیس در گوسفندان شهر مشهد، استان خراسان رضوی، ایران

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چکیده

هدف این مطالعه تعیین و شناسایی آلودگی به گونه های سارکوسیستیس در گوسفندان شهر مشهد، ایران بود. از مهر سال ۱۳۹۷ تا اردیبهشت ۱۳۹۸، مری و دیافراگم ۱۰۰ گوسفند از کشتارگاه مشهد جمع آوری گردید. ابتدا نمونه ها از نظر حضور کیست های ماکروسکوپی توسط چشم غیرمسلح بررسی شد. همچنین تمامی نمونه ها از نظر وجود گونه های سارکوسیستیس توسط تست های اسمیر فشاری بافت، هیستوپاتولوژی وPCR ارزیابی شدند. به منظور تأیید گونه های یافته شده، تعداد هشت نمونه بوسیله میکروسکوپ الکترونی عبوری و روش تعیین توالی ژن مطالعه گردید. میزان آلودگی به سارکوسیستوزیس در روش های اسمیر فشاری، هیستوپاتولوژی و PCR به ترتیب ۶۹٪ ، ۹۶٪ و ۱۰۰٪ بود. بررسی هیستوپاتولوژی وجود ماکروکیست های *S. gigantea* با دیواره ثانویه PAS مثبت را در ۲۶٪ از گوسفندان نشان داد. همچنین طبق مورفولوژی دیواره کیست، دو نوع میکروکیست شامل *S. tenella* با دیواره ضخیم مخطط و *S. arietcanis* با دیواره نازک صاف به ترتیب در ۴۷٪ و ۱۱٪ گوسفندان شناسایی شد. در مطالعه TEM، زوائد دیواره کیست های *S. gigantea* گل کلمی، *S. tenella* انگشتی و *S. arietcanis* مویی شکل بودند. بررسی مقایسه ای توالی ژن *S rRNA*، گونه های *S. tenella* ، S. gigantea و *S. arietcanis* را توسط PCR تایید نمود. نتایج مطالعه حاضرنشان داد که میزان آلودگی به گونه های سارکوسیستیس در روش PCR بسیار بالا بود. همچنین وجود گونه های *S. tenella*، *S. gigantea* و *S. arietcanis* توسط روش های هیستوپاتولوژی، TEM و تعیین توالی DNA در گوسفندان این منطقه تایید گردید.

واژگان کلیدی

گوسفند، سارکوسیستیس، مرفولوژی، فراساختار، 18SrRNA، مشهد

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همراه کردن استرادیول بنزوات با عصاره گیاه پنج انگشت در بروز القا جفت پذیری سگ ها

آلا قادری، اسماعیل آین، علیرضا کبیریان

گروه مامایی، دانشکده دامپزشکی، دانشگاه ارومیه، ارومیه، ایران.

چکیده

در این مطالعه دو روش القا جفت پذیری در سگ ها مقایسه شده است (استفاده از ویتاگنوس و ویتاگنوس استرادیول). ۱۰ قلاده سگ ماده سیکلیک، نژاد شیتزوتریر مخلوط در مرحله آنستروس انتخاب و به ۲ گروه تقسیم شدند. گروه اول (VAC) به مدت ۵ هفته ویتاگنوس دریافت کردند (۹۰ میلی گرم، روزانه، خوراکی). گروه دوم (VAC-E2) با ویتاگنوس و استرادیول درمان شدند. استرادیول بنزوات در شروع هر هفته تزریق شد (۰/۰۱ میلی گرم به ازای هر کیلوگرم، عضلانی). هر هفته نمونه خون جهت ارزیابی هورمون های استروئیدی و اسمیر مهبلی اخذ گردید. علایم و برگشت به جفت پذیری همراه با تعداد توله های به دنیا آمده ثبت شد. در گروه VAC ۶۰ و در گروه ۸۰ VAC-E2 درصد سگ ها بعد از ۵ هفته به فاز فولیکولار برگشتند. در گروه VAC-E2 علایم جفت پذیری ۷ تا ۱۰ روز و پروسه جفت گیری ۴ تا ۶ روز زودتر از گروه VAC رخ داد. میانگین تعداد توله های متولد شده ۴ تا بود. این علایم با سیتولوژی مهبل تایید شد. تفاوت معنی داری بین گروه های مورد مطالعه در سطح استرادیول و پروژسترون مشاهده نشد. تغییرات میانگین غلظت استرادیول بین هفته های ۱ و ۴، ۱ و ۵، ۲ و ۵ به طرز معنی داری مشاهده شد ($p < ۰.۰۵$). سطح پروژسترون در گروه VAC-E2 در هفته پنجم بیشتر از سایر هفته ها است. در نتیجه، استفاده از استرادیول بنزوات قبل از ویتاگنوس منجر به بهبود القا جفت پذیری در سگ ها می شود.

واژگان کلیدی

القا جفت پذیری، استرادیول بنزوات، عصاره گیاه پنج انگشت، سگ

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بررسی پارامترهای بیوشیمیایی و استرس اکسیداتیو در گاوهای هلشتاین مبتلا به تب برفکی

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چکیده

تب برفکی یک بیماری ویروسی شدید با واگیری بالا در نشخوارکنندگان زوج سم است که توسط آفتوویروس از خانواده پیکورناویریده ایجاد می شود. این بیماری در گاوها با تب بالا و ظهور تاول در محوطه دهان، بین سم ها و روی پستانک ها مشخص می شود. مطالعه حاضر برای تعیین پارامترهای بیوشیمیایی سرم گاوهای هلشاین مبتلا به تب برفکی در ناحیه شهرکرد-ایران انجام شده است. برای این منظور، خون وریدی از ۲۳ راس گاو با نشانه های درمانگاهی تب برفکی و ۲۲ راس گاو به ظاهر سالم اخذ شد. در این نمونه ها میزان فعالیت سرمی آسپارتات آمینوتراسفراز، کراتینین کیناز، کراتینین کیناز قلبی، لاکتات دهیدروژناز، گلوتاتیون پروکسیداز، سوپراکسید دیسموتاز و کاتالاز، و نیز غلظت تروپونین I، مالون دی آلدئید، گلوکز و تری گلیسیرید اندازه گیری شدند. نتایج، افزایش معنی دار فعلیت سرمی آنزیم های آسپارتات آمینوتراسفراز، کراتینین کیناز، کراتینین کیناز قلبی و لاکتات دهیدروژناز و نیز مقرار سرمی تروپونین I، مالون دی آلدئید، گلوکز و تری گلیسیرید در گاوهای مبتلا به تب برفکی در مقایسه با دام های سالم را نشان داد (p>۰/۰۵). میزان فعالیت سرمی گلوتاتیون پروکسیداز و سوپراکسید دیسموتاز در گاوهای مبتلا به تب برفکی به طور معنی داری کمتر از گاوهای سالم بود (۰/۰۵ >p) و میزان فعالیت سرمی کالاز تفاوت معنی دار بین دو گروه نشان نداد.

واژگان کلیدی

تب برفکی، گاو، پیکورناویریده، پیکورناویریده، پانکراس

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اثرتزریق ویتامین دی (کوله کلسیفرول) در نزدیکی زایش بر مقادیر برخی از شاخص های ایمنی و استرس اکسیداتیو در گاوهای شیری در دوره انتقال

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۲ قطب علمی مطالعات سقط جنین و مرگ و میر نوزاد نشخوارکنندگان، دانشکده دامپزشکی دانشگاه فردوسی مشهد، مشهد، ایران.

چکیده

مطالعات جدید به نقش ویتامین D به عنوان عامل بازدارنده و حتی درمانگر در بیماری های خودایمن، سرطان و دیابت تیپ یک و دوپرداخته است. در گاو شیری بیشتر مطالعات گذشته معطوف به نقش ویتامین D در متابولیسم کلسیم بوده و دانش ما درباره اثر ویتامین D در تنظیم ایمنی و کاهش استرس اکسیداتیو خصوصا در دوره انتقال ناچیز است. دراین مطالعه فرضیه این است که تزریق ویتامین D3 موجب بالا رفتن میزان ویتامین D در سرم می شود و به دنبال آن تنظیم مناسب ایمنی موجب کاهش فعالیت سایتوکاین های آغازگر التهاب شده و نهایتا موجب مدیریت استرس اکسیداتیو در دوره انتقال می شود. مطالعه در یک فارم تجاری با ۱۵۰۰ راس دوشا در استان تهران انجام شد. ۲۴ گاو چند شکم زایش به صورت اتفاقی در دو گروه شاهد و مداخله قرار گرفتند. در گروه مداخله ۱۲ گاو تک دوز هشت میلیون واحد ویتامین D3 را به صورت عضلانی ودر گروه شاهد ۱۲ گاو نمایه (آب مقطر) دو تا هشت روز مانده به زمان زایش دریافت کردند. نمونه های خونی در ۲۱ و ۷ روز مانده به زایش و در روزهای ۱،۳،۷،۱۵ و ۳۰ بعد از زایش اخذ شد. مقادیر۲۵(OH)D ، TNF-α، INF-γ، هاپتوگلوبین، SOD، GPx، FRAP، IL-6 وhemolysate GPx اندازه گیری شد. نتیجه نشان داد که تزریق ویتامین D3 در گروه مداخله اثر معنی داری روی مقادیر hemolysate GPx و IL-6دارد. به نظر ویتامین D سبب افزایش فعالیت آنتی اکسیدانی شده است. مطالعات بیشتری در جهت نشان دادن اثر تنظیم کنندگی ایمنی ویتامین D باید انجام شود.

واژگان کلیدی

تنظیم ایمنی، سوپر اکسید دیسموتاز، هاپتوگلوبین، ۲۵ هیدروکسی ویتامین دی

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خالص سازی و کشت سلول های رده ی سلولی سلول های ماهواره ای عضلانی گوسفند

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۳ گروه سلول های بنیادی و طب بازساختی، پژوهشکده فناوری زیستی، دانشگاه فردوسی مشهد، ایران.

چکیده

از آنجایی که سلول‌های ماهواره‌ای گوسفند از لحاظ طول عمر، تکثیر، تمایز و متابولیسم شباهت بسیاری به سلول‌های ماهواره‌ای انسان نسبت به سلول‌های رت و موش دارد، مورد توجه قرار گرفتند. بازسازی ماهیچه های اسکلتی در پاسخ به جراحات، از طریق تلفیق سلول های ماهواره ای انجام می گیرد. این سلول ها کاربرد بسیاری در مدل سازی و درمان بیماری هایی مانند نارسایی های قلبی، دیستروفی عضلانی، پیوند سلول های مغزی برای درمان میگرن، تولید داروهای جدید و غیره دارند. در این مطالعه، از عضله جنین گوسفند ۶۰-۵۰ روز، به منظور توصیف روش های جداسازی و کشت سلول های ماهواره ای، ترسیم منحنی رشد برای این سلول ها و تست عدم وجود مایکوپلاسما و باکتری استفاده شد. نمونه ها از کشتارگاه صنعتی مشهد جمع آوری شد. پس از هضم آنزیمی با کلاژناز، ترکیبی از سلول های ماهواره ای و غیر میوژنیک در کف فلاسک حاوی محیط DMEM کشت داده شدند. جهت جداسازی سلول هایی که میوژنیک نیستند مانند فیبروبلاست ها، بعد از ۳ ساعت، فلاسک ها تعویض گردیدند. پس از شش روز، سلول ها به میوبلاست تمایز پیدا کردند. با استفاده از محیط تمایز حاوی سرم اسب، سلول های میوبلاست در فلاسک مشاهده شد، که تاییدی بر وجود سلول های ماهواره ای بودند. بیان mRNA ژن PAX7 برای تأیید حضور سلول های ماهواره ای مورد استفاده قرار گرفت. همچنین، نتایج نشان داد که سلول های ماهواره ای در یک کشت متوسط حاوی ۵٪ FBS بدون تمایز رشد یافته و برای شروع تمایز به ۱۰٪ FBS نیاز دارند.

واژگان کلیدی

سلول های ماهواره ای، گوسفند ،PAX7 ،میوبلاست

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تأثیر شدت تمرین هوازی بر عضلات اسکلتی PGC–1α ، فاکتور تنظیم کننده اینترفرون ۴ و شاخص آتروژنیک در رت ها چاق صحرایی نر نژاد ویستار

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چکیده

PGC-1a گیرنده تنظیم کننده اصلی در سوخت و ساز انرژی است. در طول تمرین، بسیاری از فرآیندها مانند بیوژنز میتوکندری، متابولیسم گلوکز و متابولیسم اسیدهای چرب را تحریک می کند. همچنین باعث افزایش ظرفیت اکسیداسیون چربی می شود. هدف از پژوهش حاضر بررسی تاثیر هشت هفته تمرین هوازی با شدت های متفاوت بر سطوح PGC-1a، IRF4 و شاخص آتروژنیک در رت های چاق نژاد ویستار بود. ۲۴ سر رت نر نژاد ویستار با (وزن ۲۵۰ تا ۳۰۰ گرم، نمایه توده بدن: بیشتر از ۳۰ گرم بر سانتی متر مربع در سه گروه تمرین هوازی با شدت متوسط (۸ سر)، گروه تمرین هوازی با شدت زیاد (۸ سر) و گروه کنترل (۸ سر) تقسیم شدند. برنامه تمرین هوازی هشت هفته، پنج جلسه در هفته به مدت ۶۰ دقیقه در روز بود. سرعت دویدن در گروه شدت متوسط ۲۸ و شدت زیاد ۳۴ متر در دقیقه به ترتیب بود.سطوح PGC-1a در هر دو گروه شدت متوسط و بالا در مقایسه با گروه کنترل افزایش معنی داری یافت. همچنین، تفاوت معنی داری بین سطوح IRF۴ در هر دو گروه دیده نشد. سطوح سرمی HDL-C فقط در گروه شدت متوسط نسبت به گروه کنترل افزایش معنی دار یافت.سطوح LDL-C، TG، TC و شاخص آتروژنیک در هردو گروه تمرین هوازی با شدت متوسط و بالا نسبت به گروه کنترل کاخش معنی داری یافت.نتایج نشان می دهد که هشت هفته تمرین هوازی با دو شدت متوسط و زیاد ممکن است مسیرهای سیگنالینگ پروتئین PGC-1a (یعنی تنظیم کننده اصلی متابولیسم انرژی و بیوژنز میتوکندری) در عضله اسکلتی را فعال بسازد.

واژگان کلیدی

تمرین هوازی، IRF4، PGC-1a، شاخص آتروژنیک

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خالص سازی و تجزیه و تحلیل بیولوژیکی آنتی ژن‌های خاص (ESAT6/CFP10) مایکوباکتریوم توبرکلوزیس

ناهید مژگانی، مهدی بابائی، نفیسه شکیبا مهر، محمد محمد طاهری، نادر مصوری، آرام قائم پناه، کیومرث سلیمانی بابادی

آزمایشگاه رفرانس سل گاوی، مؤسسه تحقیقات واکسن و سرم سازی رازی، سازمان تحقیقات، آموزش و ترویج کشاورزی (تات)، تهران، ایران.

چکیده

بیماری‌زایی مایکوباکتریوم توبرکلوزیس (Mtb) مربوط به پروتئین‌های با وزن مولکولی پایین آن به طور عمده ESAT-6 و CFP10 است که برای تشخیص سل بسیار خاص و بالقوه هستند. این تحقیق بر جدا سازی، خالص سازی و تعیین خصوصیات پروتئین‌های با وزن مولکولی پایین Mtb تمرکز دارد. کشت‌های Mtb به ترتیب با حرارت در ۶۸ °C به مدت ۹۰ دقیقه و ۱۰۰ °C به مدت ۳ ساعت غیرفعال گردیدند. کشت‌های غیرفعال، فیلتر شدند و پروتئین‌های مایع رویی در دو مرحله با آمونیوم سولفات در دمای ۴ °C رسوب داده شدند. رسوبات حاصله دیالیز شدند و ژل کروماتوگرافی (G-۵۰) انجام گرفت. فراکسیون‌های به دست آمده برای تعیین غلظت پروتئین و وزن مولکولی مورد تجزیه و تحلیل قرار گرفتند. کمپلکس پروتئینی ESAT-6 و CFP10 در فراکسیون خالص شده با وسترن بلات تایید شد. از روش سنجش حساسیت خوکچه هندی برای تخمین قدرت بیماری‌زایی فراکسیون خالص شده در مقایسه با PPD استاندارد استفاده شد. حداکثر مقدار پروتئین‌های با وزن مولکولی پایین آمونیوم سولفات ۲۰٪ رسوب داده شدند. تجزیه و تحلیل SDS-PAGE باندهای پروتئینی تقریباً ۱۵-۱۰ کیلو دالتونی را نشان داد. خلوص پروتئین حدود ۹۵≤، بود که توسط SDS-PAGE تایید گردید. حضور کمپلکس ESAT-6/CFP10 با تجزیه و تحلیل وسترن بلات تأیید شد. فراکسیون‌های خالص شده هیچ واکنش متقاطعی با سویه BCG یا مایکوباکتریوم اویوم نشان ندادند. به نظر می‌رسد ESAT-6/CFP10 خالص شده با روش آمونیوم سولفات برای توسعه کیت تشخیصی برای تشخیص Mtb مناسب می‌باشد.

واژگان کلیدی

مایکوباکتریوم توبرکلوزیس، ESAT-6/CFP10، آمونیوم سولفات، کروماتوگرافی، وسترن بلات

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بررسی میزان آلودگی و تعیین انواع انگل های خارجی پرندگان زینتی در شمال سیستان و بلوچستان(جنوب شرق ایران)

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چکیده

بررسی انگل های خارجی در پرندگان اهمیت بسیار زیادی دارد زیرا سبب کاهش پرها، جراحت و آسیب پوستی، حالت های عصبی و کاهش تولید می گردند، این انگل ها هم چنین می توانند به عنوان حامل عوامل پاتوژن مطرح باشند. بررسی حاضر جهت شناسایی گونه های انگلی آلوده کننده پرندگان زینتی و درصد شیوع آلودگی به انگل خارجی در پرندگان زینتی شمال استان سیستان و بلوچستان انجام شده است. این مطالعه در سال ۱۳۹۵ در شمال استان انجام شد. بدین منظور تعدادی از پرندگان زینتی موجود در پرنده فروشی ها و باغ وحش های شهرستان های زاهدان و زابل مورد بررسی قرار گرفتند. بدن هرگونه پرنده به تفکیک و به طور کامل از لحاظ وجود انگل خارجی بررسی شد. نمونه های مشاهده شده روی بدن و پر پرندگان به وسیله برس ظریف و پنس جدا و به لوله های حاوی اتانول ۷۰ ٪ منتقل گردید. پس از جمع آوری همه نمونه ها به منظور شناسایی نوع انگل ؛ نمونه ها را در ۱۰٪ KOH شفاف نموده، سپس با استفاده از میکروسکوپ نوری و باتوجه به خصوصیات مورفولوژیکی وکلیدهای توصیفی مطرح شده برای هرگونه ، به شناسایی انگل ها پرداختیم. در مجموع ۳۱۸ پرنده مورد بررسی قرار گرفتند. از این تعداد ۷۰/۲۱ ٪ آلودگی به انگل های خارجی را نشان دادند. انگل های خارجی جدا شده شامل ۶ نوع شپش، یک نوع کنه، یک نوع مگس و یک نوع پشه می باشند.

واژگان کلیدی

انگل خارجی، پرندگان زینتی، سیستان، بلوچستان

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Abstracts (in Persian)

مطالعه مقایسه ای آناتومی مغز در خدنگ هندی خاکستری(Herpestes Edwardsii)

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چکیده

خدنگیان خانواده ای از گوشتخواران می باشند که در نواحی وسیعی از جنوب ایران یافت می شوند. مطالعات آناتومیکی و مورفولوژیکی مغز به دلیل اهمیت بالای آن در زمینه های مختلف دامپزشکی و جانور شناسی همواره مورد توجه متخصصین علوم تشریح بوده است. به دلیل کمبود اطلاعات لازم پیرامون ساختمان مغز در گوشتخواران وحشی، مطالعه ی حاضر به منظور ادراک بهتر از ویژگی های کمی و آناتومیکی آن در خدنگ هندی خاکستری انجام گرفت. بدین منظور از لاشه ی ۸ قلاده خدنگ بالغ که در نواحی مختلف استان فارس بر اثر حوادث طبیعی مرده بودند، استفاده گردید. پس از آماده سازی لاشه و تزریق ماده فیکساتیو به داخل بطن های مغزی، ساختمان مغز به دقت از جمجمه جدا شده و اندازه گیری و مشاهدات لازم بر روی قسمت های مختلف آن صورت گرفت. در این مطالعه مشخص گردید که ساختمان مغز از نمای پشتی تخم مرغی شکل بوده و حباب بویایی، شیاری های عرضی و طولی عمیق و هم چنین مخچه و ناحیه کرمینه ی نسبتا بزرگ از ویژگی های آن به حساب می آمد. با توجه به مطالعه فوق می توان اینگونه نتیجه گرفت که ویژگی های آناتومیکی مغز در خدنگ شباهت زیادی با سایر گوشتخواران داشته و در هماهنگی کامل با قابلیت های حسی و حرکتی این حیوان است.

واژگان کلیدی

آناتومی، مغز، خدنگ، مورفولوژی

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Abstracts (in Persian)

تاثیر سویه های ایرانی واکسن های کاپری پاکس ویروس بروی تیتر آنتی بادی خنثی کننده در گاو

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چکیده

ویروس بیماری لمپی اسکین، آبله بزی و آبله گوسفندی از اعضاء جنس کاپری‌پاکس ویروس‌ها است. استفاده از واکسن های کاپری‌پاکس ویروس می تواند در محافظت گاوها در برابر بیماری لمپی اسکین (LSD) کمک کند. هدف از این مطالعه مقایسه تیتر آنتی بادی واکسن‌های هترولوگ آبله بزی و گوسفندی در برابر بیماری LSD در گاو است. ۱۰۰ گوساله توسط واکسن های آبله بزی(GPV) و آبله گوسفندی (SPV) در فارم های جداگانه واکسینه شدند. تیتر آنتی بادی واکسن در روزهای ۱۴، ۲۸، ۴۵، ۹۰ و ۱۸۰ بعد از واکسیناسیون بررسی شد. میانگین دمای مقعد گوساله های واکسینه شده با GPV نسبت با گوساله‌های واکسینه شده با SPV نه تنها بالاتر بود بلکه ترشحات چشمی و آبریزش از بینی در زمان تب نیز مشاهده شد در حالیکه در گوساله های واکسینه شده با GPV هیچ علایم بالینی دیده نشد. در گروه های واکسینه اولین تیتر آنتی بادی در روز ۱۴ مشاهده شد و در روز ۲۴ الی ۴۵ بعد از واکسیناسیون به بالاترین میزان خود رسید و سپس در روزهای بعد کاهش یافت. با وجودیکه میانگین تیتر آنتی بادی در هر یک از گروه های واکسینه در روزهای بعد از واکسیناسیون تقریباً یکسان بود و از نظر آماری اختلاف معنی داری بین آنها مشاهده نشد. اما در گوساله های واکسینه شده با GPV این میزان در روز ۴۵ به سطح محافظت کنندگی خود رسیده بود و در روز ۱۸۰ نیز دارای تیتر بالاتر از SPV داشت. نتایج نشان داد که GPV به جهت القاء بهتر پاسخ ایمنی و ماندگاری بیشتر آنتی بادی در ماه های متوالی بعد از واکسیناسیون، از خاصیت ایمنی زایی بهتری برخوردار بوده و می توان بعنوان واکسن مناسب در برابر LSD بهره برد.

واژگان کلیدی

آبله بزی، آبله گوسفندی، کاپری پاکس واکسن، آنتی بادی خنثی کننده

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Abstracts (in Persian)

موردی نادر با غیاب تنه بازویی–سری مشترک مشخص در یک اسب بالغ نژاد مخلوط

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چکیده

تنها شاخه‌ای که از کمان آئورتی اسب‌های بالغ جدا می‌شود، تنه بازویی-سری مشترک است. این گزارش به توصیف یک مورد نادر از آرایش سرخرگی غیرمعمول کمان آئورتی در یک اسب ماده بالغ می‌پردازد. مشخص گردید که کمان آئورتی فاقد یک تنه بازویی-سری مشترک است و بجای آن دارای دو شاخه یعنی شاخه اول تنه بازویی-سری و شاخه دوم سرخرگ تحت ترقوه‌ای چپ بود. منشاء مستقیم سرخرگ تحت ترقوه‌ای چپ از کمان آئورتی در اسب‌ها بسیار نادر است و مورد مشابهی از آن قبلا گزارش نشده است. جنبه های رویان‌شناختی و فیلوژنتیکی این متغیر بحث می‌گردد.

واژگان کلیدی

تنه بازویی-سری مشترک، اسب، سرخرگ تحت ترقوه‌ای چپ

GUIDE FOR AUTHORS

IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

Guide for authors

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IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

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Gene names: The standard gene names, as provided by HGNC (HUGO Gene Nomenclature Committee) should be used. Gene names must be italicized. If the case of mammalian species and if gene names refer to rodent species, they must be upper case; if they refer to non-rodent species they must be written in capitals. If they refer to other species, they must written lower case. Protein names are written in capitals and are not italicized. As an example:

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Quantitative PCR: If the quantitative PCR method has been used, the related section in Materials and Methods must be written following the reference:

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr;55(4):611-22.

The following information must be provided in the section:

Protocol for DNA/RNA extraction, including quantification and determination of purity;

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An unhealthy diet, obesity and physical inactivity play a role in the onset of type 2 diabetes, but it has been shown that increased physical activity substantially reduces the risk [1], and participation in regular physical activity is one of the major recommendation of the evidence based guidelines for the primary prevention of diseases [2]. According to the 2004-05 National Health Survey, more than half a million Australians (3.5% of the population) have diabetes mellitus which had been medically diagnosed and most of these people have the Type 2 condition [3]. Gestational diabetes is also on the increase, rising steadily between 2000-01 and 2005-06 [4]. Approximately two thirds of those with diabetes have been prescribed medication [3], but it is of concern that a recent review of the literature found that many people do not take their medication as prescribed [5]. Many patients also self monitor the disease by measuring their blood glucose levels with a glucose meter but Song and Lipman [6] have concerns about how well this is managed.

References for the above example:

1. Hull J, Forton J, Thompson A. Paediatric respiratory medicine. Oxford: Oxford University Press; 2015.

2. Eckerman AK, Dowd T, Chong E, Nixon L, Gray R, Johnson S. Binan goonj: bridging cultures in Aboriginal health. 3rd ed. Chatswood, NSW: Elsevier Australia; 2010.

3. Johnson C, Anderson SR, Dallimore J, Winser S, Warrell D, Imray C, et al. Oxford handbook of expedition and wilderness medicine. Oxford: Oxford University Press; 2015.

4. McLatchie GR, Borley NR, Chikwe J, editors. Oxford handbook of clinical surgery. Oxford: Oxford University Press; 2013.

5. Petitti DB, Crooks VC, Buckwalter JG, Chiu V. Blood pressure levels before dementia. Arch Neurol. 2005 Jan;62(1):112-6.

6. Liaw S, Hasan I, Wade, V, Canalese R, Kelaher M, Lau P, et al. Improving cultural respect to improve Aboriginal health in general practice: a multi-perspective pragmatic study. Aust Fam Physician. 2015;44(6):387-92.

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Peer Review Process

PEER REVIEW PROCESS

Iranian Journal of Veterinary Science and Technology peer reviews all submitted manuscripts with contents within the scope of the journal.

Initial assessment

The submitted manuscript will be subjected to a primary review by the editor or a member of the editorial board for suitability and relevance of the findings to the scope of the journal and quality of the science presented in the paper (sufficient originality, having a message that is important to the general field of Veterinary Medicine, quality of data, novelty, English language, and overall manuscript quality) within two weeks. If the paper is evaluated to be relevant to the scope of the journal and having enough scientific rigor and novelty, it will be sent for the next stage. Otherwise, those manuscripts which are evaluated as not-appropriate in the initial review will be rejected at this stage.

Initial screen

The initial screen will be performed by the editorial office for the structure and format of the manuscript.

Peer review (double-blind)

The manuscripts which are found to be appropriate after the initial screen will be sent for external review by experts in the related field. We have prepared a checklist for reviewers that summarizes their evaluation of the manuscript. The items in this checklist are:

1. TITLE is clear and adequate
2. ABSTRACT clearly presents objects, methods, and results.
3. INTRODUCTION well-structured and provides a rationale for the experiments described.
4. MATERIALS AND METHODS are sufficiently explained and is detailed enough to be reproduced.
5. RESULTS are clearly presented and supported by figures and tables.
6. DISCUSSION properly interprets the results and places the results into a larger research context, and contains all important references.
7. Conclusions are logically derived from the data presented.
8. English Language/style/grammar is clear, correct, and unambiguous.
9. Figures and tables are of good quality and well-designed and clearly illustrate the results of the study.
10. References are appropriate.
11. Regarding this article are you concerned about any issues relating to author misconduct such as plagiarism and unethical behavior.
12. Comments on the importance of the article.

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