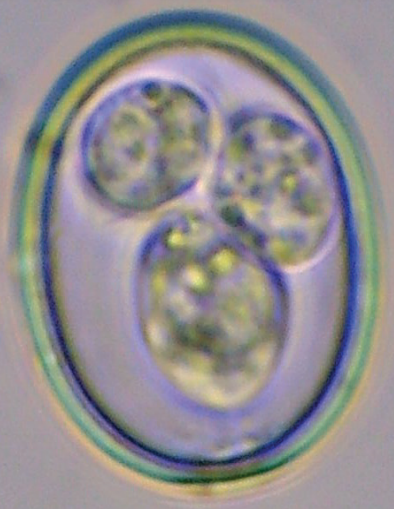
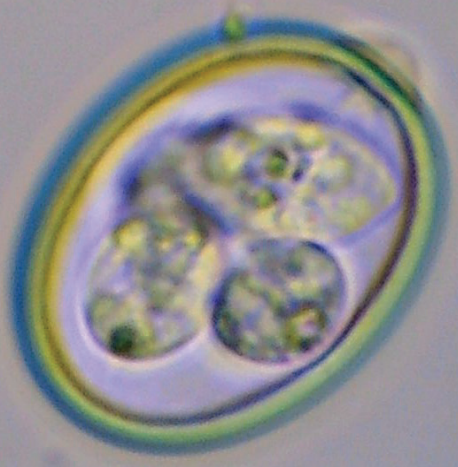
IJVST

Number 2
Volume 13
Year 2021
ijvst.um.ac.ir

Iranian Journal of Veterinary Science and Technology





Ferdowsi Univerity of Mashhad

ISSN (Print): 2008-465X
ISSN (Online): 2423-6306
Serial number: 25

In the Name of God, the Compassionate, the Merciful

Iranian Journal *of* Veterinary Science and Technology

Editorial Office:
Faculty of Veterinary Medicine,
Ferdowsi University of Mashhad,
Azadi Square, Mashhad, IRAN
P.O. Box: 1793; Postal Code: 9177948974

Tel: +98 51 3880 3742 ***Fax:*** +98 51 3876 3852
Web: ijvst.um.ac.ir ***Email:*** ijvst@um.ac.ir

FERDOWSI UNIVERSITY OF MASHHAD PRESS

GENERAL INFORMATION

ISSN Print Edition: 2008-465X
ISSN Online Edition: 2423-6306

Journal Homepage:
ijvst.um.ac.ir

Abstracting and Indexing:
Scopus, ISI Master Journal List, Zoological Record; EBSCO, Scientific Information Database (SID); Islamic World Science Citation Database (ISC); Magiran; Google Scholar; Centre for Agriculture and Biosciences International (CABI), DOAJ.

This journal has achieved the rating of
"Scientific-Research", by Commission of Evaluation of Iranian Scientific Journals, the Ministry of Science, Research and Technology, from Vol.7, No. 1, July 2015 onward.

Publication Date:
Iranian Journal of Veterinary Science and Technology (IJVST) is published 2 times a year. Volume 13 with 2 issues appear in 2021.

SCOPE

Iranian journal of Veterinary Science and Technology (IJVST) publishes important research advances in veterinary medicine and subject areas relevant to veterinary medicine including anatomy, physiology, pharmacology, bacteriology, biochemistry, biotechnology, food hygiene, public health, immunology, molecular biology, parasitology, pathology, virology, large and small animal medicine, poultry diseases, diseases of equine species, and aquaculture. Articles can comprise research findings in basic sciences, as well as applied veterinary findings and experimental studies and their impact on diagnosis, treatment, and prevention of diseases. IJVST publishes four kinds of manuscripts: Research Article, Review Article, Short Communication, and Case Report.

ON THE COVER

Eimeria ahsata oocysts in sheep. Please see paper by *Ahmadi Saleh Baberi et al.*, page 20.

Editorial Office:
Faculty of Veterinary Medicine,
Ferdowsi University of Mashhad,
Azadi Square, Mashhad, IRAN
P.O. Box: 1793; Postal Code: 9177948974

Tel: +98 51 3880 3742 ***Fax:*** +98 51 3876 3852
Web: ijvst.um.ac.ir ***Email:*** ijvst@um.ac.ir

FERDOWSI UNIVERSITY OF MASHHAD PRESS

TABLE OF CONTENTS

IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

Editorial Office:
Faculty of Veterinary Medicine, Ferdowsi University of Mashhad,
Azadi Square, Mashhad, IRAN
P.O. Box: 1793; Postal Code: 9177948974

Tel: +98 51 3880 3742 ***Fax:*** +98 51 3876 3852
Web: ijvst.um.ac.ir ***Email:*** ijvst@um.ac.ir

TABLE OF CONTENTS

IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

Editorial Office:
Faculty of Veterinary Medicine, Ferdowsi University of Mashhad,
Azadi Square, Mashhad, IRAN
P.O. Box: 1793; Postal Code: 9177948974

Tel: +98 51 3880 3742 ***Fax:*** +98 51 3876 3852
Web: ijvst.um.ac.ir ***Email:*** ijvst@um.ac.ir

Iranian Journal of Veterinary Science and Technology

Received: 2021- May-07
Accepted after revision: 2021- Jun- 08
Published online: 2021- Dec- 01

REVIEW ARTICLE

DOI: 10.22067/ijvst.2021.70605.1044

New outlook to vitamin D functions in dairy cows: non- classical roles

Saba Ahmadi Sheikhsarmast[a], Mehrdad Mohri[a,b]

[a] Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[b] Center of Excellence in Ruminant Abortion and Neonatal Mortality, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

ABSTRACT

In addition to the well-studied effects in regulating calcium and phosphorus balance, vitamin D has many non-calcemic effects that include acting as an immune modulator or an antioxidant. Cows acquire vitamin D either from photosynthesis in the skin or through swallowing fungi in the forage or vitamin D supplements. Although vitamin D deficiency is rare, today we are facing an increasing number of vitamin D deficiencies in cows due to the indoor housing away from sunlight exposure. According to the NRC recommendation, to maintain the vitamin D serum concentration in the range of 20 to 50 ng/ ml, it is necessary to administer 21,000 IU/ d of vitamin D in cattle. In addition, considering the involvement of vitamin D in various calcemic and non-calcemic effects, it seems that previously recommend levels of vitamin D supplementation have not been enough for preventing many diseases and disorders in cattle. Vitamin D toxicity may also occur due to over-supplementation of vitamin D or overgrazing in plants with high amounts of vitamin D metabolites. This review article will discuss various roles of vitamin D in dairy cattle health, normal physiology, and disease prevention.

Keywords

Calcitriol, Immune modulation, Oxidative Stress

Number of Figures: 2
Number of Tables: 0
Number of References:: 74
Number of Pages: 11

Abbreviations

TRPV6: transient receptor potential vanilloid 6
7- DHC : 7- dihydroxycholecalciferol
VDBP: vitamin D binding protein
PTH: parathyroid hormone
FGF23: fibroblast growth factor 23
DCAD: dietary cation anion difference
RANKL: receptor activator of nuclear factor kappa-B ligand
OPG: osteoprotegerin
RXR: retinoid- X receptor
iNOS: inducible nitric oxide synthase
TLR: toll like receptor

Corresponding author: *Mehrdad Mohri*
Email: *mohri@um.ac.ir*
Tel: *+98 (51)3880-3763*

Introduction

It can be said with confidence that vitamin D was one of the earliest hormones synthesized on the planet by phytoplanktons millions of years ago, possibly protecting these organisms from radiation. The ocean's environment was rich in calcium, and aquatic organisms could easily use it for their metabolic activities. As life spread from water to land, organisms faced a calcium deficiency crisis. Therefore, a strategy was created to absorb low calcium from the environment with maximum efficiency through the intestines. For unknown reasons, vitamin D gets a regulatory role in calcium absorption [8, 16].

Inscriptions on cave walls indicate that primitives praised the sun for its life-giving effects. With the industrial revolution and the development of urbanization in European countries, evidence of the vitality of sunlight appeared. People settled in building close to each other, and burning coal caused severe air pollution. Thus, the children of these cities were no longer exposed to sunlight and showed growth disorders [20].

More than a century ago, Sir Edward Mellanby discovered that the British people, especially the Scottish, were suffering from a high prevalence disease, which is probably related to their diet. Initially, the disease was known as English disease, which today is called rickets. Mellanby experimented on about 400 dogs for 5 years. He kept them away from sunlight exposure and fed them with an oatmeal diet, which was similar to the British diet at that time. After a while, the dogs showed similar symptoms to rickets. He managed to treat these dogs with cod fish liver oil. But he mistakenly called it vitamin A. Later, McCollum et al. named it Vitamin D. [1, 2, 3, and 4]. Not long after the discovery of vitamin D as an anti-rickets agent, its importance in the natural growth of cattle was revealed [32].

Vitamin D photobiology

Vitamin D has two types: Vitamin D2 or ergocalciferol, which is present in several plants that can convert ergosterol to vitamin D, and Vitamin D3 or cholecalciferol, which is derived from 7- dihydroxycholecalciferol [7- DHC] of animal products [6,40, 55]. They differ in chemical structure in a side chain [15]. Metabolites of both types of vitamin D are present in the blood of cattle, but using vitamin D3 is preferable [6, 40]. Cattle gain vitamin D from three main sources, vitamin D3 supplements through the diet, sunlight exposure, and vitamin D2 from ingesting fungus in forages [40, 51].

Exposure to sunlight is essential for the synthesis of endogenous vitamin D. Penetration of UVB photons (270- 315 nm) into the stratum basale and stratum spinosum layers converts 7- DHC in human's skin to pre-vitamin D3. This compound is unstable and immediately undergoes thermal isomerization and is converted to vitamin D [7, 8, 10]. Vitamin D formation in the skin alters with UVB exposure which may be modifiable through different factors [10, 12].

One factor relates to fur or hair coat pigmentation; the higher the melanin concentration of the skin and the darker the skin, the longer it takes to form vitamin D [7, 11].

The second factor is UVB intensity which varies through latitude, altitude, clouds, and air pollution [10]. In general, the radiation intensity is lower at higher latitudes, especially in winter, when the day length is shorter. At higher altitudes, because animals are exposed to more intense radiation for a longer period, vitamin D3 is converted to biologically neutral sterols and is excreted from the shedding of skin keratinocytes [7, 11].

The third factor is 7-DHC amounts in the skin [12]. In fur-covered animals such as rabbits and rats, the 7-DHC appears to be at the site of the sebaceous glands in the skin, where it can be exposed to radiation and swallowed by animals grooming [13]. But in cows, there were three hypotheses about the production of vitamin D in the skin. a) According to previous studies on rats, cows received the required vitamin D3 by self-grooming or grooming each other. b) Scattered-hair areas of the body, including the udder and snout, are the main sites for vitamin D synthesis. c) Vitamin D is synthesized all over the skin with hair coat. Hymøller et al. 2010 conducted an experiment on cattle. They were able to prove that in cows, vitamin D is produced throughout their body despite hair coat, and the grooming hypothesis in cows was rejected [14].

Metabolic pathway of vitamin D

Vitamin D3, produced in the skin, is transported by the vitamin D binding protein (VDBP) to be stored in adipose tissue or must be taken to the liver to become active. VDBP or transcalciferin is a type of albumin that has a high affinity to bind to various metabolites of vitamin D, including calcitriol or calcidiol, so that about 0.01% and 1% of these metabolites are free in plasma, respectively. Other functions of VDBP include connection to actin, activating macrophages, and carrying fatty acids [6]. The initial stage of hydroxylation at carbon-25 is mediated by cytochrome P450 hydroxylase enzymes such as CYP27A, CYP3A4, CYP2R1, and CYP2J3 in the liver. Due to the binding of 25-(OH) D3 to VDBP, its half-life is about 2 to 3 weeks. 25-(OH) D3 (calcidiol) is the most abundant form of vitamin D in cattle's blood and is

used to assess the status of vitamin D in the body [6, 15]. The conversion of vitamin D3 to 25-[OH) D3 is not under strict control and almost all the vitamin D3 of the body is immediately converted to 25-(OH) D3 [6,7]. The association of CYP2J2 genes in cattle with 25-(OH) D3 synthesis indicates their role in mediating hydroxylation reaction in cattle likewise [6]. In the second stage of activation, vitamin D is transported through VDBP and undergoes hydroxylation in the site of carbon-1 in proximal tubules of the kidney with 1- α- hydroxylase (CYP27B1) and converted to 1, 25-$(OH)_2$D3, which is known as calcitriol [6,7,15]. After this stage, vitamin D is taken to the target organs by VDBP to perform its functions (Figure 1).

The function of the 1-α-hydroxylase enzyme is controlled strictly by the parathyroid hormone (PTH), negative feedback of calcitriol concentration, and calcitonin. When the concentration of ionized calcium in the blood drops, PTH stimulates the production of the 1-α-hydroxylase, and the amount of calcitriol production rises. At a sufficient amount of ionized calcium, calcitonin suppresses the activity of 1-α-hydroxylase and instead increases the conversion of active vitamin D to inactive forms by increasing activity of 23,24- hydroxylase [6, 12]. Phosphorus can also affect the activity of 1-α-hydroxylase, independent of PTH function and calcium levels. A high concentration of phosphorus enhances 1-α-hydroxylase activity, while lower levels of phosphate distract calcitriol production through fibroblast growth factor 23 (FGF23) and phosphatonin [6,7]. The proportion of the ratio of 1-α-hydroxylase to 24- hydroxylase in dairy cattle during the transition period is very consequential. The higher this ratio, the easier it will be to increase the amounts of 1, 25- $(OH)_2$D3. Any factor that increases the secretion of the PTH hormone and enhances the signaling of receptors can increase this ratio. Increased sensitivity of PTH is achieved with lower dietary cation-anion difference (DCAD). Acidic conditions with low DCAD make the receptors of this hormone more sensitive in the kidney. Also, keeping the FGF23 amounts low may elevate this ratio [17].

Catabolic pathway of vitamin D

It has been shown that CYP24A1 is responsible for hydroxylation reactions in the side chain at C-

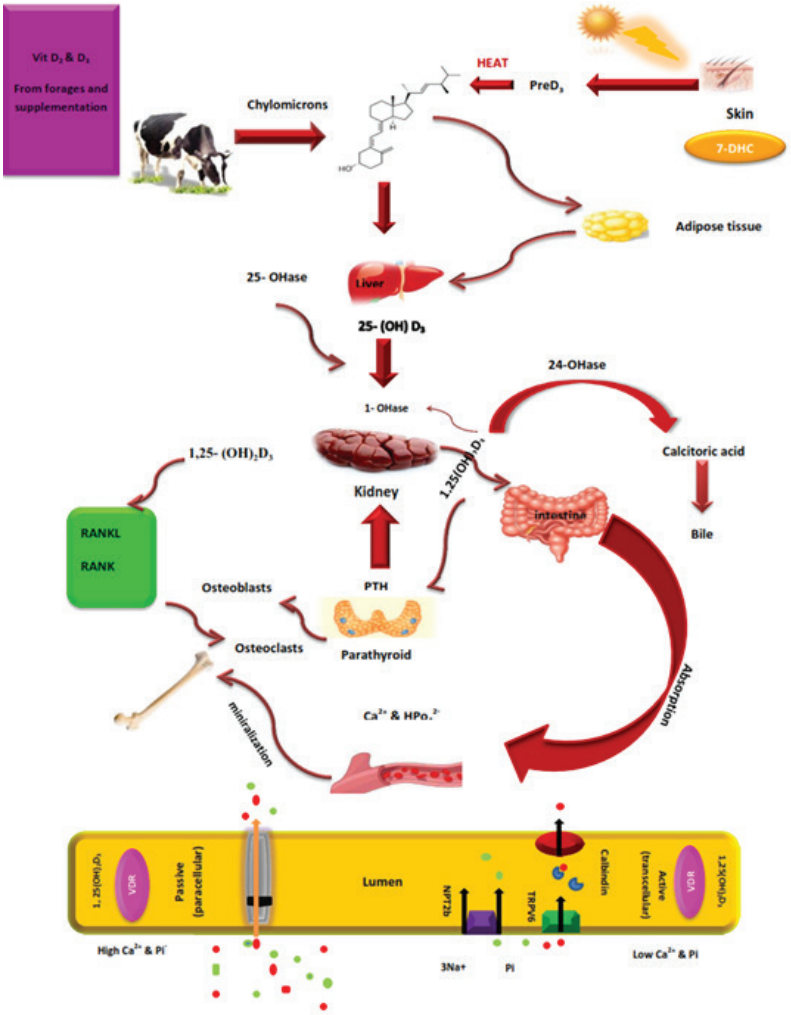


Figure 1.
The metabolism of vitamin D and its classical effects on calcium and phosphorus homeostasis.

24 and C- 23 carbon sites of either 25- (OH)D3 and 1,25- $(OH)_2$ D3. In the C- 24 oxidation pathway, 1, 25- $(OH)_2$ D3 is converted to calcitoric acid, a biliary catabolite, whereas in the second reaction1, 25- $(OH)_2$ D3, is converted to 1,25(OH) -26,23 lactone by 23-hydroxylation [5,7]. CYP24A1 is also involved in the hydroxylation of 25-(OH) D2 and 1, 25$(OH)_2$ D2 side chains and produces a series of hydroxylation products [5]. There are two VDREs in the promoter region of the CYP24A1 gene, which allow 1, 25- $(OH)_2$ D3 to regulate the expression of CYP24 via VDR and cause its catabolism. PTH and serum phosphorus levels also play a role in regulating of vitamin D catabolism pathway. Under conditions of normal calcium concentration and suppression of PTH production, CYP24A1 production is stimulated and 25-(OH] D3 is converted to 24, 25-$(OH)_2$ D3 and 1, 25- $(OH)_2$ D3 is catabolized subsequently. However, a decrease in phosphate concentration reduces the expression of CYP24A1, which leads to a decrease in 1, 25- $(OH)_2$ D3 catabolism [7].

Vitamin D functions

A substantial role of vitamin D is to preserve the concentration of calcium and phosphorus in a narrow range. These two ions are responsible for very vital functions in the body. The four main target organs for this function of vitamin D are the guts, kidneys, skeletal system, and parathyroid glands [7].

Intestine: Calcium can be transported from the guts through both transcellular and paracellular pathways. The absorption of calcium through the intestines is mediated via transient receptor potential vanilloid 6 (TRPV6) channels that are induced in the apical site of villi by 1, 25-$(OH)_2$ D3. It is revealed that these channels can interact with proteins like calmodulin, S100A10-annexin 2 complexes, and Rab11a [18]. TRPV6 channels carry calcium ions inside the cells where they join Calbindin- D9K proteins to pass across the cells. Plasma membrane ATPase (PMCA1b) and sodium-calcium exchanger (NCX1) then pump the calcium ions into the bloodstream [7]. The number of TRPV6 channels and calbindin- D9K is regulated by vitamin D to increase blood calcium levels and suppress the expression of TRPV6 leading to a decline in intestinal calcium absorption. Unlike transcellular calcium transport, paracellular calcium transport is not limited in its capacity. Paracellular calcium transport occurs through tight junctions, which are independent of 1, 25-$(OH)_2$ D3 [19]. The majority of calcium absorption of the diet is in the distal part of the guts, especially in the ileum, but the highest amount of active transport of calcium occurs in the duodenum [18]. In normal ranges of vitamin D, 30% of calcium is absorbed through the intestines, but in conditions of vitamin D deficiency, only 10 to 15% of calcium is uptaken from the diet, however, conditions such as growth, lactation, and pregnancy can increase absorption up to 60-80% [20]. Most of the phosphorus uptake occurs passively through the mechanism of diffusion throughout the intestine, but 70% of the absorption is in the small intestine. Even in severe hyperphosphatemia, dietary phosphate uptake continues and is only slightly less than normal. Albeit, phosphorus active transport is mediated by 1, 25-$(OH)_2$ D3 by increasing the number of Na+-Pi cotransporter [7,19].

Skeletal system: Longitudinal bone growth in juveniles occurs with mineralization of the bone matrix and vascular invasion. In vitamin D deficiency status, minerals no longer deposit in the matrix, leading to rickets in juveniles and osteomalacia in adults. Another function of vitamin D is to maintain serum calcium levels constant in cooperation with the parathyroid glands. Bones act as a reservoir of calcium in deficiency conditions [12]. 1, 25-$(OH)_2$ D3 has been shown to regulate the development of osteoblasts. 1, 25-$(OH)_2$ D3 elevates the expression of RANKL (Receptor activator of nuclear factor kappa-B ligand) on the surface of osteoblasts, which in turn stimulates osteoclastogenesis. Osteoclast differentiation from its precursor and maturation and bone resorption occurs with the attachment of the RANKL to the RANK (receptor activator of nuclear factor kappa-B) and Ca^{2+} ions efflux to blood flow. Production and maturation of osteoclasts are stopped by the attachment of RANKL to its antagonist osteoprotegerin (OPG) [7, 21,22].

Kidney: Approximately 65% of excreted Ca^{2+} is reabsorbed along with water and sodium in renal proximal tubules, 20% is reabsorbed through the cortical thick ascending limb of the Henle loop (CTAL). About 15% of the luminal Ca^{2+} is transported into the cells through the TRPV5 channels located at the apical region of the renal epithelial cells, where then the calbindin-D28K transports it across the cells. The Ca^{2+} ions are finally released into the bloodstream through active transport via NCX1 [23].

Vitamin D receptors

The biological functions of 1, 25-$(OH)_2$ D3 are carried out by vitamin D receptors (VDRs) [24]. The VDR is a superfamily of steroid hormones that create a heterodimer by interacting with the retinoid- X receptor (RXR). VDR/RXR heterodimers attach to the vitamin D responsive elements (VDRE) of the genome inside the nucleus [24, 25]. The expression of VDRs in various organs, including the skin keratinocytes, pancreas, guts, breast epithelial cells, prostate, activated lymphocytes, mononuclear cells, etc., indicates their extensive effects beyond calcium homeo-

stasis [12, 20].

Non- calcemic functions of vitamin D

Vitamin D modulates both innate and acquired immune systems (Figure 2). The VDR is abundantly expressed on immune cells such as B and T lymphocytes, NK cells, and antigen-presenting cells (APCs) [24]. Vitamin D can be converted to 1, 25-$(OH)_2$ D3 inside the cells of the immune system, acting locally [26, 27]. Recent studies on humans revealed that vitamin D plays an important role in immune cell mitosis, proliferation, and differentiation [26]. 1,25-$(OH)_2$ D3 enhances the production of type 2 anti-inflammatory cytokines including interleukin 4 (IL-4), interleukin 5 (IL-5), and interleukin 10 (IL-10], and decreases type 1 pro-inflammatory cytokines, for instance, tumor necrosis factor α (TNF-α), interferon γ (INF-γ), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin (IL-9], interleukin 12 (IL-12), and interleukin 17 (IL-17) [27]. 1, 25-$(OH)_2$ D3 also elevates the production of H_2O_2 which has antimicrobial and tumoricidal activity [26]. In humans, vitamin D can also have inhibitory effects on inflammatory and autoimmune diseases, including multiple sclerosis (MS), rheumatoid arthritis (RA), diabetes mellitus type 1, psoriasis, lupus erythematosus, inflammatory bowel disease (IBD), asthma, respiratory tract infections (RTI), etc. [27]. However, the effects of vitamin D on the human immune system cannot be generalized to other species, because the target organ of innate immunity in cattle is different from humans, while the acquired immunity of humans, mice and cow has many similarities [37]. For example, cathelicidin antimicrobial peptide [CAMP), which is stimulated by vitamin D, is unique to primates [28].

Studies in cattle have demonstrated that 1, 25-$(OH)_2$ D3 is in association with the innate immune system [28]. According to studies of Merriman et al. (2015) and Nelson et al. (2012), in bovine macrophages, which are the main source of calcitriol, toll-like receptor (TLR) activates 1-α-hydroxylase by pathogen's peptidoglycan, lipopeptide, and lipopolysaccharide recognition, which eventually leads to vitamin D-related immune responses. The responses

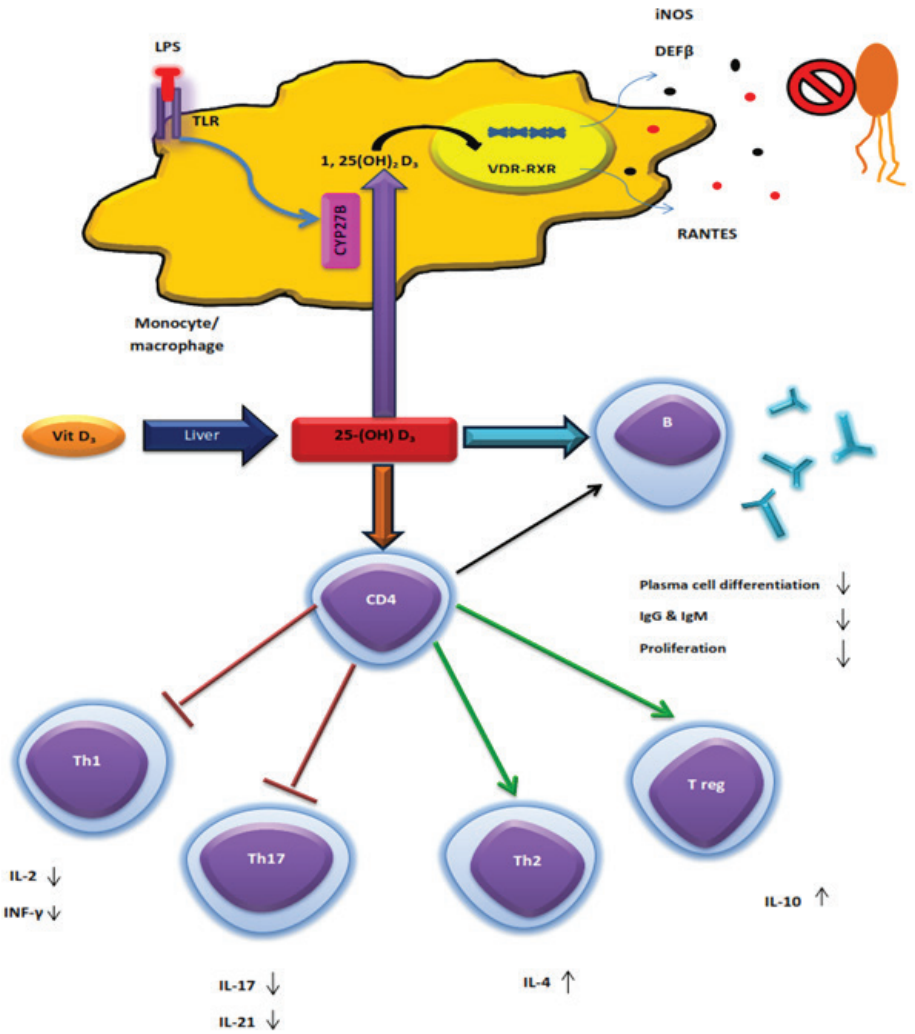


Figure 2.
The effects of vitamin D on innate and adaptive immune responses.

Ahmadi Sheikhsarmast & Mohri, IJVST 2021; Vol.13, No.2
DOI:10.22067/ijvst.2021.70605.1044

include inducible nitric oxide (iNOS), RANTES, and five ß-defensins (DEFB3, DEFB4, DEFB6, DEFB7, and DEFB10) which are related to 1, 25- $(OH)_2$ D3 levels *in vitro* [6, 29]. It has previously been proved that vitamin D has an inhibitory effect on the production of IL-4, IL-17, and INF-γ [63, 74]. Hassanabadi et al. showed that prepartum vitamin D injection has an increasing effect on IL-6 levels in dairy cows [50]. In contrast, Xu et al. (2021) showed that vitamin D supplementation has an inhibitory effect on IL-6 production [48].

1,25-$(OH)_2$ D3 may suppress the proliferation of mammary gland epithelial cells through cell cycle regulators, such as p21 and p27P21 [6]. However, a later *in vivo* study conducted by Merriman et al. (2016) demonstrated that vitamin D leads to an increase in iNOS and DEFB7 in mammary glands, while other ß-defensins were not affected [29]. Elevated induction of iNOS in bovine udder induces strong bactericidal effects in macrophages. Likewise, ß-defensins located in the udder, have potent antimicrobial effects against common mastitis-related bacteria [30]. Lippolis et al. proved this claim and by injecting intermammary 25-(OH) D3, they showed that mammary glands' immunity was significantly increased against *Streptococcus uberis,* and somatic cell count (SCC) was reduced in milk (31).

The results of the study of Martinez et al. (2018) were in agreement with previous findings. They showed that high levels of calcidiol and calcitriol in cattle's blood amplify the innate immune system and reduce the risk of periparturient diseases. 25-(OH) D3 elevates the number and activity of neutrophils with bactericidal properties and may prevent retained placenta and the establishment of bacteria in the uterus [33]. In the retained placenta, the immune system is unable to identify semi-allogeneic fetal tissues [34]. Thus, boosting innate immunity with vitamin 25-(OH) D3 may have inhibitory effects on the retained placenta and metritis [35]. Because, in cattle, bacteria settle in the uterus after parturition [36]. Calcidiol prevents metritis by its effects on immune cells and secretion of antimicrobial peptides [37].

Studies show that 25-(OH) D3 levels are decreased during the transition period in cattle, thus susceptibility to oxidative stress and diseases are enhanced. In general, calving causes an inflammatory condition, and the highest amount of Haptoglobin and C-reactive protein was recorded in Holstein Friesian cattle during the first month of calving compared with prepartum and late lactation [38]. Systemic inflammatory conditions like parturition and oxidative stress, deplete vitamin D metabolites due to elevated intracellular hydroxylation of 25- (OH)D3 to 1,25-$(OH)_2$ D3 [39]. Also, increased milk production in the mammary glands and cholesterogenesis reduce the amount of 25- (OH) D3 [40].

Calcitriol has been proved to stimulate the production and secretion of prolactin from the pituitary gland, decidua, and immune cells in rats and endometrium in humans [41, 42]. In dairy cattle, prolactin is not necessary for milk yield and has permissible impacts on steroids, but a prolactin surge occurs before calving [43], indicating that it is necessary for milk production [44]. Calcitriol also stimulates the expression of RANKL, which is an important paracrine factor in alveologenesis induced by progesterone [45]. The prepartum calcitriol administration in cows elevates the absorption of IgG through the mammary cells and raises its amount in the colostrum [33]. It is probably due to the increased production of IL-10, which leads to increased secretion of immunoglobulins from plasmablasts [47]. The results of a study conducted by Hassanabadi et al showed that injection of a single dose of vitamin D in dairy cows leads to an increase in glutathione peroxidase (GSH-PX) in hemolysate [50]. The findings of Xu et al. were consistent with this result. Xu et al. (2021) reported that vitamin D administration in cows can elevate the amounts of total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), and GSH-PX [48]. The results of a survey indicated that vitamin D is a potent antioxidant factor in cell membranes. Therefore, administration of vitamin D declines the levels of malondialdehyde (MDA) and thiobarbituric acid reactive substance (TBARS), which are indicators of oxidative damage to cells [49].

John's disease or paratuberculosis is an inflammatory disease of the guts caused by *Mycobacterium avium* subsp. paratuberculosis. According to research by Sorge et al., there is a direct relationship between the severity of the disease and vitamin D levels. They found a significant difference between the vitamin D levels of healthy and sick cows. They mentioned three explanations for it. The first reason, cows with lower vitamin D levels are more susceptible to paratuberculosis infection. The second reason is that in the development of paratuberculosis, the absorption of vitamin D from the intestine is decreased. The last reason is that most of the vitamin D in the body is used to modulate the hyperactive immune response in paratuberculosis. The prevalence pattern of this disease is similar to Crohn's disease in humans and the incidence is high in coordinates with less radiation [26].

Requirements

Dairy cattle gain the required vitamin D, either by eating forages that contain vitamin D2 and consuming vitamin D3 supplements or from direct sun exposure, which produces vitamin D3 endogenously [51,

52]. Cattle can get significant amounts of vitamin D2 from forages such as alfalfa, which contains 2,500 IU of vitamin D2 /Kg of DM, and silage, which contains 500 IU of vitamin D2 /Kg of DM [53, 54]. However, vitamin D3 is the main form in blood circulation [55]. Due to the inefficient metabolism of vitamin D2 [56, 57] and raising cows indoors away from sunlight, the likelihood of vitamin D deficiency is high [26]. Thus, NRC recommends administering 21,000 IU/d vitamin D3 in dairy cattle to maintain 25(OH) D3 levels between 20 and 50 ng/mL and regulate calcium and phosphorus homeostasis [58]. Although the amount of vitamin D intake in most dairy cows is about 1.5 to 2.5 times the amount recommended by the NRC, the average vitamin D is about 60 to 70 ng/mL [32]. Dairy cows also experience a decrease in vitamin D levels in the postpartum period. At early lactation, cows are more susceptible to oxidative stress and metabolic disorders, which is probably due to vitamin D insufficiency or deficiency. A threshold of 30 ng/ml has been suggested in human studies to improve immune functions but is not yet conclusive [59]. In cattle, the optimal amount of vitamin D has not been determined for the proper functioning of the immune system. Nelson et al. (2012) reported that vitamin D improves performance in macrophages of the immune system *in vitro* up to 100 ng/ml. For instance, there was no difference between calves with 175 ng of 25-(OH) D3 and those with 30 ng of 25-(OH) D3 against the respiratory syncytial virus (RSV) [6].

Deficiency

Vitamin D deficiency in cattle along with calcium and phosphorus imbalance causes rickets in calves due to lack of calcium deposition in the growing bone matrix and osteomalacia in adult cows due to calcium loss from developed bone [60]. Clinical symptoms of vitamin D deficiency include loss of appetite, gastrointestinal upset, stiffness in gait, severe weakness, difficulty in breathing, irritability, and sometimes tetany and seizures. Swelling and erosions on joints lead to difficulty in motion, arching of the back, and bending of the legs [60, 61]. Calves born to mothers with vitamin D deficiency may be malformed, weak, or even dead [62]. The metacarpal and metatarsal bones begin to thicken, and as the disease progresses, the anterior limbs bend forward or to the sides. In advanced cases of vitamin D deficiency, long bone deformity occurs as a result of normal muscle tension. Beading appearance occurs at the junction of the ribs in the sternum due to the enlargement of bone and accumulation of cartilage [61]. Eating is difficult due to the softness and thickening of the mandible. In older cattle, the bones are very fragile, which can lead to posterior paralysis with vertebral fractures. Decreased milk production and lack of estrus are observed in vitamin D deficient dairy cattle [58]. The probability of vitamin D deficiency in beef cattle is very low unless a diet poor in vitamin D is consumed and housed away from sunlight. In this case, the symptoms of deficiency appear in less than 6 to 10 months [64]. In general, calving rates are very low in deficient herds, and newborns are often very weak and die immediately after birth [60].

Milk fever is a metabolic disorder that occurs due to excessive demand for calcium periparturient period [52, 65]. Milk fever begins about 3 days after parturition and continues with depression, general paralysis, circulatory collapse, coma, and death. The most important feature of the disease is a decrease in calcium levels to values between 3 to 7 mg/dl [12]. Milk fever is more likely to occur in older cows than in heifers [66]. Older cows show reduced production and reduced response to calcitriol. There are also fewer osteoclasts to respond to calcitriol and increased plasma calcium levels through bone dissolution [65]. They also have lower levels of 1-α hydroxylase [52, 65]. We also face with decreased number of VDRs and the activity of osteoblasts in the periparturient period [67, 68]. But a low-calcium, adequate- phosphorus prepartum diet followed with a high calcium diet postpartum can prevent milk fever [52]. A low-calcium diet induces calcitriol production through PTH [69, 70].

Toxicity

Vitamin D toxicity may occur due to overfeeding with calcinogenic herbs or taking high doses of vitamin D supplements, leading to calcification in soft tissues. 400 ng/ mL of vitamin D in plasma could be safe [71, 72]. According to the NRC recommendation, cows can tolerate 2200 IU D3/kg for 60 days and 2,500 IU D3/kg for shorter periods. Hibbs et al. determined that for the inhibition of milk fever without any toxicity feeding with high doses of vitamin D could be more effective than parental administration. Administration of 20 to 30 million IUD2 for 3 to 8 days prepartum was able to reduce 80% of milk fever cases while prolonging the duration of treatment to 20 days prepartum led to toxicity [73]. Calcinogenic plants include Solanum malacoxylon, Cestrum diurnum, Trisetum flavescens, and Nierembergia veitchii. These plants contain 1, 25-$(OH)_2$ D3 or its glycosides. These glycosides are activated through microbial digestion in the rumen. Clinical signs of calcinosis include weight loss, increased respiratory rate, tachycardia, impaired mobility, fertility problems, and decreased survival. However, some calcinogenic plants can be useful in preventing hypocalcemia [26].

Conclusion

In summary, it should be noted that beyond its classical roles in calcium and phosphorus homeostasis, vitamin D is an immunomodulatory agent and has protective effects against oxidative stress. These functions are important in preventing numerous diseases, especially peripartum diseases in cattle. Therefore in future studies, it is essential to determine the optimal concentration of vitamin D for the best function of the immune system and reduction of oxidative stress that minimizes the economic burden of disease in the dairy cattle industry.

Authors' Contributions

SAS: Investigation; Writing-original draft; MM: Conceptualization; Supervision; Writing-review & editing.

Competing Interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

References

1. DeLuca HF. History of the discovery of vitamin D and its active metabolites. BoneKEy reports. 2014; 3: 479.
2. Dobson RC, Ward G. Vitamin D physiology and its importance in dairy cattle: a review. Journal of dairy science. 1974;57 (9):985-91.
3. Norman AW, Roth J, Orci L. The vitamin D endocrine system: steroid metabolism, hormone receptors, and biological response (calcium binding proteins). Endocrine Reviews. 1982; 3(4):331-66.
4. Norman AW. Vitamin D: the calcium homeostatic steroid hormone. 1st ed. New York: Academic Press, 1979.
5. Jones G, Prosser DE, Kaufmann M. 25-Hydroxyvitamin D-24-hydroxylase (CYP24A1): its important role in the degradation of vitamin D. Archives of biochemistry and biophysics. 2012; 523(1):9-18.
6. Nelson CD, Merriman KE. Vitamin D metabolism in dairy cattle and implications for dietary requirements. 25th Annual Florida Ruminant Nutrient Symposium. 2014: 78-90.
7. Dittmer KE, Thompson KG. Vitamin D metabolism and rickets in domestic animals: a review. Veterinary Pathology. 2011 ;48(2):389-407.
8. Holick MF, Chen TC, Lu Z, Sauter E. Vitamin D and skin physiology: AD-lightful story. Journal of Bone and Mineral Research. 2007; 22(S2):V28-33.
9. Norman AW. Sunlight, season, skin pigmentation, vitamin D, and 25-hydroxyvitamin D: integral components of the vitamin D endocrine system. 1998: 1108-1110.
10. Hagenau T, Vest R, Gissel TN, Poulsen CS, Erlandsen M, Mosekilde L, Vestergaard P. Global vitamin D levels in relation to age, gender, skin pigmentation and latitude: an ecologic meta-regression analysis. Osteoporosis International. 2009;20(1):133-40.
11. Holick MF. The cutaneous photosynthesis of previtamin D3: a unique photoendocrine system. Journal of Investigative Dermatology. 1981;77(1):51-8.
12. DSM. Vitamin D. https://www.dsm.com/anh/en_US/products/vitamins/vitamin-nutrition-compendium/ruminants/vitamin-d.html. 2021/28/5
13. Carpenter KJ, Zhao L. Forgotten mysteries in the early history of vitamin D. The Journal of nutrition. 1999;129(5):923-7.
14. Hymøller L, Jensen SK. Vitamin D3 synthesis in the entire skin surface of dairy cows despite hair coverage. Journal of dairy science. 2010; 93(5):2025-9.
15. Wikvall K. Cytochrome P450 enzymes in the bioactivation of vitamin D to its hormonal form. International journal of molecular medicine. 2001 Feb 1;7(2):201-9. Wikvall K. Cytochrome P450 enzymes in the bioactivation of vitamin D to its hormonal form. International journal of molecular medicine. 2001; 7(2):201-9.
16. Holick M.F. Vitamin D and Health: Evolution, Biologic Functions, and Recommended Dietary Intakes for Vitamin D. In: Holick M. (eds) Vitamin D. Nutrition and Health. Humana Press. 2010.
17. Horst RL, Goff JP, Reinhardt TA. Adapting to the transition between gestation and lactation: differences between rat, human and dairy cow. Journal of mammary gland biology and neoplasia. 2005; 10(2):141-56.
18. Christakos S, Dhawan P, Porta A, Mady LJ, Seth T. Vitamin D and intestinal calcium absorption. Molecular and cellular endocrinology. 2011; 347(1-2):25-9.
19. Christakos S, Lieben L, Masuyama R, Carmeliet G. Vitamin D endocrine system and the intestine. BoneKEy reports. 2014; 3: 496.
20. Holick MF. Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. The American journal of clinical nutrition. 2004; 80(6):1678S-88S.
21. Anderson PH, Atkins GJ. The skeleton as an intracrine organ for vitamin D metabolism. Molecular aspects of medicine. 2008; 29(6):397-406.
22. Suda T, Ueno Y, Fujii K, Shinki T. Vitamin D and bone. Journal of cellular biochemistry. 2003; 88(2):259-66.

23. Goltzman D, Mannstadt M, Marcocci C. Physiology of the calcium-parathyroid hormone-vitamin D axis. Vitamin D in Clinical Medicine. 2018;50:1-3.

24. Nagpal S, Na S, Rathnachalam R. Noncalcemic actions of vitamin D receptor ligands. Endocrine reviews. 2005; 26(5):662-87.

25. DeLuca HF. Overview of general physiologic features and functions of vitamin D. The American journal of clinical nutrition. 2004; 80(6):1689S-96S.

26. Hodnik JJ, Ježek J, Starič J. A review of vitamin D and its importance to the health of dairy cattle. Journal of Dairy Research. 2020; 87(S1):84-7.

27. Colotta F, Jansson B, Bonelli F. Modulation of inflammatory and immune responses by vitamin D. Journal of autoimmunity. 2017 Dec 1;85:78-97.

28. Merriman KE, Kweh MF, Powell JL, Lippolis JD, Nelson CD. Multiple β-defensin genes are upregulated by the vitamin D pathway in cattle. The Journal of steroid biochemistry and molecular biology. 2015; 154:120-9.

29. Merriman KE, Poindexter MB, Kweh MF, Santos JE, Nelson CD. Intramammary 1, 25-dihydroxyvitamin D3 treatment increases expression of host-defense genes in mammary immune cells of lactating dairy cattle. The Journal of steroid biochemistry and molecular biology. 2017; 173:33-41.

30. Selsted ME, Tang YQ, Morris WL, McGuire PA, Novotny MJ, Smith W, Henschen AH, Cullor JS. Purification, primary structures, and antibacterial activities of beta-defensins, a new family of antimicrobial peptides from bovine neutrophils. Journal of Biological Chemistry. 1993; 268(9):6641-8.

31. Lippolis JD, Reinhardt TA, Sacco RA, Nonnecke BJ, Nelson CD. Treatment of an intramammary bacterial infection with 25-hydroxyvitamin D 3. PLoS One. 2011; 6(10):e25479.

32. Nelson CD, Lippolis JD, Reinhardt TA, Sacco RE, Powell JL, Drewnoski ME, O'Neil M, Beitz DC, Weiss WP. Vitamin D status of dairy cattle: Outcomes of current practices in the dairy industry. Journal of dairy science. 2016; 99(12):10150-60.

33. Martinez N, Rodney RM, Block E, Hernandez LL, Nelson CD, Lean IJ, Santos JE. Effects of prepartum dietary cation-anion difference and source of vitamin D in dairy cows: Health and reproductive responses. Journal of dairy science. 2018;101(3):2563-78.

34. Davies CJ, Hill JR, Edwards JL, Schrick FN, Fisher PJ, Eldridge JA, Schlafer DH. Major histocompatibility antigen expression on the bovine placenta: its relationship to abnormal pregnancies and retained placenta. Animal reproduction science. 2004; 82:267-80.

35. Kimura K, Goff JP, Kehrli Jr ME, Reinhardt TA. Decreased neutrophil function as a cause of retained placenta in dairy cattle. Journal of dairy science. 2002; 85(3):544-50.

36. Elliott L, McMahon KJ, Gier HT, Marion GB. Uterus of the cow after parturition: bacterial content. American Journal of Veterinary Research. 1968; 29(1):77-81.

37. Nelson CD, Reinhardt TA, Lippolis JD, Sacco RE, Nonnecke BJ. Vitamin D signaling in the bovine immune system: a model for understanding human vitamin D requirements. Nutrients. 2012; 4(3):181-96.

38. Dębski B, Nowicki T, Zalewski W, Ochota M, Mrowiec J, Twardoń J. Evaluation of acute phase proteins in clinically healthy dairy cows in perinatal period and during lactation. Polish journal of veterinary sciences. 2016; 19(3): 519-523.

39. Heaney RP, Armas LA. Quantifying the vitamin D economy. Nutrition reviews. 2015; 73(1):51-67.

40. Holcombe SJ, Wisnieski L, Gandy J, Norby B, Sordillo LM. Reduced serum vitamin D concentrations in healthy early-lactation dairy cattle. Journal of dairy science. 2018; 101(2):1488-94.

41. Delvin EE, Gagnon L, Arabian A, Gibb W. Influence of calcitriol on prolactin and prostaglandin production by human decidua. Molecular and cellular endocrinology. 1990;71(3):177-83.

42. Díaz L, Martínez-Reza I, García-Becerra R, González L, Larrea F, Méndez I. Calcitriol stimulates prolactin expression in non-activated human peripheral blood mononuclear cells: breaking paradigms. Cytokine. 2011;55(2):188-94.

43. Ingalls WG, Convey EM, Hafs HD. Bovine serum LH, GH, and prolactin during late pregnancy, parturition and early lactation. Proceedings of the Society for Experimental Biology and Medicine. 1973;143(1):161-4.

44. Akersr MR, Bauman DE, Capuco AV, Goodman GT, Tucker AH. Prolactin regulation of milk secretion and biochemical differentiation of mammary epithelial cells in periparturient cows. Endocrinology. 1981;109(1):23-30.

45. Macias H, Hinck L. Mammary gland development. Wiley Interdisciplinary Reviews: Developmental Biology. 2012; 1(4):533-57.

46. Reinhardt TA, Stabel JR, Goff JP. 1, 25-dihydroxyvitamin D3 enhances milk antibody titers to Escherichia coli J5 vaccine. Journal of dairy science. 1999; 82(9):1904-9.

47. Defrance T, Vanbervliet B, Briere F, Durand I, Rousset F, Banchereau J. Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. The Journal of experimental medicine. 1992; 175(3):671-82.

48. Xu HJ, Jiang X, Zhang CR, Ma GM, Wang LH, Zhang QY, Zhang YG. Effects of dietary 25-hydroxyvitamin D3 on the lactation performance, blood metabolites, antioxidant and immune function in dairy cows. Livestock Science. 2021; 248:104497.

49. Wiseman H. Vitamin D is a membrane antioxidant Ability to inhibit iron-dependent lipid peroxidation in liposomes compared to cholesterol, ergosterol and tamoxifen and relevance to anticancer action. FEBS letters. 1993; 326(1-3):285-8.

50. Hassanabadi M, Mohri M, Seifi HA. Effects of single injection of vitamin D3 on some immune and oxidative stress characteristics in transition dairy cows. Iranian Journal of Veterinary Science and Technology. 2020; 12(2):25-35.

51. Hymøller L, Jensen SK. 25-Hydroxycholecalciferol status in plasma is linearly correlated to daily summer pasture time in cattle at 56 N. British journal of nutrition. 2012;108(4):666-71.

52. Horst RL, Goff JP, Reinhardt TA. Calcium and vitamin D metabolism in the dairy cow. Journal of dairy science. 1994;77(7):1936-51.

53. Wallis GC, Kennedy GH, Fishman RH. The vitamin D content of roughages. Journal of Animal Science. 1958;17(2):410-5.

54. Horst RL, Reinhardt TA, Russel JR, Napoli JL. The isolation and identification of vitamin D2 and vitamin D3 from Medicago sativa (alfalfa plant). Archives of Biochemistry and Biophysics. 1984; 231(1):67-71.

55. Horst RL, Littledike ET. Comparison of plasma concentrations of vitamin D and its metabolites in young and aged domestic animals. Comparative biochemistry and physiology. B, Comparative biochemistry. 1982; 73(3):485-9.

56. Hymøller L, Jensen SK. Vitamin D2 impairs utilization of vitamin D3 in high-yielding dairy cows in a cross-over supplementation regimen. Journal of dairy science. 2011; 94(7):3462-6.

57. Sommerfeldt JL, Napoli JL, Littledike ET, Beitz DC, Horst RL. Metabolism of orally administered [3H] ergocalciferol and [3H) cholecalciferol by dairy calves. The Journal of nutrition. 1983; 113(12):2595-600.

58. National Research Council. Nutrient Requirements of Dairy Cattle. 7th Revised Edition. NATIONAL ACADEMY PRESS, Washington, D.C. 2001.

59. F Gunville C, M Mourani P, A Ginde A. The role of vitamin D in prevention and treatment of infection. Inflammation & Allergy-Drug Targets (Formerly Current Drug Targets-Inflammation & Allergy)(Discontinued). 2013; 12(4):239-45.

60. Frye TM, Williams SN, Graham TW. Vitamin deficiencies in cattle. Veterinary Clinics of North America: food animal practice. 1991;7(1):217-75.

61. National Research Council. Nutrient Requirements of Dairy Cattle. 6th Revised Edition. NATIONAL ACADEMY PRESS, Washington, D.C. 1996.

62. Rupel IW, Bohstedt G, Hart EB. Vitamin D in the nutrition of the dairy calf. Agricultural Experiment Station, University of Wisconsin. Research bulletin. 1933; No. 115.

63. Nelson CD, Nonnecke BJ, Reinhardt TA, Waters WR, Beitz DC, Lippolis JD. Regulation of Mycobacterium-specific mononuclear cell responses by 25-hydroxyvitamin D 3. PLoS One. 2011 Jun 28;6(6):e21674.

64. Wallis GC. Vitamin-D deficiency in dairy cows. Research Bulletins of the South Dakota Agricultural Experiment Station (1887-2011). 372.

65. Goff JP, Reinhardt TA, Horst RL. Enzymes and factors controlling vitamin D metabolism and action in normal and milk fever cows. Journal of Dairy Science. 1991; 74(11):4022-32.

66. Shappell NW, Herbein JH, Deftos LJ, Aiello RJ. Effects of dietary calcium and age on parathyroid hormone, calcitonin and serum and milk minerals in the periparturient dairy cow. The Journal of nutrition. 1987; 117(1):201-7.

67. Goff JP, Reinhardt TA, Horst RL. Milk fever and dietary cation-anion balance effects on concentration of vitamin D receptor in tissue of periparturient dairy cows. Journal of dairy science. 1995; 78(11):2388-94.

68. Naito Y, Shindo N, Sato R, Murakami D. Plasma osteocalcin in preparturient and postparturient cows: Correlation with plasma 1, 25-dihydroxyvitamin D, calcium, and inorganic phosphorus. Journal of Dairy Science. 1990; 73(12):3481-4.

69. Kichura TS, Horst RL, Beitz DC, Littledike ET. Relationships between prepartal dietary calcium and phosphorus, vitamin D metabolism, and parturient paresis in dairy cows. The Journal of Nutrition. 1982; 112(3):480-7.

70. Green HB, Horst RL, Beitz DC, Littledike ET. Vitamin D metabolites in plasma of cows fed a prepartum low-calcium diet for prevention of parturient hypocalcemia. Journal of Dairy Science. 1981;64(2):217-26.

71. Tomkins NW, Elliott R, McGrath JJ, Schatz T. Managing plasma P concentrations in beef heifers with a slow release vitamin D supplementation. Animal Production Science. 2020; 60(5):610-7.

72. Celi P, Williams S, Engstrom M, McGrath J, La Marta J. Safety evaluation of dietary levels of 25-hydroxyvitamin D3 in growing calves. Food and Chemical Toxicology. 2018; 111:641-9.

73. Hibbs JW, Conrad HR. Milk fever in dairy cows. VII. Effect of continuous vitamin D feeding on incidence of milk fever. Journal of dairy science. 1976; 59(11):1944-6.

74. Waters WR, Nonnecke BJ, Rahner TE, Palmer MV, Whipple DL, Horst RL. Modulation of Mycobacterium bovis-specific responses of bovine peripheral blood mononuclear cells by 1, 25-dihydroxyvitamin D3. Clinical and diagnostic laboratory immunology. 2001; 8(6):1204-12.

How to cite this article
Ahmadi Sheikhsarmast S, Mohri M. New outlook to vitamin D functions in dairy cows: non- classical roles. Iran J Vet Sci Technol. 2021;13(2): 1-13.
DOI: https://doi.org/10.22067/ijvst.2021.70874.1051
URL: https://ijvst.um.ac.ir/article_40175.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Apr- 28
Accepted after revision: 2021- Jul- 12
Published online: 2021- Dec-01

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2021.69966.1038

The decreasing effect of troxerutin on the level of pro-inflammatory cytokines in rats with sepsis caused by the experimental cecal puncture

Ali Shaker[a], Siamak Kazemi-Darabadi[a], Yaser Jafari-Khataylou[b]

[a] Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran.
[b] Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran.

ABSTRACT

Sepsis is the main mortality factor in patients undergoing surgery and its treatment currently includes cardiac resuscitation and reducing the immediate risk of infection. Troxerutin is a common compound in vegetables, fruits, and seeds and has several biological activities, including anti-platelet, anti-serotonin, antioxidant, and anti-inflammatory effects. Accordingly, we hypothesized that it can decrease interleukin 1 (IL-1) and tumor necrosis factor-alpha (TNF-α) levels in the serum of rats with sepsis. Twenty-four adult male Sprague-Dawley rats were used in this study. The rats were equally and randomly divided into 3 groups: sham operation group, control group, and treatment group. Both the control and treatment groups underwent surgical cecal ligation and perforation. Troxerutin (130 mg/kg) was injected subcutaneously twice a day to the animals of the treatment group for 3 days or until the animals' death. Surviving rats were euthanized after 1.5 ml of blood samples were taken 3 days after the cecal ligation and perforation. IL-1 and TNF-α were measured by the blood serum ELISA assay. The differences in mortality rates were significant between the control group and the other two groups ($p = 0.008$). The results showed a significant increase in IL-1 and TNF-α in the control group compared to the sham group ($p < 0.05$). In addition, the levels in the treatment group significantly decreased compared to the control group ($p < 0.05$). In conclusion, our results indicate that troxerutin could increase survival in rats developing septic shock by reducing pro-inflammatory cytokines including IL-1 and TNF-α.

Keywords

Immune response, laboratory animals, peritonitis, septic shock, troxerutin

Number of Figures: 3
Number of Tables: 1
Number of References:: 45
Number of Pages: 8

Abbreviations

CLP: Cecal ligation and perforation
IL-1: Interleukin 1
IL-1β: Interleukin 1-beta
TNF-α: Tumor necrosis factor-alpha
IL-6: Interleukin 6
IL-12: Interleukin 12
IFN-γ: Interferon-gamma
NO: Nitric oxide

Corresponding author:
Siamak Kazemi-Darabadi
Email: s.kazemi@tabrizu.ac.ir
Tel: +98 (41)3637-8743

Introduction

Sepsis is caused by bacteria or their endotoxins and is the main contributor to mortality in patients undergoing surgery. When endotoxins enter the body, significant changes occur in the immune response and organ function [1]. Endotoxins are the lipopolysaccharide membrane of gram-negative bacteria. They are released during bacterial cell lysis, creating a strong response in the body that leads to various components of immune cell activation with subsequent septic shock [2]. The interaction of lipopolysaccharides with receptors on different cells induces the production of many pro-inflammatory factors including interferon-gamma (IFN-γ) nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), as well as interleukins 1-beta (IL-1β), 6 (IL-6), and 12 (IL-12) [3]. Studies have shown that TNF-α and IL-1 are the first cytokines to be produced in large quantities in response to lipopolysaccharides, and this is the main cause of the many effects of lipopolysaccharides. These cytokines and chemokines enhance the host response to bacterial infection [4]. In particular, TNF-α, which is released by macrophages, acts as a stimulant for cytokine cascade and ultimately leads to lethal septic shock. However, anti-inflammatory cytokines are also abundant during sepsis. The latter cytokines reduce pro-inflammatory cytokines production so that they could help the animal to overcome sepsis and endotoxin-related shock to some extent [3]. However, if cytokines are released too much, they could have detrimental consequences for the body. Recent studies have shown that large amounts of cytokines, particularly IL-1, TNF-α, and IFN-γ, are produced in septic shock and related injury, which mediate many of the detrimental effects of septic shock [5].

Treatment for sepsis currently includes cardiac resuscitation and management of the immediate risk of infection. Intravenous fluid therapy, vasopressor medications, and oxygen therapy are the main resuscitation options. Immediate intravenous antibiotic therapy is performed to reduce potential pathogens. The only currently available immunotherapeutic treatment is the short-term use of hydrocortisone in patients with resistant septic shock [6]. The use of corticosteroids in septic shock has been extensively studied [7]. Preliminary studies have shown that high doses of corticosteroids are not helpful for septic shock and can be harmful. In a study on 499 patients, hydrocortisone did not improve survival in patients with septic shock, and it was concluded that discontinuation of corticosteroids if the patient is unresponsive to treatment should be considered due to the potential risks of infection, hypoglycemia, and myopathy [8].

The failure to find new effective treatments, together with advances in finding the biological characteristics of sepsis, has been one of the greatest desperations of the past few decades [9]. Two types of materials could be used for this purpose: materials that disrupt the primary cytokine cascade (such as anti-inflammatory cytokines and anti-lipopolysaccharides) and those that prevent dysregulated coagulation (including activated protein C and antithrombin) [10]. Activated protein C was recently approved despite being withdrawn from the market by the manufacturer due to concerns about safety and efficacy [11]. There is currently no comprehensive evidence for the effectiveness of anti-cytokines in the treatment of sepsis [6].

Troxerutin (C33H42O19), or vitamin P4, is a compound derived from rutin flavonoid. This substance is available in vegetables, fruits, and seeds with a range of biological activities [12]. It has anti-serotonin and anti-platelet properties. The use of troxerutin in the treatment of vascular diseases such as phlebitis or capillary hemorrhage has been extensively studied [13,14]. Antioxidant and anti-inflammatory effects of troxerutin have also been reported in other studies. It crosses the blood-brain barrier and thus affects the central nervous system. Troxerutin can prevent the activation of nuclear factor kappa B (NF-κB) signaling [15]. NF-κB could increase pro-inflammatory cytokines expression and intensify the inflammatory response. It has been suggested that the anti-inflammatory effect of troxerutin in ischemia/reperfusion injuries of diabetic myocardium may be because of a reduction in TNF-α activity, resulting in NF-κB blocking [16]. A recent in vivo study suggests that troxerutin reverses the inflammatory response by inhibiting elastase. This protease activates by TNF-α and contributes to inflammation [17]. Due to the good solubility of troxerutin in water, it is easily absorbed from the gastrointestinal tract and exerts its protective effects on tissues without cell toxicity [18]. Troxerutin has a potential effect on the treatment of diabetes mellitus and Alzheimer's disease, which is partly due to its antioxidant activity. It has been reported that it reduces damage to various tissues such as the brain, liver, and kidneys by improving anti-

Abbreviations-Cont'd

NF-κB: Nuclear factor kappa B
ELISA: Enzyme-Linked Immunosorbent Assay
TMB: Tetramethylbenzidine
ANOVA: Analysis of variance
SEM: Standard error of the mean
S: Sham
C: Control
T: Treatment

oxidant levels [19]. The immunoprotective and anti-aging effects of troxerutin are also known [20]. Consequently, we hypothesized that troxerutin might prevent elevated serum levels of IL-1 and TNF-α serum levels in rats with sepsis.

Results

None of the rats of the sham (S) group (0%, n = 0) died during the 3 days. 25% (n = 2) of the animals in the control (C) group died a day after surgery and 37.5% (n = 3) died after two days, thus, only 37.5% (n = 3) in this group survived after 3 days. In addition, the remaining rats were secluded and lethargic, with no desire to move or eat. Lack of grooming and response to the external stimuli were the other clinical symptoms. These manifestations indicated peritonitis-induced septic shock [21]. Only 12.5% (n = 1) of the treatment (T) group died on postoperative day 2 and 87.5% (n = 7) of them survived. Survivors were alert and ambulatory. The differences in mortality rates were significant between groups C and the other two groups ($p = 0.008$). Because the survived rats were euthanized 3 days after cecal ligation and perforation (CLP), we considered these animals as censored after experiment termination. Therefore, the mean survival days presented in Table 1 are calculated for 3 days after CLP, and the actual survival of the animals cannot be determined. As seen in Table 1, most of the control group rats died during the experiment, and they also died earlier (with lower survival days) than the rats in the treatment group.

The results of pro-inflammatory cytokines analysis showed a significant increase in IL-1 and TNF-α in group C (1438.66 ± 23.81 pg/ml and 1358.42 ± 21.89 pg/ml, respectively) compared to the group S (17.32

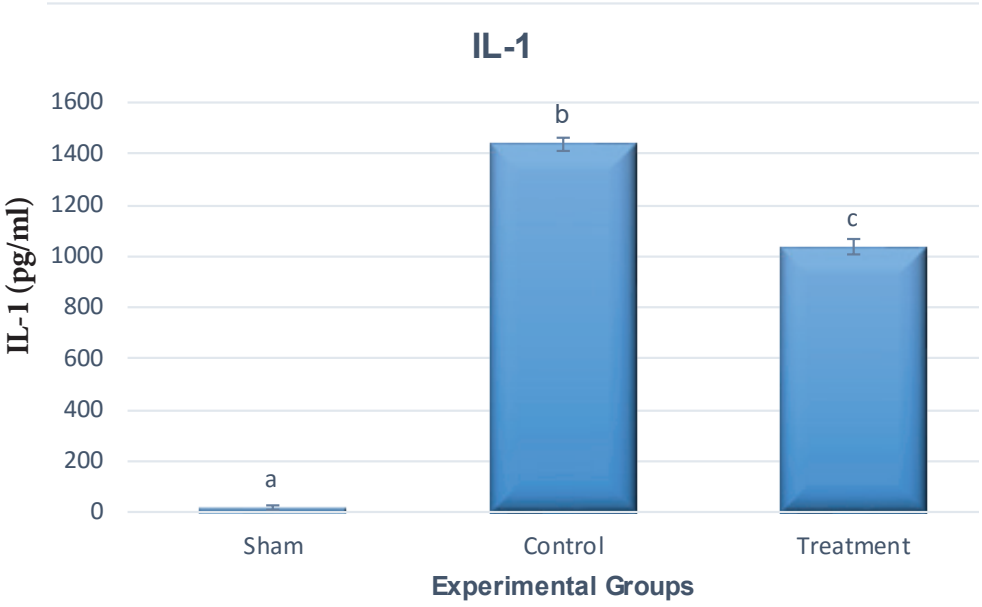


Figure 1.
IL-1 levels (mean ± SEM) in the serum of rats in three experimental groups. Different letters indicate a significant difference ($p < 0.05$).

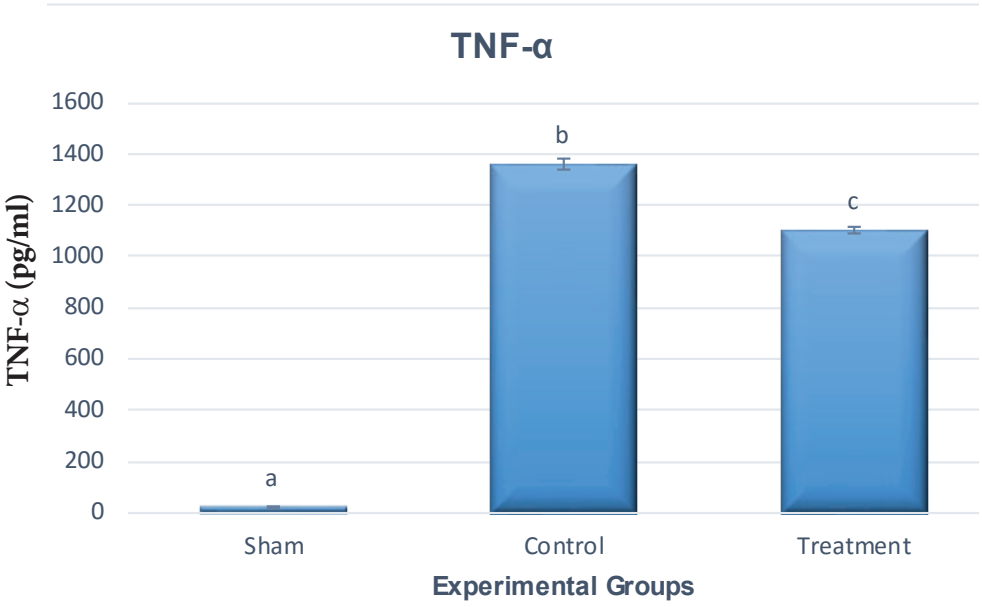


Figure 2.
TNF-α levels (mean ± SEM) in the serum of rats in three experimental groups. Different letters indicate a significant difference ($p < 0.05$).

± 7.93 pg/ml and 20.31 ± 4.72 pg/ml, respectively) ($p < 0.05$). In addition, the measured cytokine levels in group T (1036 ± 27.72 pg/ml and 1100.42 ± 15.22 pg/ml, respectively) were significantly lower than in those in group C ($p < 0.05$) (Figures 1 and 2).

Table 1.
Survival days and survial rates of the rats in different experimental groups 3 days after cecal ligation and perforation (CLP).

Groups	Survival days (mean ± SEM)	Survival rate (%)
Sham	3.00 ± 0.00	100
Control	2.12 ± 0.29*	37.5*
Treatment	2.87 ± 0.12	87.5

*: significant difference between groups ($p < 0.05$).

Discussion

Although short-term mortality in patients with septic shock has decreased in recent years, it remains a major problem. The mortality rate could vary from 35% to 70%, depending on several factors such as age, gender, comorbidities, acute respiratory involvement, or renal failure [22]. However, there is very little data for the long-term mortality in patients with septic shock. One report estimated that about 20% of hospital survivors die within the first year [23]. The short-term mortality rate in our study was also 62.5% in the control group which is in line with other studies [24,25,26] but the survival of the treatment group was significantly improved and the short-term mortality in this group was significantly improved by 12.5%. This indicates that troxerutin could prevent septic shock-related death, at least in the early stages of peritonitis development. Severe septic shock affects the central nervous system and causes drowsiness or delirium. Non-focal encephalopathy, polyneuropathy, and myopathy are common findings in these patients [6]. This could explain why the surviving rats in group C were recluse and lethargic while the others were alert and ambulatory.

Although severe inflammation was previously believed to be the cause of the clinical symptoms of sepsis, Bone et al. (1997) reported that compensatory anti-inflammatory response syndrome can arise from an initial inflammatory response [27]. On the other hand, we currently know that a more complex and prolonged host response is provoked by infection. Both the infection elimination and the tissue recovery, and secondary infection can be the result of pro-and anti-inflammatory mechanisms [28]. Pro-inflammatory responses to kill invading pathogens are liable for tissue damage, organ dysfunction, and early mortality, while anti-inflammatory reactions that limit local and systemic tissue damage are involved in the development of secondary infections in sepsis [6]. Some types of receptors (namely C-type lectin, toll-like, nucleotide-binding oligomerization domain-like, and retinoic acid-inducible gene 1–like receptors) could bind to bacterial lipopolysaccharides, resulting in immune cell activation and upregulation of inflammatory gene transcription [29] and the release of pro-inflammatory cytokines including IL-1 and TNF-α [3]. These cytokines stimulate the synthesis of phospholipase A2, inducible cyclooxygenase, 5-lipoxygenase, and acetyltransferase, which contribute to the synthesis of prostaglandins and leukotrienes and platelet-activating factor which lead to further inflammation, vasomotor tone alteration, and increasing blood flow and vascular permeability [22]. Additionally, these cytokines attract monocytes, neutrophils, T cells, and macrophages to the area to confront infection, but they also play a role in the pathogenesis of severe inflammation at the same time. Tamayo et al. (2011) reported that both pro-and anti-inflammatory (IL-10) cytokines are present in septic shock [30].

Troxerutin reduces enzymes and proteins inside cells including cyclooxygenase in some tissues [31]. The anti-inflammatory properties of troxerutin in a wide variety of organs such as kidneys, liver, brain, and heart are well understood. For instance, Najafi et al. (2018) found that troxerutin prevents myocardial arrhythmias in rats by inhibiting inflammatory cytokines and reducing inflammation arising from ischemia/reperfusion [16]. More recently, Jafari-Khataylou and coworkers (2021) reported that troxerutin reduced inflammation and histopathological lesions and improved antioxidant activity and survival rate in mice injected with lipopolysaccharides [32]. Despite some methodological differences, our results are in agreement with this study. Although their study is the only report available in the literature on the influence of troxerutin on peritoneal sepsis, there is extensive data on its protective and anti-inflammatory properties in a wide variety of organs. Shan et al. (2018) had previously found that this substance improves kidney function against inflammatory damage by blocking certain signaling pathways [33]. The results of Hoseinidoost and coworkers (2019) suggested that troxerutin can hinder the adverse effects of maternal high fat diet on their offspring through pro-inflammatory cytokines inhibition [34]. Lu et al. (2013) had recommended troxerutin for the prevention and therapy of cognitive deficits resulting from brain inflammatory response [35]. Zhang and coworkers (2015) found that troxerutin can be beneficial in the prevention and treatment of liver inflammation

[36]. All of the reports mentioned had emphasized the ameliorating effect of troxerutin against inflammatory reactions. Troxerutin could mediate its anti-inflammatory function by changing inflammation-related microRNAs (miRs) expression, including miR-146a and miR-155 [37]. A study by Yavari et al. (2016) in diabetic rats demonstrated that the anti-inflammatory effect of troxerutin on the NF-κB-mediated pathway is related to its effect on the miR-146a restoration [38].

Our results are also in accordance with other studies that investigate the effects of other natural substances in peritonitis or septic shock. Ozer and coworkers (2010) reported that *Nuphar lutea* leaf extract (NUP) has an anti-inflammatory effect in two acute septic shock models in mice by inhibiting the NF-κB pathway, modulating cytokine production and ERK phosphorylation [39]. Qin et al. (2016) investigated the effects of Micheliolide, a sesquiterpene lactone isolated from *Michelia compressa* with anti-inflammatory effects in the acute peritonitis mouse model and found that this plant terpenoid inhibits lipopolysaccharide-induced inflammatory response via NF-κB and PI3K/Akt pathways [40]. More recently, the anti-inflammatory effect of Xuebijing, a Chinese herbal medicine, in murine CLP model has been reported [41]. Accordingly, it seems that natural products may play a significant role in ongoing and future studies on discovering new treatments for peritonitis and septic shock.

We did not use laboratory diagnostics or organ dysfunction tests to confirm peritonitis, which could be a limitation of this study. It is now accepted that polymorphonuclear leukocytes in the peritoneal fluid are the cornerstone of diagnosing peritonitis, while the role of other biochemical tests is quite controversial [42]. However, we used a standard animal model for peritonitis and subsequent septic shock. In addition, the cytokines measured in the control group convinced us that peritonitis and septic shock had occurred in the rats. Clinical manifestations also demonstrated this condition.

In summary, our results demonstrate that troxerutin could increase patient survival in rats developing peritonitis and septic shock by reducing pro-inflammatory cytokines including IL-1 and TNF-α. Further studies are needed to find out whether troxerutin also prevents the synthesis of other anti-inflammatory cy-

Materials & Methods

Animals

this research was approved by the Regional Research Ethics Committee of the University of Tabriz (approval ID: IR.TABRIZU.REC.1398.008). The study complies with the Declaration of Helsinki (DoH). Twenty-four adult (2 months of old) male Sprague-Dawley rats were used in this study. The animals were given separate cages. The environment had a 12-hour light/dark cycle and controlled temperature and humidity conditions. The rats were allowed to stay in their new place for a week to acclimate, with free access to the semi-synthetic pellets and tap water. Individual rats were randomly divided into three equal groups (n = 8) based on online random number generator (available at:https://www.graphpad.com/quickcalcs/randomN1.cfm) including sham (S), control (C), and treatment (T).

Experimental sepsis and treatment

General anesthesia was induced by intraperitoneal injection of 10% ketamine (100 mg/kg, Alfasan, Woerden, Netherlands) and 2% xylazine (20 mg/kg, Alfasan, Woerden, Netherlands) in all rats, and their ventral abdomen was shaved from the xiphoid process of the sternum to the pubis. Celiotomy was performed through routine midline skin and linea alba incisions. The cecum of group S was just exteriorized, manipulated, and returned to the peritoneal cavity. In contrast, cecal ligation and perforation (CLP) was performed in groups C and T. For this purpose, the cecum was identified and exteriorized from the peritoneal cavity. Next, a simple ligature was placed just below the ileocecal valve using 3-0 silk suture material (SUPA, Tehran, Iran). The procedure was completed by piercing the cecum twice with an 18 G needle [43,44] (Figure 3a). Slight pressure was applied to the perforated cecum to ensure leakage of its contents (Figure 3b). The cecum was returned into the peritoneal cavity and the abdominal wall and skin were closed in two layers.

Troxerutin (Merck, Darmstadt, Germany) was dissolved in sterile normal saline and injected subcutaneously into the animals of group T twice daily at a dose of 130 mg/kg [20] for 3 days or until the animals died. At the same time, the rats in groups S and C received the same volume of normal saline solution. The animals were closely monitored for their general status and all symptoms related to peritonitis and septic shock (including loss of appetite and lethargy) were noted [45]. Because rats died on different days after surgery, mean survival days were also calculated in addition to the ultimate survival rate. The longer the mean survival days, the longer the animals survive in the three days of the study. On the other hand, lower survival days indicate that the rats have died earlier in that period. However, the survival rate indicates the percentage of animals that were still alive after 3 days of the experiment, without reflecting when the lost ones have died. Surviving rats were generally anesthetized and weighed 3 days after CLP. They were euthanized under deep general anesthesia after taking approximately 1.5 ml of blood samples from their heart and subjected to a subsequent cervical dislocation. Blood samples were also taken from dying rats on day 2 immediately after death.

Estimation of cytokine levels by Enzyme-Linked Immunosorbent Assay (ELISA)

Blood samples were collected from individual rats in Eppendorf tubes and allowed to clot by leaving them undisturbed at room temperature for about 30-60 minutes. The clot was removed by centrifuging at 3200 rpm for 10 minutes. The resulting supernatant serum was kept in a refrigerator at -80 °C for future ELISA assay for pro-inflammatory cytokines IL-1 and TNF-α. The Sandwich ELISA was carried out according to the manufacturer's instructions (Bender Med Systems GmbH, Vienna, Austria). Briefly, the capture antibodies specific for IL-1 and TNF-α of the rat were coated on 96-well plates. The plates were incubated overnight at 4 °C. The day after, the plates were washed 5 times with the buffer solution (0.01 M PBS, pH = 7.0 and 0.05% Tween 20). They were blocked for one hour with assay diluent of the kit and washed again 5 times. After the samples and the respective standards had been poured into the wells, the plates were incu-

bated for two hours at room temperature. In the next step, the antibodies were added. Then, the detection antibodies were added for one hour. The wells were washed again. Avidin-HRP was added for 30 minutes and washed. Adding TBM substrate and 15 minutes of incubation developed the color. Finally, the reaction was stopped with 2N sulfuric acid and the amount of absorbance was determined at 540 nm using a microplate reader (Hiperion, Model: MPR4+, Medizintechnik GmbH & Co. KG, Roedermark, Germany). A standard graph was used to calculate the relative levels of cytokines in the samples. The examiner was blind about the grouping of the samples.

Statistical analysis

The data were analyzed with the Minitab statistical software (version 16.2.0, Minitab Inc, State College, PA, USA). The examination of normal distribution was accomplished with the Kolmogorov-Smirnov test and the assumption of equal variance by Levene's test. Cytokines data were analyzed with one-way analysis of variance (ANOVA) followed by *Tukey's* post hoc test. Survival was analyzed with the *Chi*-Square method using the survival analysis tool of the software. Results are presented as percentage (%) for survival rate and mean ± standard error of the mean (SEM) for survival days and ELISA tests. The *p*-value less than 0.05 was considered statistically significant.

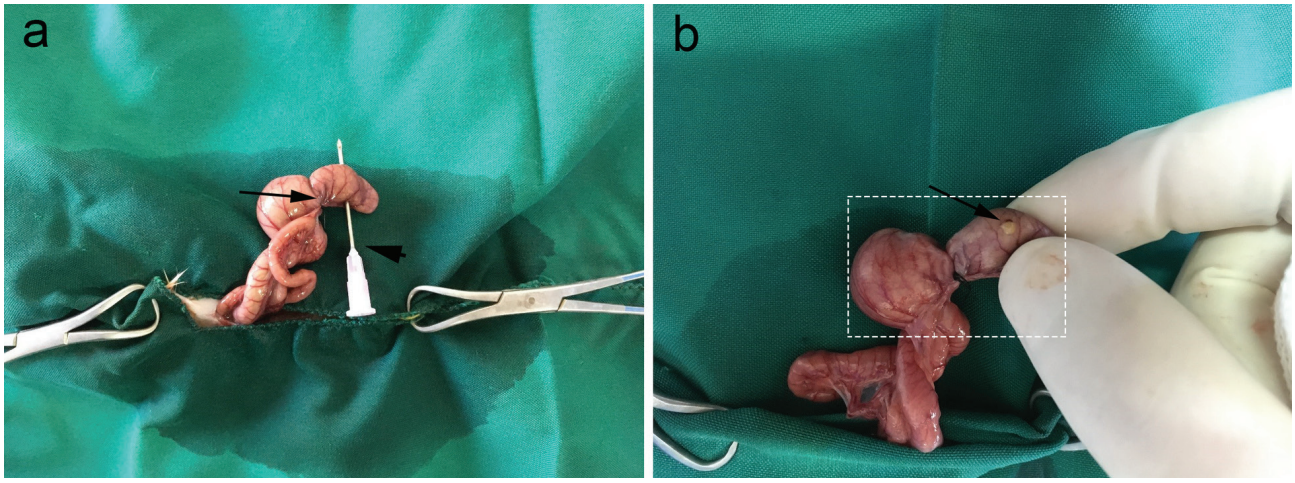


Figure 3.
a. Cecal ligation and perforation (CLP) in rats of control and treatment groups. A simple ligature is placed just below the ileocecal valve (arrow) using 3-0 silk suture material after exteriorizing the cecum from the peritoneal cavity and the cecum is perforated twice by an 18 G needle (arrowhead).
b: Slight pressure is applied to the perforated cecum of the rat to ensure leakage of its contents (arrow) after cecal ligation and perforation. The rectangle shows the cecum.

Authors' Contributions

S.K.D. and Y.J.K. conceived and planned the experiments. S.K.D. and A.S. carried out the experiments. S.K.D. and A.S. planned and carried out the simulations. Y.J.K and A.S. contributed to sample preparation. S.K.D. and Y.J.K. contributed to the interpretation of the results. S.K.D. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript

Acknowledgements

The authors would like to express their gratitude to Vice-Chancellor for Research and Technology, University of Tabriz, for approving and funding this research.

Competing Interests

The authors declare that there is no conflict of interest.

References

1. Yeh SL, Lai YN, Shang HF, Lin MT, Chen WJ. Effects of glutamine supplementation on innate immune response in rats with gut-derived sepsis. British Journal of Nutrition. 2004; 91(3):423-9.

2. Marton A, Kolozsi C, Kusz E, Olah Z, Letoha T, Vizler C, Pecze L. Propylene-glycol aggravates LPS-induced sepsis through production of TNF-α and IL-6. Iranian Journal of Immunology. 2014; 11(2):113-22.

3. Carrillo-Vico A, Lardone PJ, Naji L, Fernández-Santos JM, Martín-Lacave I, Guerrero JM, Calvo JR. Beneficial pleiotropic actions of melatonin in an experimental model of septic shock in mice: regulation of pro-/anti-inflammatory cytokine network, protection against oxidative damage and anti-apoptotic effects. Journal of Pineal Research. 2005; 39(4):400-8.

4. Zhang D, Chen L, Li S, Gu Z, Yan J. Lipopolysaccharide (LPS) of Porphyromonas gingivalis induces IL-1β, TNF-α and IL-6 production by THP-1 cells in a way different from that of Escherichia coli LPS. Innate Immunity. 2008; 14(2):99-107.

5. Polat G, Ugan RA, Cadirci E, Halici Z. Sepsis and Septic

Shock: Current Treatment Strategies and New Approaches. The Eurasian Journal of Medicine. 2017; 49(1):53-8.

6. Angus DC, Van der Poll T. Severe sepsis and septic shock. New England Journal of Medicine. 2013; 369:840-51.

7. Mason PE, Al-Khafaji A, Milbrandt EB, Suffoletto BP, Huang DT. Corticus: The end of unconditional love for steroid use? Critical Care. 2009; 13(4):309.

8. Sprung CL, Annane D, Keh D, Moreno R, Singer M, Freivogel K, Weiss YG, Benbenishty J, Kalenka A, Forst H, Laterre PF. Hydrocortisone therapy for patients with septic shock. New England Journal of Medicine. 2008; 358(2):111-24.

9. Angus DC. The search for effective therapy for sepsis: back to the drawing board? Journal of the American Medical Association. 2011; 306(23):2614-5.

10. Webster NR, Galley HF. Immunomodulation in the critically ill. British Journal of Anaesthesia. 2009; 103(1):70-81.

11. Ranieri VM, Thompson BT, Barie PS, Dhainaut JF, Douglas IS, Finfer S, Gårdlund B, Marshall JC, Rhodes A, Artigas A, Payen D. Drotrecogin alfa (activated) in adults with septic shock. New England Journal of Medicine. 2012; 366(22):2055-64.

12. Dehnamaki F, Karimi A, Pilevarian AA, Fatemi I, Hakimizadeh E, Kaeidi A, Allahtavakoli M, Rahmani MR, Khademalhosseini M, Bazmandegan G. Treatment with troxerutin protects against cisplatin-induced kidney injury in mice. Acta Chirurgica Belgica. 2019; 119(1):31-7.

13. Schuller-Petrovic S, Wolzt M, Böhler K, Jilma B, Eichler HG. Studies on the effect of short-term oral dihydroergotamine and troxerutin in patients with varicose veins. Clinical Pharmacology & Therapeutics. 1994; 56(4):452-9.

14. Badalzadeh R, Layeghzadeh N, Alihemmati A, Mohammadi M. Beneficial effect of troxerutin on diabetes-induced vascular damages in rat aorta: histopathological alterations and antioxidation mechanism. International Journal of Endocrinology and Metabolism. 2015; 13(2):e25969.

15. Gui Y, Li A, Chen F, Zhou H, Tang Y, Chen L, Chen S, Duan S. Involvement of AMPK/SIRT1 pathway in anti-allodynic effect of troxerutin in CCI-induced neuropathic pain. European Journal of Pharmacology. 2015; 769:234-41.

16. Najafi M, Noroozi E, Javadi A, Badalzadeh R. Anti-arrhythmogenic and anti-inflammatory effects of troxerutin in ischemia/reperfusion injury of diabetic myocardium. Biomedicine & Pharmacotherapy. 2018; 102:385-91.

17. Vidhya R, Anuradha CV. Anti-inflammatory effects of troxerutin are mediated through elastase inhibition. Immunopharmacology and Immunotoxicology. 2020; 42(5):423-35.

18. Babri S, Mohaddes G, Feizi I, Mohammadnia A, Niapour A, Alihemmati A, Amani M. Effect of troxerutin on synaptic plasticity of hippocampal dentate gyrus neurons in a β-amyloid model of Alzheimer s disease: an electrophysiological study. European Journal of Pharmacology. 2014; 732:19-25.

19. Shan Q, Zhuang J, Zheng G, Zhang Z, Zhang Y, Lu J, Zheng Y. Troxerutin reduces kidney damage against BDE-47-induced apoptosis via inhibiting NOX2 activity and increasing Nrf2 activity. Oxidative Medicine and Cellular Longevity. 2017.

20. Wang Y, Wei S, Chen L, Pei J, Wu H, Pei Y, Chen Y, Wang D. Transcriptomic analysis of gene expression in mice treated with troxerutin. PloS One. 2017; 12(11):e0188261.

21. Redondo-Calvo FJ, Montenegro O, Padilla-Valverde D, Villarejo P, Baladrón V, Bejarano-Ramírez N, Galán R, Gómez LA, Villasanti N, Illescas S, Morales V. Thiosulfinate-Enriched Allium sativum Extract as an Adjunct to Antibiotic Treatment of Sepsis in a Rat Peritonitis Model. Applied Sciences. 2021; 11(11):4760.

22. Annane D, Bellissant E, Cavaillon JM. Septic shock. Lancet. 2005; 365(9453):63-78.

23. Annane D, Sébille V, Charpentier C, Bollaert PE, François B, Korach JM, Capellier G, Cohen Y, Azoulay E, Troché G, Chaumet-Riffaut P. Effect of treatment with low doses of hydrocortisone and fludrocortisone on mortality in patients with septic shock. Journal of the American Medical Association. 2002; 288(7):862-71.

24. Temmesfeld-Wollbrück B, Brell B, Dávid I, Dorenberg M, Adolphs J, Schmeck B, Suttorp N, Hippenstiel S. Adrenomedullin reduces vascular hyperpermeability and improves survival in rat septic shock. Intensive Care Medicine. 2007; 33(4):703-10.

25. Taniguchi T, Kurita A, Kobayashi K, Yamamoto K, Inaba H. Dose-and time-related effects of dexmedetomidine on mortality and inflammatory responses to endotoxin-induced shock in rats. Journal of Anesthesia. 2008; 22(3):221-8.

26. Chang L, Du JB, Gao LR, Pang YZ, Tang OS. Effect of ghrelin on septic shock in rats. Acta Pharmacologica Sinica. 2003; 24(1):45-9.

27. Bone RC, Grodzin CJ, Balk RA. Sepsis: a new hypothesis for pathogenesis of the disease process. Chest. 1997; 112(1):235-43.

28. Van der Poll T, Opal SM. Host–pathogen interactions in sepsis. Lancet Infectious Diseases. 2008; 8(1):32-43.

29. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010; 140(6):805-20.

30. Tamayo E, Fernández A, Almansa R, Carrasco E, Heredia M, Lajo C, Goncalves L, Gómez-Herreras JI, de Lejarazu RO, Bermejo-Martin JF. Pro-and anti-inflammatory responses are regulated simultaneously from the first moments of septic shock. European Cytokine Network. 2011; 22(2):82-7.

31. Zhang ZF, Fan SH, Zheng YL, Lu J, Wu DM, Shan Q, Hu B. Troxerutin protects the mouse liver against oxidative stress-mediated injury induced by D-galactose. Journal of Agricultural and Food Chemistry. 2009; 57(17):7731-6.

32. Jafari-Khataylou Y, Emami SJ, Mirzakhani N. Troxerutin attenuates inflammatory response in lipopolysaccharide-induced sepsis in mice. Research in Veterinary Science. 2021; 135:469-78.

33. Shan Q, Zheng GH, Han XR, Wen X, Wang S, Li MQ, Zhuang J, Zhang ZF, Hu B, Zhang Y, Zheng YL. Troxerutin protects kidney tissue against BDE-47-induced inflammatory damage through CXCR4-TXNIP/NLRP3 signaling. Oxidative Medicine and Cellular Longevity. 2018;1-11.

34. Hoseindoost M, Alipour MR, Farajdokht F, Diba R, Bayandor P, Mehri K, Rad SN, Babri S. Effects of troxerutin on inflammatory cytokines and BDNF levels in male offspring of high-fat diet fed rats. Avicenna Journal of Phytomedicine. 2019; 9(6):597.

35. Lu J, Wu DM, Zheng YL, Hu B, Cheng W, Zhang ZF, Li MQ. Troxerutin counteracts domoic acid–induced memory deficits in mice by inhibiting CCAAT/enhancer binding protein β–mediated inflammatory response and oxidative stress. The Journal of Immunology. 2013; 190(7):3466-79.

36. Zhang ZF, Zhang YQ, Fan SH, Zhuang J, Zheng YL, Lu J, Wu DM, Shan Q, Hu B. Troxerutin protects against 2, 2', 4, 4'-tetrabromodiphenyl ether (BDE-47)-induced liver inflammation by attenuating oxidative stress-mediated NAD+-depletion. Journal of Hazardous Materials. 2015; 283:98-109.

37. Liu F, Huang Y, Fu P. Involvement of inflammation-related microRNAs: miR-155 and miR-146a in diabetic nephropathy: Implication in inflammation mediated endothelial injury. Nephrology Dialysis Transplantation. 2012; 27:34-5.

38. Yavari R, Badalzadeh R, Alipour MR, Tabatabaei SM. Modulation of hippocampal gene expression of microRNA-146a/microRNA-155-nuclear factor-kappa B inflammatory signaling by troxerutin in healthy and diabetic rats. Indian Journal of Pharmacology. 2016; 48(6):675.

39. Ozer J, Levi T, Golan-Goldhirsh A, Gopas J. Anti-inflammatory effect of a Nuphar lutea partially purified leaf extract in murine models of septic shock. Journal of Ethnopharmacology. 2015; 161:86-91.

40. Qin X, Jiang X, Jiang X, Wang Y, Miao Z, He W, Yang G, Lv Z, Yu Y, Zheng Y. Micheliolide inhibits LPS-induced inflammatory response and protects mice from LPS challenge. Scientific Reports. 2016; 6(1):1-3.

41. Chen X, Feng Y, Shen X, Pan G, Fan G, Gao X, Han J, Zhu Y. Anti-sepsis protection of Xuebijing injection is mediated by differential regulation of pro-and anti-inflammatory Th17 and T regulatory cells in a murine model of polymicrobial sepsis. Journal of Ethnopharmacology. 2018; 211:358-65.

42. Lippi G, Danese E, Cervellin G, Montagnana M. Laboratory diagnostics of spontaneous bacterial peritonitis. Clinica Chimica Acta. 2014; 430:164-70.

43. Hubbard WJ, Choudhry M, Schwacha MG, Kerby JD, Rue III LW, Bland KI, Chaudry IH. Cecal ligation and puncture. Shock. 2005; 24:52-7.

44. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA. Immunodesign of experimental sepsis by cecal ligation and puncture. Nature Protocols. 2009; 4(1):31-6.

45. Murando F, Peloso A, Cobianchi L. Experimental abdominal sepsis: sticking to an awkward but still useful translational model. Mediators of Inflammation. 2019:1-8.

How to cite this article
Shaker A, Kazemi-Darabadi S, Jafari-Khataylou Y. The decreasing effect of troxerutin on pro-inflammatory cytokines levels in rats with sepsis caused by the experimental cecal puncture. Iran J Vet Sci Technol. 2021; 13(2): 12-19.
DOI: https://doi.org/10.22067/ijvst.2021.69966.1038
URL: https://ijvst.um.ac.ir/article_40440.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Jul- 06
Accepted after revision: 2021- Aug- 10
Published online: 2021- Dec-01

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2021.71247.1057

Parasitological and pathological findings of coccidiosis in an experimental infection caused by *Eimeria ahsata* in lambs

Nader Ahmadi Saleh Baberi[a], Iraj Karimi[b], Hossein Nourani[c], Hamidreza Azizi Alavije[b], Gholamreza Razmi[c]

[a] Graduate student of the Veterinary Parasitology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[b] Department of Pathobiology, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran.
[c] Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

ABSTRACT

This study was conducted to investigate the pathogenesis process of *E. ahsata* and its morphological, pathological, and distribution of lesions in the involved tissues during 42 days of infection. Twelve lambs were randomly divided into two groups including the control and the infected groups after confirmation of their health. The animals in the experiment group were orally infected with 1×10^5 sporulated oocysts. From 7 days after inoculation (DAI), the feces were sampled and oocysts per gram of feces (OPG) were individually examined for each lamb. . At 7, 14, 21, 28, 35, and 42 DAI, one lamb from each group was necropsied and the abomasum, small and large intestine, mesenteric lymph nodes, spleens, and livers were grossly investigated. From 21 to 42 DAI, mild to severe clinical lesions such as congestion and edema were seen on the mucosal surface of the small intestine associated with white and small foci about 1-2 mm, especially jejunum and ileum. From 7 DAI to the end of the study various stages of the parasite life cycle, infiltration of inflammatory cells, epithelial hyperplasia of villi, and destruction of villi epithelium were seen. The results showed that *E. ahsata* was pathogenic in lambs and the macro and microscopic lesions were mostly seen in the jejunum.

Keywords

Eimeria ahsata; Sheep; Pathology; OPG

Number of Figures: 6
Number of Tables: 2
Number of References:: 30
Number of Pages: 9

Abbreviations

OPG: oocysts per gram
DAI: days after inoculation
FS: fecal score
DPI: days post inoculation

Corresponding author: *Gholamreza Razmi*
Email: *razmi@um.ac.ir*
Tel: *+98 (51)3876-3853*

Introduction

Coccidiosis is one of the most common parasitic diseases, caused by the genus *Eimeria spp.* in the alimentary system of sheep in the world [1,2]. The disease is more common in lambs aged 4-6 months [3]. The rearing stressor conditions such as weaning, transportation or transfer to a new pen, malnutrition, overcrowded population, and unsuitable weather play an important role in the incidence of the disease in sheep and goats [4]. The genus *Eimeria* causes the death of a large number of host intestinal cells and enterocytes leading to reduced absorbance of the critical electrolytes and nutrients [5]. The most common clinical signs of the disease are diarrhea, weight loss, anemia, rough hair coat, and weakness [5]. In addition, the disease reduces the production of meat and milk products and increases the mortality rate. The mechanism and grading of tissue damage depend on Eimeria species, the number of oocysts ingested, stress condition, age, physical condition, genetic sensitivity, and host immune system. Due to the sensitivity of young animals, the clinical form of the disease is reported at this age [6]. In the small ruminant, the induced hyperplasia by coccidia results in the thickness of the intestinal wall leading to poor absorbance of nutrients, diarrhea, and dehydration [4]. The mild to moderate histopathologic changes can be associated with the thickness of intestinal mucosa as well as the formation of the plague or the nodules with 1-2 mm in diameters in size [4]. In some *Eimeria* species, large schizonts are considerably seen. The most common clinical lesions of coccidiosis in young sheep and goats are non-pedunculated whitish nodules on the intestinal mucosa. These plaques are adhered to each other in advanced infection [4]. To date, 12 intestinal and 1 abomasal *Eimeria* species including *E. crandallis, E. bakuensis, E. weybridgensis, E. ovinoidalis, E. intricate, E. gilruthi, E. pallid, E. faurei, E.parva, E. marsica, E.granulose, E. bakuensis*, and *E. ahsata* have been identified in sheep [3].

Among the above species, *E. ovinoidalis*, *E. ahsata, E, crandallis,* and *E. bakuensis* are considered serious pathogens [3]. Regarding high prevalence of *E. ahsata* in sheep of different areas of Iran [7,8,9], this experimental study was conducted to study the pathogenesis of this parasite and to evaluate the morpho-pathology and distribution of lesions in lambs.

Results

Clinical signs

There were no clinical signs till 20 DAI in all animals. Diarrhea was the first clinical sign that was observed in two lambs at 21 DAI, which led to anorexia, weakness, dehydration, mucosal paleness and weight loss. The fecal samples consistency of these lambs were semiliquid (Fecal score=2) at 21 DAI and watery diarrhea (fecal score= 3) at 35 DAI (Table 1). No clinical signs were seen in other lambs in infected and control groups and fecal samples consistency were normal till the end of experiment.

OPG rate

The level of oocysts per gram of feces (OPG) of each lamb after the pre-patent period that was varying from 7 to 42 DAI are shown in Table 1. Two lambs with diarrhea had high OPG.

Body weight

Based on the results of the present study, the lambs in the case group had less weight gain than the control group and in the studied model, a significant difference was observed between the two groups ($p < 0.05$) (Table. 2).

Histopathological findings

Gross lesions. There was no considerable gross lesion in the sacrificed lambs at 7 and 14 DAI. Mild to severe lesions were seen in the jejunum and with less intensity in the ileum at 21 DAI. The congestion and edematous state of small intestine mucosa, especially, in the jejunum and ileum were identified. The small intestinal mucosal thickness associated with congestion and white nodules with 1-2 mm in diameter were detected on the internal surface of mucosal jejunum and ileum at 28 DAI. In addition to white nodules were creased on the small intestinal mucosal in particular jejunum at day 35 of infection (Figure 1).

At 42 DAI, the advance and diffuse glandular lesions associated with mucosal thickness and creasing from serosal surface of jejunum with less intensity in the ileum were noted (Figure 2). There was no gross lesion in the abomasum, liver, and spleen of all animals. The enlargement of mesenteric lymph nodes was the commonly detected lesion on all infected animals at 21, 28, 35, and 42 DAI. No gross lesions were observed in the gastrointestinal tract in lambs of the control group from the beginning to the end of the study.

Microscopic lesions. At 7 DAI, vascular congestion of mucosal and submucosal surfaces associated with different stages of the parasite life cycle including micro and macrogametes, and schizonts were seen in the jejunum and ileum. The epithelial and lymph tissues hyperplasia, infiltration of inflammatory cells such as eosinophilic cells in the lamina properia and villous tip as well as denuded villous tip and hyperplasia were seen in the ileum and jejunum.

At 14 DAI, the various stages of the parasite life

Table 1.
The changes of OPG and fecal score in each lamb of infected group during experimental infection with *E. ahsata* at a dose of 1×10^5

Lamb	7 DAI		14 DAI		21 DAI		28 DAI		35 DAI		42 DAI	
	OPG	FS	OPG	FS	OPG	FS	OPG	FS	OPG	FS	OPG	FS
1	0	1	-		-		-		-		-	
2	0	1	1200	1	-		-		-		-	
3	0	1	1000	1	38,000	2	-		-		-	
4	0	1	800	1	15,000	1	8400	1	-		-	
5	0	1	1000	1	33,000	2	30,000	3	45,000	3	-	
6	0	1	1000	1	10,000	1	6400	1	8000	1	3000	1

OPG: Oocyst per gram
DAI: Day(s) after infection
FS: Fecal score

cycle were seen in more parts of the small intestine. The vascular congestion of mucosal and submucosal, epithelial and lymph tissues hyperplasia, infiltration of inflammatory cells, and eosinophilic infiltration in the lamina properia and villous tip as well as denuded villous tip and hyperplasia was seen in the small intestine. The second-generation schizont and merozoites were detected within villi epithelial cells of the small intestine. Other stages of parasite life cycle such as progamonts, the developed micro and macrogametes, and a few oocysts were also detected within villi epithelial and crypts of duodenum, jejunum, and ileum. Most lesions were observed in the jejunum. A few first-generation schizonts were seen in the livers, spleens, and mesenteric lymph nodes.

Table 2.
Comparison of the body weight (Kg) of lambs in the infected and control groups

Weight (Kg)		
DPI	Control group	Test group
0	23.2 ± 0.87	23.60 ± 1.2
7	23.3 ± 0.62	23.7 ± 1.1
14	23.2 ± 0.53	22.32 ± 0.93
21	24.65 ± 0.85*	21.62 ± 1.13*
28	25.7 ± 1.04 *	21.66 ± 1.09*
35	27.2 ± 1.30	-
42	27.3 ± 1.32	-

*$p < 0.05$ was accepted as statistically significant.
DPI: days post inoculation

At 21 DAI, microscopic intestinal lesions were widely detected. Infiltration of lymphocytes and eosinophils in lamina properia with less intensity in small intestine submucosa. The various forms of the parasite including gamonts and a large number of oocysts were seen in different parts of the small intestine in particular the jejunum (Figure 3). The infiltration of lymphocytes was also seen in the ileum. The round micro containing a large number of basophilic and clear nuclei as well as the round macro-gametocytes containing a large number of eosinophilic granules were seen. There was no lesion in the cecum, colon, liver, spleen, and mesenteric lymph nodes.

At 35 DAI, The microscopic lesions were widely detected in the ileum and jejunum and some cases in the colon. The losses of epithelial surface, infiltration of eosinophils, micro and macrogametes and oocysts associated with lymphatic hyperplasia, mucosal thickness resulting from papillary hyperplasia, and infiltration of inflammatory cells especially eosinophils in the lamia properia were seen (Figure 4). Microscopically, nodular hyperplasia and noted white pulp were seen in the spleen. The liver and mesenteric lymph nodes were reported as normal and hyperplasic particularly in the cortex, respectively.

At 42 DAI, congested livers, spleen, and mesenteric lymph nodes were reported. In the jejunum, losses of the integrity of the lieberkühn gland, infiltration of inflammatory cells, micro and macrogametes, and the developed oocysts were seen. In the ileum, the presence of oocysts, villous epithelial hyperplasia, losses of integrity, infiltration of eosinophils, plasma cells, macrophages, micro, and macrogametes was seen (Figure 5). There was no lesion in the abomasum, rectum, and colon.

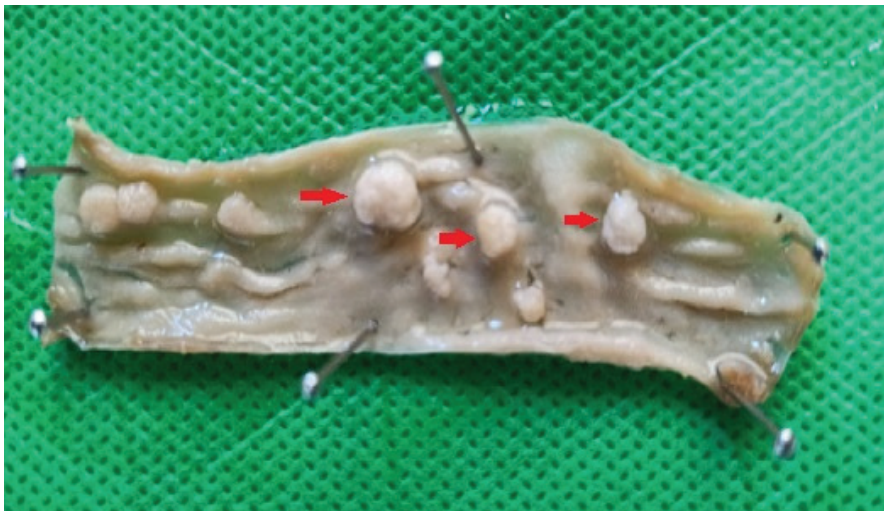


Figure 1.
The whitish nodules (arrows) on mucosal surface of the jejunum at 35 DAI

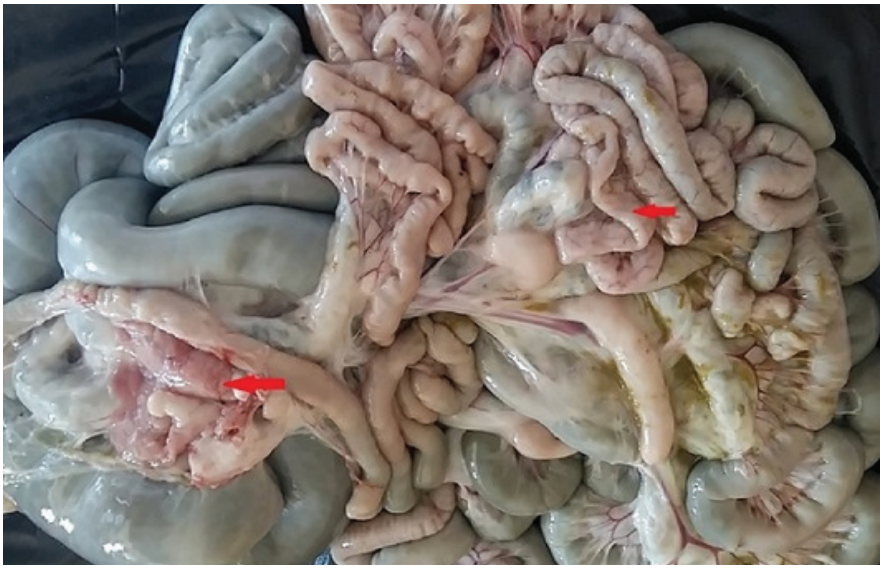


Figure 2.
Cerebriform or gyrate pattern and depressions on the serosal surface of jejunum (arrows) at 42 DAI.

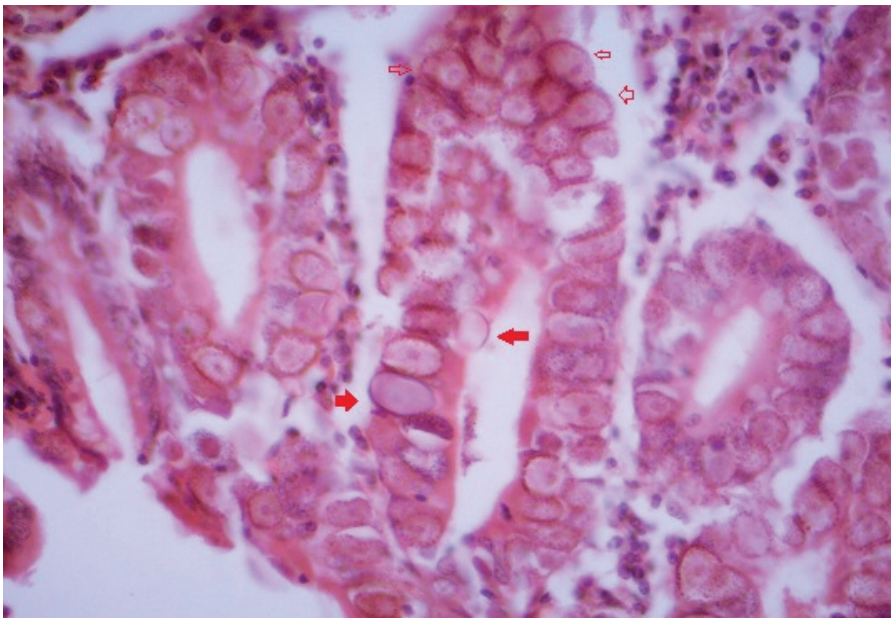


Figure 3
Histopatholoical section of jejunum at 21 DAI. There are a large number of gamonts (hollow arrows), and oocysts (soild arrows) within the epithelial tissue of intestinal glands. H&E, ×400

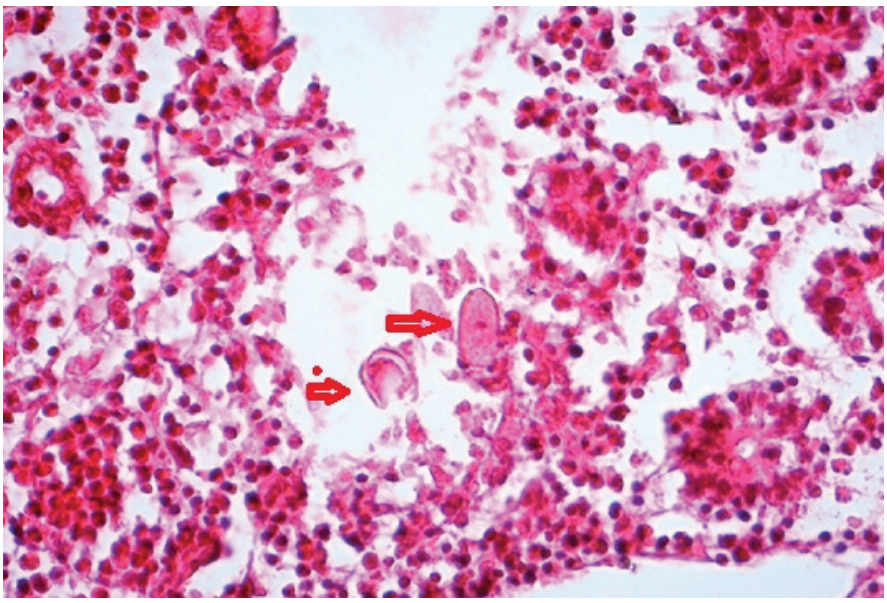


Figure 4
Histopathological section of jejunum at 35 DAI. The presence of oocysts (arrows) associated with infiltration of eosinophils and other inflammatory cells, creasing of villi. H&E, ×400

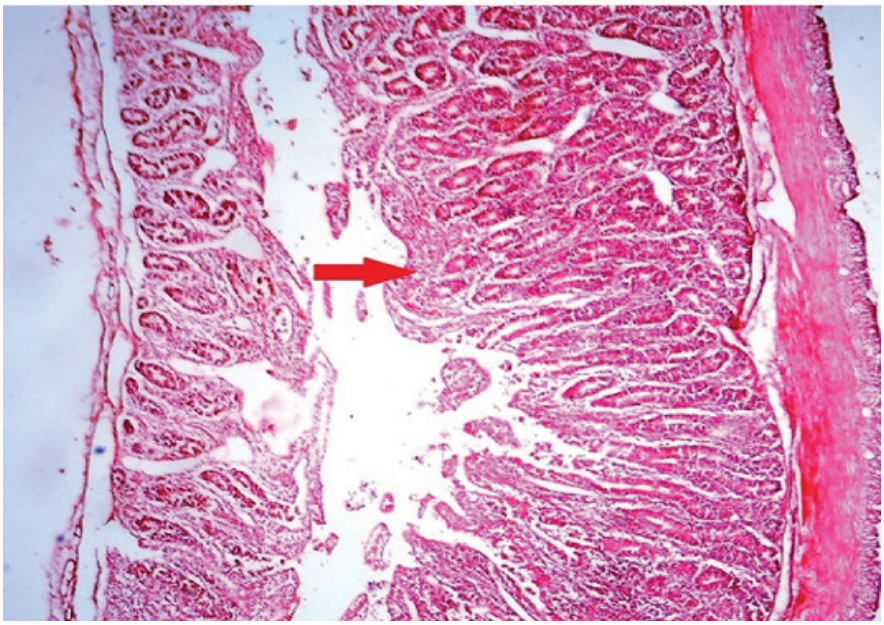


Figure 5.
Histology of the jejunum at 42 DAI with the presence of oocysts, infiltration of eosinophils and other inflammatory cells, associated with partial epithelial hyperplasia and hyperemia and hemorrhage (arrow). H&E, ×100

Discussion

Eimeria ahsata has been known as the most common Eimeria species in the sheep in Iran, Spain, and China [8,15,5]. In the present study, the pathogenicityof E. ahsata as one of the common species in lambswas experimentally investigated. The first detection of oocysts was at 14 DAI in fecal samples of the infected group which are consistent with the prepatent period of E. ahsata about 12 to 18 days in an experimental study [16]. Clinical signs appeared with diarrhea, anorexia, dehydration, and weakness in two infected lambs at 21 DAI. Few studies have been performed on the pathogenicity of E. ahsata. Smith et al (1960) showed that oral occultation with 1×105 E. ahsata oocysts caused diarrhea, loss of appetite, and listlessness at 15-16 DAI and death in some lambs at 23-32DAI[17]. Mahart and Sherrick (1965) showed the low pathogenicity of E. ahsata in feedlot lambs[18]. Cathpole et al (1976) compared the pathogenesis of four Eimeria species in lambs for 4 weeks. They reported no clinical signs in the lambs when E. ahsata oocysts were given 10 to 1000 oocysts per day in a week, whereas E. ovinoidalis caused diarrhea in lambs. The difference in clinical signs severity in experimental studies may be related to infective dose, age, sheep breed, infective dose, and concomitant in-

fections[1,19]. The OPG of diarrheic lambs was higher than non-diarrheic lambs, indicating a positive relationship between oocyst excretion rate and diarrhea. Many experimental and field studies have demonstrated a positive correlation between the total OPG of Eimeria and diarrhea in dairy cattle [13,20,21].

In the present study, the white to gray nodules and pinpoint to large nodules on the mucosal surface of the small intestine in particular jejunum and some parts of ileum were noted from at 21 DAI to the end of the study. Nodular lesions in the intestinal mucosa indicate the accumulation of different stages of the parasite that induced enterocyte hyperplasia in the intestinal wall[1]. The morphology and distribution

pattern of them throughout the intestine depends on the infected Eimeria species [3]. Cerebriform or gyrate patterns and depressions were seen on the serosal surface of the jejunum. This gross lesion was reported in ovine and caprine coccidiosis which resulted in the projection of proliferative nodules in intestinal mucosa toward the serosa intestine [3,22,23]. The nodule formation and thickening of the intestinal wall can cause a reduction in food absorption, diarrhea, and dehydration. emaciation, serous atrophy of fat [3].

Histopathological examination showed that E.ahsata mainly invaded the jejunum and the ileum. The main lesions were mild to severe hyperplasia epithelial cells with the presence of intracellular developmental stages of E.ahsata with infiltration of lymphocytes and

eosinophils in lamina properia. It seems that the presence of intracellular stages of the coccidia especially pro gamont is caused by hyperplastic mucosa in lambs [12]. Recent in vitro findings, have shown that crypt

cell hyperplasia is initiated by T-cell activation[24]. Intracellular parasites would represent an appropriate stimulus to responsive lymphocytes. An experimental study showed an increased intraepithelial lymphocytes population due to activation of T cells in the distal jejunum and ileum of lambs 13 days after infection with coccidian[25]. The infiltration of eosinophils in the small intestinal mucosa may be associated.

The obtained clinical and histopathological findingsindicate the pathogenicity of E. ahsata in the lambs. The lesions caused by E. ahsata were mostly seen in the jejunum. The gross lesions were congestion and white nodules on the internal surface of the mucosal jejunum and ileum. The presence of various stages of the parasite life cycle within the villi and crypts associated with infiltration of inflammatory cells in particular eosinophils and lymphocytes were seen histologically. It seems that the gametogenic stage consisting gametocytes and oocysts had a major role in the destruction of villi and crypts. with the release of histamine from damaged intestinal cells in parasitic infection. Other studies have been reported an ileoileal intussusception associated with proliferative [26]. The mild to severe hyperplasia in the crypt and villi of the small and large intestine in naturally occurring coccidiosis in sheep [26]. In addition,

necrosis, denuding of villi and intestinal gland

epithelium, congestion, infiltration of inflammatory cells associated with various stages of Eimeria such as micro and macrogamete and oocysts in the small intestinal mucosa has been also reported in infected lambs [27]. For comparison, similar microscopic lesions such as hyperplasia of epithelial cells of villi and crypts of jejunum and ileum, remarkable infiltration of lymphocytes and eosinophils have been reported in kids that experimentally infected by E. arloingi [22].

It seems that the tissue damage intensity depends on the Eimeria species, the number of the inoculated oocysts, host immune system, age genetic, nutrition, and stress [28,29].

The gain weight in the present study was significantly decreased in the infected group compared to the control group. The loss of body weight associated with clinical coccidiosis is mainly due to loss of nutrients as a result of parasite-induced mucosal lesions and, to a lesser degree, to alterations of intestinal digestion and absorption of nutrients [30]. Subclinical coccidiosis may also lead to reduced growth, uneven *lamb size, and a higher food conversion ratio [31].*

Materials & Methods

Isolation of E. ahsata

Before the start of the experiment, The fecal samples of 70 refereed lambs with diarrhea from the School of Veterinary Medicine were examined by the Mac-Master method [10]. A portion of each positive sample (3 gram) was mixed in 42 ml of phosphate buffer solution (PBS) and filtered through the sieve (Azman co. Iran) to omit the large particles. The filtered suspension was centrifuged at 2000 rpm for 5 min and the sediment was mixed with 2.5% (w/v) aqueous potassium dichromate solution (1:5) in Petri dishes and kept in a climate chamber. It was aerated continuously for twenty days at 27 ºC. The rate of sporulated oocysts was determined by microscopic examination when more than 90% of oocysts were sporulated, and they were stored at 4˚C until used. The frequency of the different *Eimeria* species with special regard to the pathogenic species was determined based on the morphological characteristics of the oocysts and related to the OPG counts[10,11]. Fifty-three samples were positive for *Eimeria spp*. The *E.ahsata* was the most prevalent (79%) species and other species included *E. ovinoidalis* (1%), *E. bakuensis* (1%), *E. granulosa* (2%), *E. crandalis* (7%), *E. faurei* (18%), and *E. intricata* (25%) less prevalent in the fecal samples of diarrheic sheep. The fecal samples containing 95 -100 % *E.ahsata* and more than 500 OPG were chosen for the experiment [11]. *E.ahsata* oocyst was ovoid with non-round polar cap, yellowish-brown, no residual body, and large steady body with sporocyst residuum, 33.4 × 22.6 µm

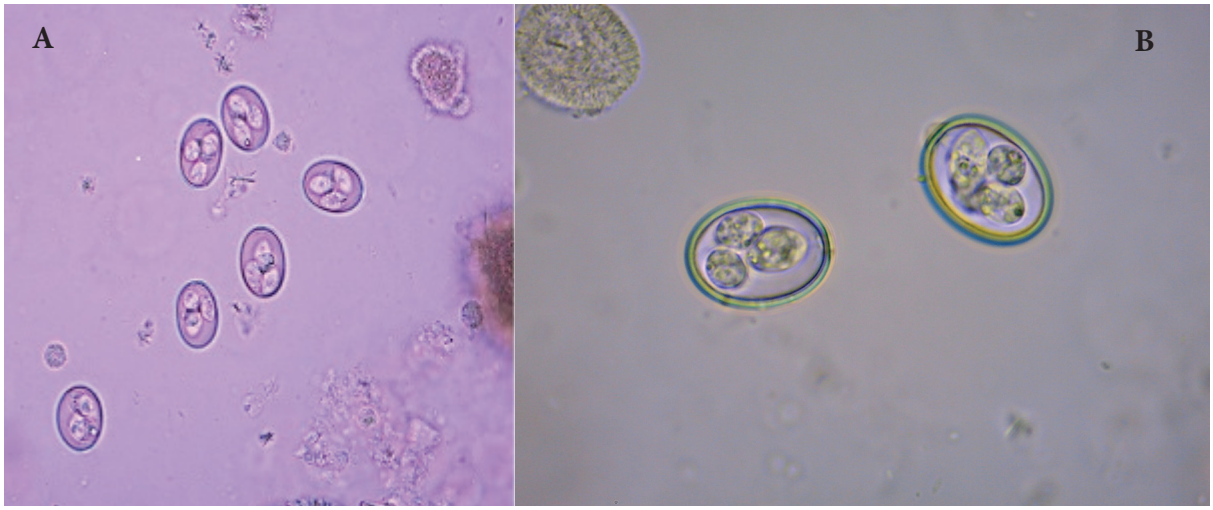


Figure 6.
A. Sporulated *E. ahsata* oocysts, ×100. B. Sporulated *E. ahsata* oocyst, ×400

(Figure 6).

Experimental examination

Twelve female lambs (Ovis aires), 2 months old were obtained from a non-infected herd raised under hygienic conditions. The lambs were transferred to the Research Center in the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad. After clinical examination and confirmation of their health, the lambs were placed in individual pens in a protective environment for three weeks, to adapt to the diet and new environment. Lambs were fed daily with a standard diet consisting of alfalfa hay and concentrate during the study period. In addition, the fecal samples of lambs were examined three times a week to ensure not to have coccidia. Thereafter, the coccidia-free lambs were randomly divided into two equal infected and control groups. Before inoculation of the lambs, the oocytes suspension was washed with PBS solution by repeated centrifugation at 2000 rpm for 5 min until removal of potassium dichromate. Finally, the volume of the sediment was increased to 300 ml by adding distilled water, and the number of oocysts was calculated for each ml of suspension by Mac- Master methods. A single inoculum of an aqueous suspension 1×10^5 sporulated oocysts (50 mL) was given to each lamb with a stomach tube [12]. The lambs of the control group received distilled water (50 mL per animal). During the study, clinical signs and parasitological findings including anemia, diarrhea, body condition, and OPG of each animal were evaluated. The fecal sample of each lamb was collected directly from the rectum in the morning at 7, 14, 21, 28, 35, and 42 DAI. The score of consistency of feces was assessed as follows: Normal to pasty (1), semiliquid to liquid (2), watery (3), hemorrhagic, and/or with tissue (4) [13]. In addition, the number of oocysts per gram (OPG) was counted using the Mac-Master method. Body weights were assessed by weighting each lamb on a scale of kilograms at 7, 14, 21, 28, 35, and 42 DAI before euthanasia.

Necropsy and histopathology

At 7, 14, 21, 28, 35, and 42 DAI, a lamb from each group randomly was euthanized by intravenous sodium pentobarbitone solution and underwent necropsy for clinical evaluation of elementary system[14]. For microscopic examination, the tissue samples from the duodenum, jejunum, ileum, cecum, colon, abomasum, mesenteric lymph nodes, liver, and spleen were harvested and fixed in 10 % formalin buffer (Merck, Germany). The samples from small and large intestines were chosen with 10 cm intervals from the beginning of the duodenum to the end of the colon for microscopic examination. The fixed samples were embedded in paraffin, sectioned at 5µm, and routinely stained using hematoxylin and eosin (H&E).

Statistical analysis

The SPSS software, version 22 (SPSS Inc., Chicago, USA) was used for data analysis. The *student's t*-test was used for investigating the effects of sampling time on the O.P.G and weight in two groups. *p*-values less than 0.05 were considered significant.

Ethics approval and consent to participate

The experiment on animals in the present study was approved by the Ethics Committee of Ferdowsi University of Mashhad (Approval ID: IR.UM.REC.1399.072).

Authors' Contributions

NASB: Methodology, Software, Formal analysis, investigation, Writing-Original draft preparation. IK: Supervision, Methodology, investigation, Resources, Writing- Reviewing and Editing. HN: Validation. HAA: Validation, Resources. GR: Supervision, Conceptualization, Visualization, Resources, Writing- Reviewing and Editing.

Acknowledgements

The research leading to these results was funded by a grant (No. 3.49852) from the Research Council of the Ferdowsi University of Mashhad, Iran

Competing Interests

The authors declare that they have no competing interests

References

1. Molina JM, Ruiz A. Coccidiosis in sheep. In: J.P.Dubey (Eds) .Coccidiosis in livestock, poultry, companion animals and human. Taylor & Francis Group: CRC Press; 2017.

2. Bangoura B, Bardsley KD. Ruminant Coccidiosis. Vet Clin North Am Food Anim Pract. 2020; 36: 187-203.

3. Taylor MA, Coop RL, Wall RL. Veterinary Parasitology 3rd ed. Blackwell Publishing, 2007.

4. Khodakaram-Tafti A, Hashemnia M. An overview of intestinal coccidiosis in sheep and goats. Revue Méd.Vét. 2017; 168: 1-3.

5. Wang CR, Xiao JY, Chen AH , Chen J, Wang Y, Gao JF, Zhu XQ. Prevalence of coccidial infection in sheep and goats in northeastern China.Vet.Parasitol. 2010; 174: 213-217.

6. Jolley WR, Bardsley KD. Ruminant coccidiosis. Vet. Clin. Food Anim. 2006; 22: 613-621.

7. Heidari H. Identification of Eimeria species in sheep of Hamedan suburb, Iran J Vet Res. 2011; 66: 165-167.

8. Hashemnia M, Rezaei F, Chalechale A, Kakaei S, Gheichivand S. Prevalence and intensity of Eimeria infection in sheep in western Iran. Int J Livest Res. 2014; 4:107-112.

9. Mirzaei M, Dahmardeh E. The Prevalence of Eimeria Species in Sheep in Zabol city, Iran. Iran Vet J. 2016; 11:98-105.

10. Eckert J, Taylor M, Catchpole J, D. Licois, P. Coudert, Bucklar H. Identification of Eimeria species and strains: Morphological characterization of oocysts. In:J. Eckert, R. Braun, M.W. Shirley, P. Coudert, P. (Eds) COST 89/820 Biotechnology—Guidelines on Techniques in Coccidiosis Research. European Commission Directorate-General XII, Science, Research and Development: Agriculture Biotechnology, Luxembourg, 1995. pp.103–119.

11. Joachim A, Altreuther G, Bangoura B, Charles S, Daugschies A, Hinney B, Lindsay DS, Mundt HC, Ocak M, S. Sotiraki. W A A V P guideline for evaluating the efficacy of anticoccidials in mammals (pigs, dogs, cattle, and sheep). Vet .Parasitol. 2018; 253: 102–119.

12. Gregory MW, Catchpole J. Ovine coccidiosis: the pathology of Eimeria crandallis infection. Inter.J.Parasitol.1978; 17:1099-1111.

13. Bangoura B, Daugschies A. Parasitological and clinical parameters of experimental Eimeria zuernii infection in calves and influence on weight gain and haemogram. Parasitol Res. 2007; 100:1331–1340.

14. Reilly JS. Euthanasia of Animals Used for Scientific Purposes. Anzccart press, 2001.

15. Hidalgo-Argüello MR, Cordero-Del-Campillo M. Epizootiology of Eimeria ahsata coccidiosis in León (Spain). Vet.Parasitol. 1988; 27: 183-191.

16. Catchpole J, Norton C, Joyner L. Experiments with defined multispecific coccidial infections in lambs. Parasitol. 1979; 72: 137-147.

17. Smith MN, Davis LR, Bowman GW. The pathogenicity of Eimeria ahsata, a coccidium of Sheep. J.Protozool. 1960; 7: (Suppl.), 8.

18. Mahart JL, Sherrick GW. Coccidiosis due to E.ahsata in feedlot lambs in Illinois. J. Am. Vet. Med. Ass. 1965; 146: 1415–1416.

19. Chartier C, Paraud C. Coccidiosis due to Eimeria in sheep and goats, a review, Small Rumin. Res. 2012; 103: 84-92.

20. Stockdale PHG, Bainborough AR, Bailey CB, Niilo LM. Some pathophysiological changes associated with infection of Eimeria zuernii in calves. Can J Comp Med.1981; 45: 34–37.

21. Enemark HL, Dahl J, Enemark JM. Eimeriosis in Danish dairy calves--correlation between species, oocyst excretion and diarrhoea. Parasitol Res. 2013; 112: 169–176.

22. Hashemnia M, Khodakaram-Tafti A, Razavi SM, Nazifi, S. Experimental caprine coccidiosis caused by Eimeria arloingi: morphopathologic and electron microscopic studies. Vet. Res.Com. 2012; 361:47–55.

23. Khodakaram Tafti A, Mansourian M. Pathologic lesions of naturally occurring coccidiosis in sheep and goats. Comp Clin Pathol. 2008; 17: 87–91.

24. Buret A, Gall DG, Nation PN, Olson ME. Intestinal protozoa and epithelial cell kinetics, structure and function. Parasitol Today.1990; 6:375–380.

25. Aleksandersen M, Landsverk T, Gjerde B, Helle O. Scarcity of γδ T Cells in intestinal epithelia containing coccidia despite general increase of epithelial lymphocytes. Vet .Pathol. 1995; 32:504-512.

26. Khodakaram.A. Ileoileal intussusception associated with coccidiosis in sheep. B. J.Vet.Med. Series B, 1995;46 : 659–663.

27. Taghavi-Razavizadeh A, Nourani H, Razmi G, Zarif MBH. A Report of unusual diffuse nodular lesions in intestines of Lambs with Coccidiosis. Vet ClinPathol.2016; 39: 251-254.

28. Daugschies A, Bürger HJ, Akimaru M. Apparent digestibility of nutrients and nitrogen balance during experimental infection of calves with Eimeria bovis. Vet Parasitol.1998; 77: 93-102

29.] Jubb KVF, Kennedy PC, Palmer N. Pathology of domestic animals. Academic Press Inc,2007.

30. Daugschies A, Najdrowski M. Eimeriosis in cattle: current understanding. J Vet Med Series B.2005; 52: 417-427.

31) de la Fuente C, Cuquerella M, Carrera L, Alunda JM. Effect of subclinical coccidiosis in kids on subsequent trichostrongylid infection after weaning. Vet Parasitol.1993; 45: 177-83.

How to cite this article
Ahmadi Saleh Baberi N, Karimi I, Nourani H, Azizi Alavije HR, Razmi GR. Parasitological and pathological findings of coccidiosis in an experimental infection caused by *Eimeria ahsata* in lambs. Iran J Vet Sci Technol. 2021; 13(2): 20-28.
DOI: https://doi.org/10.22067/ijvst.2021.71247.1057
URL: https://ijvst.um.ac.ir/article_40441.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- May- 30
Accepted after revision: 2021- Aug- 28
Published online: 2021- Dec- 11

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2021.70595.1045

Assessment of some inflammatory cytokines and immunologic factors in dairy cows with subclinical ketosis

Negar Karimi[a], Hesam A. Seifi[a,b], Mohammad Heidarpour[a,b]

[a] Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[b] Center of Excellence in Ruminant Abortion and Neonatal Mortality, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

ABSTRACT

Altered cytokine profile and weakened immunity along with clinical or subclinical ketosis (SCK) are among the remarkable challenges around parturition. Therefore, the present study aimed to compare some inflammatory cytokines and immunologic factors between two groups of healthy and SCK cows. Serum specimens were collected from 30 clinically healthy dairy cows on the early dry period (EDP), one week before expected calving (-1w), and one week postpartum (+1W). The animals were divided into the two groups of healthy (N = 20) and SCK (N = 10) based on serum β-hydroxybutyrate cut-off of 1.2 mmol/L on +1W. The concentrations of immunoglobulin G (IgG), interleukin-4 (IL-4), IL-10, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and haptoglobin (Hp) were measured by enzyme-linked immunosorbent assay. The data were statistically analyzed by mixed analysis of variance and independent samples *t*-test using the SPSS software. The findings demonstrated that the overall levels of IL-4 (p = 0.033), IL-10 (p = 0.049), TNF-α (p = 0.028), and Hp (p = 0.018) were significantly higher in the SCK group than the control group. Furthermore, the interaction of time × SCK had a significant influence on IL-4 (p = 0.028) and Hp (p = 0.022) levels. It was revealed that IL-4 (p = 0.008), IL-10 (p = 0.009), TNF-α (p = 0.01), and Hp (p = 0.002) were all significantly higher in the SCK group than the control group on +1W. In conclusion, SCK in dairy cattle might have a relationship with immunologic and inflammatory changes around calving.

Keywords

Immunologic factors, Inflammation, Ketosis, Subclinical ketosis

Number of Figures: 2
Number of Tables: 2
Number of References:: 37
Number of Pages: 8

Abbreviations

NEB: negative energy balance
BHB: β-hydroxybutyrate
SCK: subclinical ketosis
EDP: early dry period
IgG: immunoglobulin G
IL: interleukin
IFN: interferon
TNF: tumor necrosis factor
Hp: haptoglobin
ELISA: enzyme-linked immunosorbent assay
ANOVA: analysis of variance
NEFA: non-esterified fatty acids

Corresponding author: *Mohammad Heidarpour*
Email: *heidarpour@um.ac.ir*
Tel: *+98 (51)3876-3852*

Introduction

One of the most considerable challenges around calving is the debilitated immune responses, which have been reported to be correlated with the higher incidence of diseases during this period [1]. Furthermore, inflammatory markers have been reported to raise after calving [2, 3], and the postpartum inflammatory condition was revealed to have a relationship with the elevated risk of diseases and reduced milk production [4].

On the other hand, pregnancy and lactation are accompanied by remarkable metabolic demands around parturition. The NEB occurs at this period as the result of the maximum nutritional requirements of the fetus, lactation initiation, and decline in dry matter intake [5, 6]. A physiological adaptation to overcome NEB is the mobilization of fat from adipose tissue. However, an imbalance might take place in hepatic carbohydrate and fat metabolism in case of excessive fat mobilization. The consequence of this series of events is an augmentation in the blood concentrations of ketone bodies, namely BHB, acetoacetate, and acetone [7]. The SCK is known as hyperketonemia in the absence of the clinical manifestations of ketosis [8]. The gold standard for SCK diagnosis is BHB measurement in blood serum or plasma due to the stability of this marker [9].

Inflammation and disturbed immunity are among the highlights of the periparturient period in dairy cattle [1, 10]. On the other hand, high levels of ketone bodies have been shown to induce a proinflammatory state [11] and inflammatory responses might be more intense in ketotic cows than healthy animals [12]. Moreover, cows with ketosis were reported to experience immune suppression postpartum [13]. The SCK has a relatively high prevalence in different parts of the world, as well as Iran [14]. Therefore, the present study aimed to compare the levels of several immunologic and inflammatory variables between healthy and SCK cows. Furthermore, the alterations of these factors around calving, as the time of SCK occurrence and diagnosis, were compared between the two groups.

Results

The descriptive statistics of all variables in both groups and the *p*-values of ANOVA are presented in Table 1. The results of mixed ANOVA demonstrated that sampling time had a significant effect on IL-4 ($p = 0.02$) and IFN-γ ($p = 0.02$) with their highest levels found on +1W and EDP, respectively. It was observed that SCK as the grouping variable significantly influenced IL-4 ($p = 0.033$), IL-10 ($p = 0.049$), TNF-α ($p = 0.028$), and Hp ($p = 0.018$) as all these factors were higher in the SCK group than the control group. Moreover, time × SCK interaction imposed a significant impact on the levels of IL-4 ($p = 0.028$) and Hp ($p = 0.022$).

According to the results of the *t*-tests, the two groups had no significant difference on the EDP and -1W. However, the levels of IL-4 ($p = 0.008$), IL-10 ($p = 0.009$), TNF-α ($p = 0.01$), and Hp ($p = 0.002$) were all significantly higher in the SCK group than the control group on +1W (Figure 1). Furthermore, the difference in IgG ($p = 0.05$) and IFN-γ ($p = 0.05$) tended to be significant between the two groups at this time (Figure 2).

Discussion

Our findings indicated that overall, SCK cows had significantly higher levels of serum IL-4, IL-10, TNF-α, and Hp, compared to healthy cows. Furthermore, the alterations in the serum concentrations of IL-4 and Hp during the study period had a significant difference between the control and SCK groups. It was revealed that after calving, which is a critical time for SCK diagnosis, the levels of all the studied immunologic and inflammatory markers were higher in the subjects with SCK than in the healthy animals. The latter difference was significant for IL-4, IL-10, TNF-α, and Hp.

It is believed that the diminished viability of white blood cells in SCK results from the negative impacts of metabolic changes in this disease [15]. Moreover, white blood cells isolated from cows affected by ketosis had a weaker chemotaxis capacity, compared to healthy subjects [16]. In another study, exposure of bovine milk leukocytes to diverse BHB levels *in vitro* led to altered cell membranes, disturbed oxidative activity, reduced phagocytosis, and decreased chemotaxis of these cells [17]. Hoeben et al. found that butyric acid could inhibit respiratory burst in bovine blood polymorphonuclear leukocytes [18]. Some researchers suggest that controlling body condition score and lipid deposition in dry period resulting in lower mobilization of NEFA postpartum can diminish the dysfunction of leukocytes after calving [19].

Energy requirements for evoking the immune responses around calving may be involved in the exacerbation of NEB [15] coupled with increased lipid mobilization and subsequent hyperketonemia during this period. On the other hand, nutrients and metabolites are known to play role in distinct features of the immune system [20]. The main source of energy for leucocytes is glucose [21], which is also one of the key components of ketosis pathophysiology. In other words, the role of glucose in both metabolic and immune systems might lead to a relationship between

Table 1.
Mean ± SE of inflammatory cytokines and immunologic factors in subclinical ketosis and healthy cows

Variable	SCK	Healthy	*p*-value		
			Group	Time	Group × Time
IL-4 (ng/L)	40.26 ± 7.62	19.35 ± 5.39	0.033	0.02	0.028
IL-10 (ng/L)	452.64 ± 61.36	298.09 ± 43.38	0.049	0.087	0.079
TNF-α (ng/L)	944.5 ± 114.91	618.89 ± 81.25	0.028	0.136	0.223
IFN-γ (pg/mL)	522.91 ± 57.54	398.97 ± 40.69	0.09	0.02	0.476
Hp (µg/mL)	185.21 ± 29.85	93 ± 21.1	0.018	0.216	0.022
IgG (µg/mL)	67.18 ± 10.82	42.77 ± 7.65	0.076	0.316	0.613

SCK, subclinical ketosis; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; Hp, haptoglobin; IgG, immunoglobulin G.

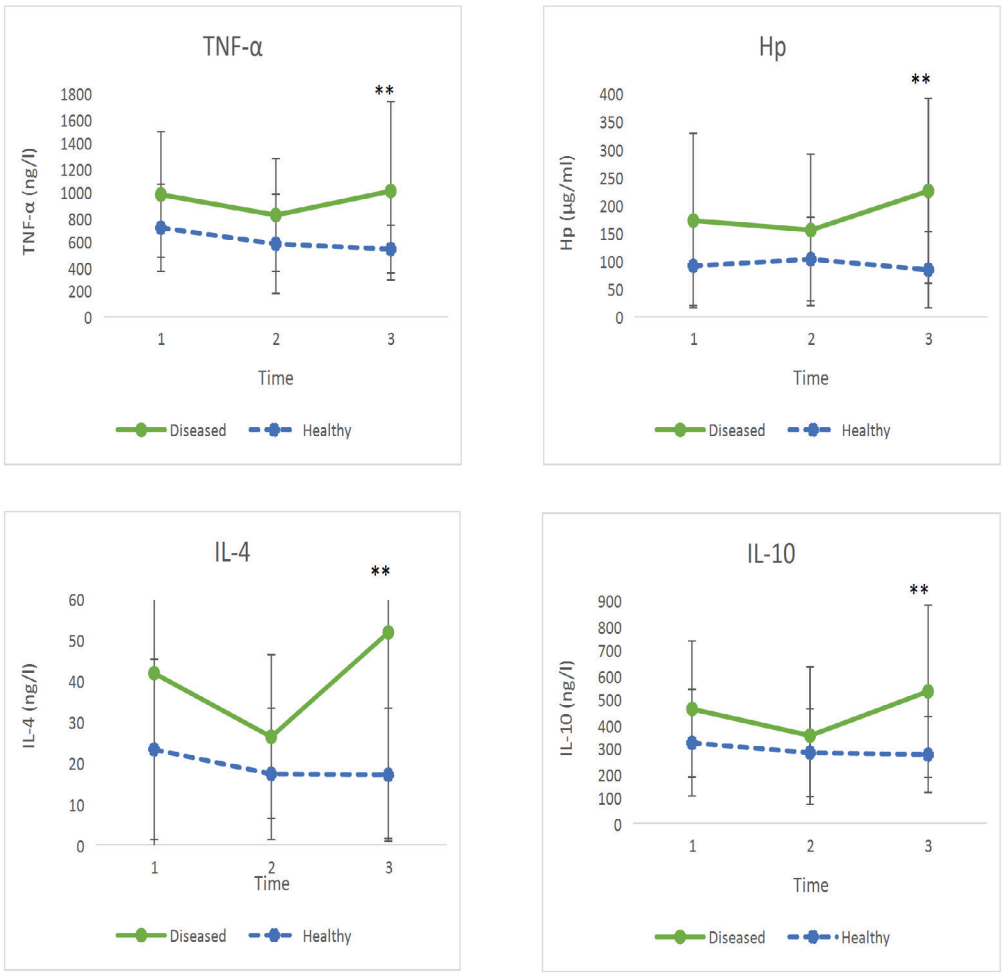


Figure 1.
Concentration (mean ± standard error of the mean) of serum IL-4, IL-10, TNF-α, and Hp in both healthy (line) and SCK (dash) groups on the EDP, -1W, and +1W.
**, Significant difference ($p < 0.05$) between groups; SCK, subclinical ketosis; EDP, early dry period; -1W, one week before expected calving; +1W, one week postpartum; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; Hp, haptoglobin.

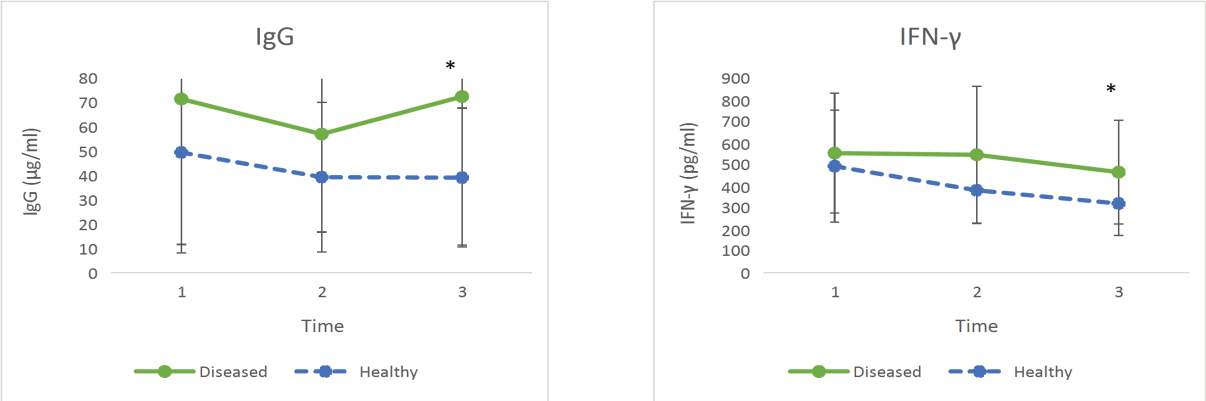


Figure 2.
Mean concentrtation (mean± standard error of the mean) of serum IgG and IFN-γ in both healthy (line) and SCK (dash) groups on the EDP, -1W, and +1W;
*, Differences that tend to be significant ($p = 0.05$) between the groups; SCK, subclinical ketosis; EDP, early dry period; -1W, one week before expected calving; +1W, one week postpartum; IFN, interferon; IgG, immunoglobulin G.

these two systems as changes in glucose due to metabolic alterations can cause changes in immune-related variables. As a result, the mentioned impacts may imply the role of SCK and clinical ketosis in the changes of the immune system and higher incidence of local and systemic infections around calving concomitant with clinical ketosis and SCK.

We found that the cows of the SCK group had a significantly higher TNF-α level than the healthy animals. It has been revealed that the high concentrations of acetoacetate in hepatocytes may upregulate and elevate the secretion of proinflammatory cytokines TNF-α, IL-1, and IL-6 [22]. Moreover, the exposure of hepatocytes to BHB has been shown to induce the NF-κB signaling pathway and upregulate TNF-α [11]. In this regard, Sun et al. also reported greater NF-κB activity, as well as more abundant mRNA of TNF-α, IL-1, and Il-6 in the mammary tissue of SCK cows than the animals with normal BHB [23]. These authors concluded that ketosis and overload of BHB may bring about oxidative stress that causes inflammation and immunologic dysfunction [23]. Some other researchers reported that high BHB and palmitic acid, as a component of NEFA, evoke inflammation in the bovine endometrial cells via triggering NF-κB signaling [24]. In a study by Ohtsuka et al., TNF-α was significantly higher in cows with severe fatty liver, compared to mild cases. In addition, serum TNF-α was correlated with insulin resistance in cattle with fatty liver [25]. The TNF-α was reported to induce insulin resistance in the animal models of obesity through affecting insulin receptors [25] and insulin resistance is known to contribute to hyperketonemia and ketosis occurrence. Furthermore, hyperketonemia in humans could induce the secretion of TNF-α by cultured monocytes and raise the concentration of this cytokine in the blood of patients with diabetes. It was justified by cellular oxidative stress caused by acetoacetate and cAMP deficiency in these patients [26].

The level of Hp, as one of the major acute phase proteins in cows, in the present study was significantly higher in SCK cows than healthy subjects during the study period and also postpartum. Moreover, the trend of changes in Hp concentration had a significant difference between the two groups. The period around parturition in dairy cattle is commonly characterized by proinflammatory status [27]. High BHB and acetoacetate levels, which are known as the markers of ketosis, cause inflammatory response through activating the NF-κB signaling pathway [11]. Periparturient inflammation was observed in ketotic cows evident by the high levels of IL-6 [28]. In line with our results, Abuajamieh et al. found that the rise in both Hp and serum amyloid A, as acute phase proteins and markers of inflammation, was significantly higher in cows with ketosis than healthy animals. These authors concluded that the augmented concentrations of inflammatory markers in the peripheral blood of ketotic cases both pre- and postpartum might be suggestive of a relationship between ketosis development and inflammation [12]. Likewise, Mezzetti et al. (2019) stated that higher positive acute phase proteins in ketotic cattle after calving, in comparison with healthy animals indicate a more remarkable inflammation at this time in cows with ketosis [15]. It has been suggested that more severe NEB is associated with the more intense inflammatory condition around parturition [3].

Although our findings showed that time alone had a significant impact on IFN-γ, the effects of SCK

and SCK × time on this variable were not significant. In contrast, Filar et al. and Kandefer et al. demonstrated that the leukocytes retrieved from the milk and blood of cows with ketosis produced significantly lower amounts of IFN-γ, compared to the control animals [29, 30]. These controversial findings might result from the differences between in vitro and in vivo conditions. The IFN-γ is believed to have the potential to induce SCK through downregulating peroxisome proliferation-activated receptor (PPAR)-γ [31], which plays role in adipocyte biology and contributes to keeping tissues, such as adipose tissue, sensitive to insulin [32, 33]. On the other hand, we observed that IL-4, which acts as one of the key regulators of humoral and adaptive immunity, was significantly higher in the SCK cases than in the healthy cows. Data concerning IL-4 alterations in ketosis are limited in the literature. While IFN-γ is mainly generated by Th1 cells (Cho et al. 2012), IL-4 is more produced by Th2 cells (Cho et al. 2012; Lastra et al. 2009). Consequently, the changes in IL-4 could to some extent indicate the possible impact of SCK on T cell populations.

We observed that the postpartum level of IL-10 was significantly higher in the SCK group than in the control animals. Similar results have been found in cows, as well as children and mice with diabetic ketoacidosis [34-36]. The IL-10 is regarded as an anti-inflammatory cytokine that has the potential to regulate both innate and acquired immune reactions by suppressing TNF-α, IFN-γ, IL-1, IL-4, IL-5, and IL-6 synthesis [34]. Consequently, it augments following inflammatory conditions and elevations in the concentrations of the mentioned cytokines.

In conclusion, the findings of the current study revealed that SCK might lead to some degrees of inflammatory conditions. This was evident by the higher concentrations of IL-4, IL-10, TNF-α, and Hp in cows with SCK than healthy subjects, especially after parturition as the critical time for SCK diagnosis. Considering the limited data about the impact of ketosis on IL-4, further evaluation of this cytokine in ketotic conditions is recommended. Furthermore, the effect of ketone bodies on T cell populations can be investigated in vitro.

Materials & Methods

Animals and Setting

The present investigation was approved by the Animal Welfare Committee of the Ferdowsi University of Mashhad with the code of 3/44869 under the institutional, national, and international guidelines. This cross-sectional study was carried out on 30 clinically healthy multiparous subjects selected from a commercial dairy herd of 3000 Holstein cows and 970 lactating animals in Neyshabur, Iran. The records of the herd showed an average milk production of 40 L. All cows were fed twice a day and had free access to water during the study. The animals received anionic salt during the far-off and close up periods. Ingredients and nutritional composition of the dry and lactation periods diets are presented in Table 2. The investigated herd had loose pens and outside yards. The cows were dried approximately 80-60 days before the expected parturition and were transferred to separated pens at this time. All the studied subjects had normal easy calving without any clinical abnormalities during the study. Finally, 10 cows with serum BHB levels of >1.2 mmol/L on +1W were categorized as the SCK group [37] and the other 20 animals were considered as healthy controls.

Sample Collection and Laboratory Analyses

Blood specimens were taken on the EDP, one week before the expected calving (-1W), and one week postpartum (+1W). The samples were collected through coccygeal venipuncture into commercial evacuated tubes, which had a clot activator. The blood specimens were centrifuged at 1800 g for 15 min and the sera were collected instantly. All the serum samples were delivered to the laboratory on ice packs and were stored at -80°C until further analyses.

The serum specimens were thawed at room temperature prior to laboratory analyses. Serum BHB concentrations were measured by a colorimetric commercial kit (Randox Laboratories Ltd., Antrim, UK) using a biochemical autoanalyzer (Mindray, BS-200, Shenzhen, China). The immunologic variables, including IgG, IL-4, IL-10, IFN-γ, TNF-α, and Hp were evaluated in the serum specimens by ELISA (Bioassay Technology Laboratory, Shanghai, China) according to the instructions of the manufacturer. An ELISA automatic washer (BioTek, ELx-50, Winooski, USA) and an ELISA reader (BioTek, ELx-800, Winooski, USA) were utilized.

Statistical Analysis

All the data were statistically analyzed using SPSS software version 22 (IBM, USA). The cows in the present study were assigned to the two groups of SCK (N=10) and control (N=20) based on a serum BHB cut-point of 1.2 mmol/L. The normality of data distribution was evaluated based on the Shapiro-Wilk test, skewness, and kurtosis indicating that none of the variables had a normal distribution. Consequently, all the factors were modified by logarithmic transformation. A 3 (time) × 2 (SCK) mixed ANOVA was carried out to evaluate the effects of time, SCK, and interactions between these factors on all immunologic markers. Pairwise comparisons for time and factor interactions were completed utilizing Bonferroni adjustment. In case a significant impact was observed for SCK or time × SCK on a variable, the variable was compared between the two groups of SCK and control for all three times separately by the independent samples t-test. p-value < 0.05 and 0.05 ≤ p-value < 0.1 were considered statistically significant and tending to be significant for all tests, respectively.

Authors' Contributions

NK contributed to study designing, sample preparation, laboratory experiments, data analysis, results interpretation, and manuscript drafting. MH conceived the study design and contributed to laboratory experiments, data analysis, and manuscript review. HAS contributed to study designing and manuscript review.

Acknowledgements

The authors would like to thank the Research Deputy of Ferdowsi University of Mashhad for the finan-

Table 2.
Ingredients and nutritional composition (% DM unless noted) of diets fed to cows during dry and lactation periods

Item	Far-off	Close-up	Fresh cow
Ingredients			
Alfalfa hay	8.55	6.3	8.25
Corn silage	76.7	64.68	42.38
Sugar beet pulp	-	-	6.64
Wheat straw	3.54	2.45	0.92
Alfalfa silage	-	-	2.29
Sugar beet molasses	-	-	9.16
Barley grain	0.88	6.14	11.14
Corn grain, ground, dry	3.17	9.04	6.03
Soybean meal	0.98	4.3	4.97
Cottonseed	-	0.69	2.96
Wheat grain	1.88	-	-
Meat meal	3.36	-	-
Fish meal	-	2.45	3.3
Roasted whole soybean seeds	-	1.85	-
Urea	0.21	-	-
Salt	0.06	-	0.18
Calcium carbonate	0.26	0.54	0.23
Sodium bicarbonate	-	-	0.73
Dicalcium phosphate	-	-	0.09
Magnesium oxide	0.06	0.03	0.23
Anionic salt (Magnesium sulfate)	-	1.05	-
Mineral-vitamin supplement [1]	0.21	0.48	0.26
Toxin binder	**0.14**	-	0.14
Energy and nutrients			
Dry matter (kg)	11.9	12.5	20.4
Net energy for lactation (Mcal/kg)	1.32	1.5	1.61
Neutral detergent fiber	44.7	28.6	29.1
Non-fiber carbohydrates	30.2	38.5	37.5
Ether extract	2.1	3.6	4.3
Crude protein (%)	12.1	14.7	16.7
Rumen degradable protein (%)	7.5	9.5	10.7
Rumen un-degradable protein (%)	4.6	5.2	6
Calcium	0.75	0.97	0.86
Phosphorus	0.35	0.37	0.48

[1] Anionic pre-fresh supplement: 250,000 IU/Kg vit A, 40,000 IU/Kg vit D3, 40,000 IU/Kg vit E, 40 mg/Kg biotin, 12 g/Kg niacin, 168 g/Kg Ca, 65 g/Kg Mg, 1300 mg/Kg Mn, 2210 mg/Kg Zn, 600 mg/Kg Cu, 10 mg/Kg Co, 8 mg/Kg Se, 12 mg/Kg I, 52 g/Kg S, 120 g/Kg Cl

cial support of this study with grant number 3/44869. Moreover, we extend our gratitude to Dr. Samuel Kia for cooperating in the sampling procedure.

Competing Interests

The authors declare no conflict of interests.

References

1. Jahan N, Minuti A, Trevisi E. Assessment of immune response in periparturient dairy cows using ex vivo whole blood stimulation assay with lipopolysaccharides and carrageenan skin test. Vet Immunol Immunopathol. 2015;165(3-4):119-26.

2. Mullins CR, Mamedova LK, Brouk MJ, Moore CE, Green HB, Perfield KL, et al. Effects of monensin on metabolic parameters, feeding behavior, and productivity of transition dairy cows. J Dairy Sci. 2012;95(3):1323-36.

3. Bertoni G, Trevisi E, Han X, Bionaz M. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. J Dairy Sci. 2008;91(9):3300-10.

4. Bradford BJ, Yuan K, Farney JK, Mamedova LK, Carpenter AJ. Invited review: Inflammation during the transition to lactation: New adventures with an old flame. J Dairy Sci. 2015;98(10):6631-50.

5. Bell AW. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J Anim Sci. 1995;73(9):2804-19.

6. Bertoni G, Trevisi, E., Lombardelli, R. Some new aspects of nutrition, health conditions and fertility of intensively reared dairy cows. Italian Journal of Animal Science. 2009;8:491-518.

7. Nielsen NI, Ingvartsen, K.L. Propylene glycol for dairy cows. A review of the metabolism of propylene glycol and its effects on physiological parameters, feed intake, milk production and risk of ketosis. Animal Feed Science and Technology. 2004;115:191-213.

8. Andersson L. Subclinical ketosis in dairy cows. Vet Clin North Am Food Anim Pract. 1988;4(2):233-51.

9. Duffield T. Subclinical ketosis in lactating dairy cattle. Vet Clin North Am Food Anim Pract. 2000;16(2):231-253.

10. Loor JJ, Bertoni G, Hosseini A, Roche JR, Trevisi E. Functional welfare – using biochemical and molecular technologies to understand better the welfare state of peripartal dairy cattle. Anim Prod Sci. 2013; 53:931–53.

11. Shi X, Li X, Li D, Li Y, Song Y, Deng Q, et al. β-Hydroxybutyrate activates the NF-κB signaling pathway to promote the expression of pro-inflammatory factors in calf hepatocytes. Cell Physiol Biochem. 2014;33(4):920-32.

12. Abuajamieh M, Kvidera SK, Fernandez MV, Nayeri A, Upah NC, Nolan EA, et al. Inflammatory biomarkers are associated with ketosis in periparturient Holstein cows. Res Vet Sci. 2016;109:81-5.

13. Holtenius K, Persson Waller K, Essén-Gustavsson B, Holtenius P, Hallén Sandgren C. Metabolic parameters and blood leukocyte profiles in cows from herds with high or low mastitis incidence. Vet J. 2004;168(1):65-73.

14. Asl AN, Nazifi S, Ghasrodashti AR, Olyaee A. Prevalence of subclinical ketosis in dairy cattle in the Southwestern Iran and detection of cutoff point for NEFA and glucose concentrations for diagnosis of subclinical ketosis. Prev Vet Med. 2011;100(1):38-43.

15. Mezzetti M, Minuti A, Piccioli-Cappelli F, Amadori M, Bionaz M, Trevisi E. The role of altered immune function during the dry period in promoting the development of subclinical ketosis in early lactation. J Dairy Sci. 2019;102(10):9241-58.

16. Suriyasathaporn W, Daemen AJ, Noordhuizen-Stassen EN, Dieleman SJ, Nielen M, Schukken YH. Beta-hydroxybutyrate levels in peripheral blood and ketone bodies supplemented in culture media affect the in vitro chemotaxis of bovine leukocytes. Vet Immunol Immunopathol. 1999;68(2-4):177-86.

17. Cerone SI, Sansinanea AS, García MC. Effects of beta-hydroxybutyric acid on bovine milk leukocytes function in vitro. Gen Physiol Biophys. 2007;26(1):14-9.

18. Hoeben D, Heyneman R, Burvenich C. Elevated levels of beta-hydroxybutyric acid in periparturient cows and in vitro effect on respiratory burst activity of bovine neutrophils. Vet Immunol Immunopathol. 1997;58(2):165-70.

19. Mezzetti M, Bionaz M, Trevisi E. Interaction between inflammation and metabolism in periparturient dairy cows. J Anim Sci. 2020;98(Suppl 1):S155-s74.

20. Ingvartsen KL, Moyes K. Nutrition, immune function and health of dairy cattle. Animal. 2013;7 Suppl 1:112-22.

21. Sordillo LM. Nutritional strategies to optimize dairy cattle immunity. J Dairy Sci. 2016;99(6):4967-82.

22. Li Y, Ding H, Wang X, Liu L, Huang D, Zhang R, et al. High levels of acetoacetate and glucose increase expression of cytokines in bovine hepatocytes, through activation of the NF-κB signalling pathway. J Dairy Res. 2016;83(1):51-7.

23. Sun X, Tang Y, Jiang C, Luo S, Jia H, Xu Q, et al. Oxidative stress, NF-κB signaling, NLRP3 inflammasome, and caspase apoptotic pathways are activated in mammary gland of ketotic Holstein cows. J Dairy Sci. 2021;104(1):849-61.

24. Li P, Li L, Zhang C, Cheng X, Zhang Y, Guo Y, et al. Palmitic Acid and β-Hydroxybutyrate Induce Inflammatory Responses in Bovine Endometrial Cells by Activating Oxidative Stress-Mediated NF-κB Signaling. Molecules. 2019;24(13).

25. Ohtsuka H, Koiwa M, Hatsugaya A, Kudo K, Hoshi F, Itoh N, et al. Relationship between serum TNF activity and insulin resistance in dairy cows affected with naturally occurring fatty liver. J Vet Med Sci. 2001;63(9):1021-5.

26. Jain SK, Kannan K, Lim G, McVie R, Bocchini JA, Jr. Hyperketonemia increases tumor necrosis factor-alpha secretion in cultured U937 monocytes and Type 1 diabetic patients and is apparently mediated by oxidative stress and cAMP deficiency. Diabetes. 2002;51(7):2287-93.

27. Loor JJ, Bertoni G, Hosseini A, Roche JR, Trevisi E. Functional welfare - using biochemical and molecular technologies to understand better the welfare state of peripartal dairy cattle. Animal Production Science. 2013;53:931-53.

28 Filar J, Kandefer-Szerszeń M, Szuster-Ciesielska A, Rzeski W. Effects of cold treatment and ketosis induced by starvation on interferon production in leukocytes of lactating cows. Dtsch Tierarztl Wochenschr. 1992;99(5):210-3.

29. Kandefer-Szerszen M, Filar J, Szuster-Ciesielska A, Rzeski W. Suppression of interferon response of bovine leukocytes during clinical and subclinical ketosis in lactating cows. Dtsch Tierarztl Wochenschr. 1992;99(11):440-3.

30. Floyd ZE, Stephens JM. Interferon-gamma-mediated activation and ubiquitin-proteasome-dependent degradation of PPARgamma in adipocytes. J Biol Chem. 2002;277(6):4062-8.

31. Waite KJ, Floyd ZE, Arbour-Reily P, Stephens JM. Interferon-gamma-induced regulation of peroxisome proliferator-activated receptor gamma and STATs in adipocytes. J Biol Chem. 2001;276(10):7062-8.

32. Choi SH, Chung SS, Park KS. Re-highlighting the action of PPARγ in treating metabolic diseases. F1000Res. 2018;7.

33. Zarrin M, Wellnitz O, van Dorland HA, Bruckmaier RM. Induced hyperketonemia affects the mammary immune response during lipopolysaccharide challenge in dairy cows. J Dairy Sci. 2014;97(1):330-9.

34. Hoffman WH, Burek CL, Waller JL, Fisher LE, Khichi M, Mellick LB. Cytokine response to diabetic ketoacidosis and its treatment. Clin Immunol. 2003;108(3):175-81.

35. Close TE, Cepinskas G, Omatsu T, Rose KL, Summers K, Patterson EK, et al. Diabetic ketoacidosis elicits systemic inflammation associated with cerebrovascular endothelial cell dysfunction. Microcirculation. 2013;20(6):534-43.

36. Ospina PA, Nydam DV, Stokol T, Overton TR. Evaluation of nonesterified fatty acids and beta-hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. J Dairy Sci. 2010;93(2):546-54.

How to cite this article

Karimi N, Seifi HA, Heidarpour M. Assessment of some inflammatory cytokines and immunologic factors in dairy cows with subclinical ketosis. Iran J Vet Sci Technol. 2021;13(2): 29-36.
DOI: https://doi.org/10.22067/ijvst.2021.70595.1045
URL:https://ijvst.um.ac.ir/article_40463.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- May- 28
Accepted after revision: 2021-Sep-11
Published online: 2021- Dec- 18

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2021.69380.1028

Evidence for an interaction between cannabinoidergic and dopaminergic systems with melanocortin MC3/MC4 receptors in regulating food intake of neonatal chick

Mohammad Bameri[a], Morteza Zendehdel[b], Bita Vazir[a], Ahmad Asghari[c], Negar Panahi[a]

[a] Department of Basic Sciences, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[b] Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
[c] Department of Clinical Science, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran.

ABSTRACT

The current study aimed to see how the central dopaminergic and cannabinoidergic mechanisms affect melanocortin-induced food intake in neonatal layer chickens. In this regard, 9 experiments were designed. In experiment 1, chicks were injected with a control solution, MTII (2.5, 5, and 10 ng). In experiment 2, control solution, L-DOPA (125 nmol), MTII (10 ng), and L-DOPA + MTII were applied to the birds. Experiments 3-9 were similar to experiment 2, except birds injected with 6-OHDA (150 nmol), SCH23390 (5 nmol), AMI-193 (5 nmol), NGB2904 (6.4 nmol), L-741,742 (6 nmol), SR141716A (6.25 µg), and AM630 (5 µg) instead of L-DOPA. Then, cumulative food intake was recorded at 30, 60, and 120 min following injection. According to the results, in comparison with the control group, dose-dependent hypophagia was observed in 3-h food-deprived neonatal layer chickens following ICV injection of MTII (2.5, 5, and 10 ng) ($p < 0.05$). ICV injection of L-DOPA and SR141716A increased hypophagia induced by MTII in chickens ($p < 0.05$), while 6-OHDA greatly suppressed MTII- induced hypophagia ($p < 0.05$). In addition, SCH23390 and AMI-193 greatly weakened the MTII-induced hypophagia in neonatal layer chickens ($p < 0.05$). However, NGB2904, L-741742, and AM630 had no role in hypophagia induced by MTII ($p > 0.05$). These results demonstrated that melanocortin-induced hypophagia in the neonatal layer chickens is likely mediated by D1, D2, and CB1 receptors.

Keywords

Dopamine, Cannabinoid, Melanocortin, Food intake, Layer chicken

Number of Figures: 9
Number of Tables: 0
Number of References:: 49
Number of Pages: 9

Abbreviations

ICV: Intracerebroventricular
CNS: Central nervous system
CB: Endocannabinoids receptors
GPCRs: G protein-coupled receptor subtypes
DA: Dopamine
ARC: Arcuate nucleus
VMH: Ventromedial hypothalamus
PVN: Periventricular nucleus

Corresponding author: *Morteza Zendehdel*
Email: *zendedel@ut.ac.ir*
Tel: *+98 (21)6111-7186*

Introduction

One of the most complicated aspects of animals is appetite regulation that modulates a large number of parts in the brain for cooperating with signals received from the peripheral organs [1]. The appetite is regulated by various neurotransmitters through complex neurological pathways in the central nervous system (CNS) [2]. Endocannabinoids and their receptors (CB1 and CB2) belong to the G protein-coupled receptor subtypes (GPCRs) with a role in several physiological functions in the brain, including locomotion nociception, learning and memory, food intake, and energetic metabolism [3,4]. Besides, it has a regulatory role in immune regulation, endocrine processes, cardiovascular system, emesis, and brain development [5]. The CB1 receptors are expressed in the presynaptic terminals of the brain, and CB2 receptors were previously found on immune system cells and organs in the peripheral nervous system (PNS), but they are also expressed in CNS [6]. Moreover, both CB1 and CB2 receptors have a role in food intake regulation in rats [7]. However, little is known about the contribution of endocannabinoids to feeding behavior in domestic fowl. Some studies claim that just the CB1 receptors have a regulatory role in appetite in broilers [8, 9]. Recently, a number of studies revealed that both CB1 and CB2 receptors have hyperphagic effects in the neonatal layer chickens [4, 10, 48, 49]

Dopamine is the main catecholamine neurotransmitter expressed in several nuclei of the brain, such as the hypothalamus, substantia nigra, and ventral tegmental area. At least five different dopamine receptor subtypes have been discovered so far (D1-D5) [11]. At least five distinct GPCRs of dopamine affect its mediatory effects [11]. The receptors D1 and D2 are found frequently than others in the brain. The dopaminergic system is involved in a variety of physiological functions, including appetite regulation, locomotor activity, emotion, and cognition [12]. Food intake reduces through D1 and D2 receptors in rats [13]. Besides, in meat-type chickens, hypophagia induced by dopamine is mediated by D1 receptors, while others (D2-D4) may have no role in appetite regulation [1, 14]. Thus, it is evident that no single neuropeptide regulates central feeding behavior and that there are interactions between a broad distributed neural network and other neurotransmitters to assess feeding status [15, 49].

The melanocortin is a neurotransmitter for which five subtypes (MC1R-MC5R) have been identified to date [16]. It has a prominent role in physiologic functions, including grooming, thermoregulation, learning, and energy balance regulation [17]. The melanocortin receptors have been identified in the bird's brain. Only the melanocortin-3 (MC3R) and melanocortin-4 (MC4R) subtypes of melanocortin receptors are responsible for the central regulation of food intake [17]. The MC3R and MC4R are mainly found in the arcuate nucleus (ARC), ventromedial hypothalamus (VMH), and periventricular nucleus (PVN) regions of the hypothalamus. According to the related published studies, the ICV injection of the MC3 and MC4 receptors reduces the food intake in rats [18].

Dopaminergic neurons interact with the actions of cannabinoids in the CNS. Dopaminergic neuronal cell bodies are located in the substantia nigra and ventral tegmental area (VTA) and projected to the caudate-putamen (CP) and nucleus accumbens (NA). The effector neurons in these structures are regulated by dopaminergic and cannabinoidergic mechanisms [3]. Motor effects of CB1 agonists are linked to intracellular responses elicited by D1 and D2 receptors in the striatal projection neurons [19]. The melanocortin and dopamine mechanisms have interactions by which several significant physiological functions are regulated [20]. In the ventral tegmental area, the MC3R, and MC4R are located in dopaminergic neurons innervated by proopiomelanocortin (POMC) neurons in the arcuate nucleus [21]. MC4R plays a critical role in regulating D1 and D2 receptors in the nucleus accumbens [20]. Activation of the dopaminergic neurons in the ventral tegmental area by melanocortin and the dopamine-mediated effects of reward and reinforcement associated with the food intake is suppressed by the dopamine D1 and D2 receptors in the nucleus accumbens [21]. Studies reveal that cannabinoids, dopamine, and melanocortin are interconnected in CNS; however, there is little data on how their interactions affect the regulation of food intake in poultry. Therefore, the current work aimed to see how the central dopaminergic and cannabinoidergic mechanisms affect melanocortin-induced food intake in neonatal layer chickens.

Abbreviations-Cont'd

VTA: Ventral-tegmental area
POMC: Proopiomelanocortin
FD3: 3-h food-deprived
BW: Body weight
ACTH: Adrenocorticotropic hormone
α-MSH: α-Melanocyte-stimulating hormone
NPY: Neuropeptide Y

Results

In experiment No.1, a dose-dependent hypophagia was observed following the MTII (2.5, 5, and 10 ng) ICV injection in the 3-h food-deprived neonatal layer chickens 120 min following the injection in comparison with the control group ($p < 0.05$) (Fig. 1).

In experiment No.2, the MTII (10 ng) ICV injection reduces food intake considerably in the 3-h food-deprived layer chickens ($p < 0.05$), whereas in comparison with the control group ($p > 0.05$), the L-DOPA (125 nmol) ICV injection has no significant effects on the cumulative food intake. Furthermore, the MTII+L-DOPA co-injection greatly increased hypophagia induced by MTII in the chickens ($p < 0.05$) (Fig. 2).

In experiment No.3, hypophagia was observed following the MTII (10 ng) ICV injection in the 3-h food-deprived layer chickens ($p < 0.05$). On the other hand, in comparison with the control group ($p > 0.05$), the 6-OHDA (150 nmol) ICV injection has no significant effects on the cumulative food intake. Also, the MTII+6-OHDA co-injection greatly reduced hypophagia induced by MTII in 3-h food-deprived layer chickens at 30, 60, and 120 min following the injection ($p < 0.05$) (Fig. 3).

As shown in Figure 4, the MTII (10 ng) ICV injection greatly reduces cumulative food intake in the 3-h food-deprived layer chickens ($p < 0.05$). In comparison with the control group ($p > 0.05$), the SCH23390 (5 nmol) ICV injection has no significant effects on the cumulative food intake. The MTII+SCH23390 co-injection greatly weakened hypophagia induced by

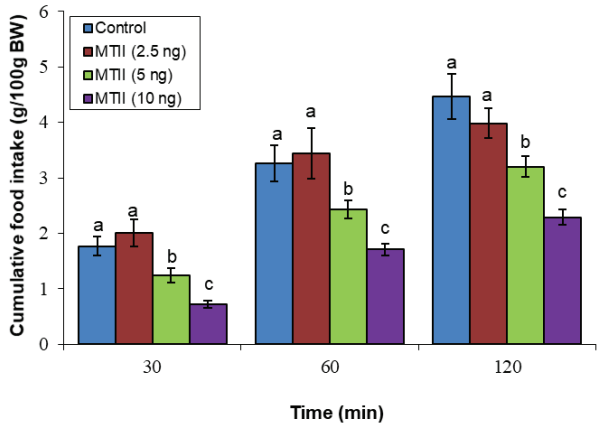


Figure 1.
Effect of intracerebroventricular injection of the MTII (MC3/MC4 receptors agonist) on food intake in the neonatal layer chickens. Data are expressed as mean ± SEM. Different letters (a, b, and c) indicate significant differences between treatments at each time ($p < 0.05$).

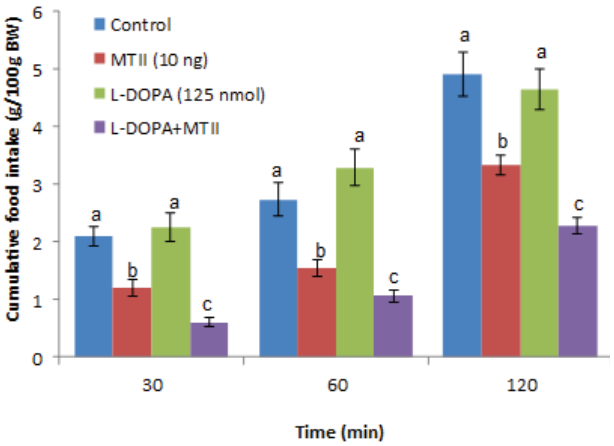


Figure 2.
Effect of intracerebroventricular injection of L-DOPA (dopamine precursor) on hypophagia-induced by MTII (MC3/MC4 receptors agonist) in the neonatal layer chickens. Data are expressed as mean ± SEM. Different letters (a, b, and c) indicate significant differences between treatments at each time($p < 0.05$).

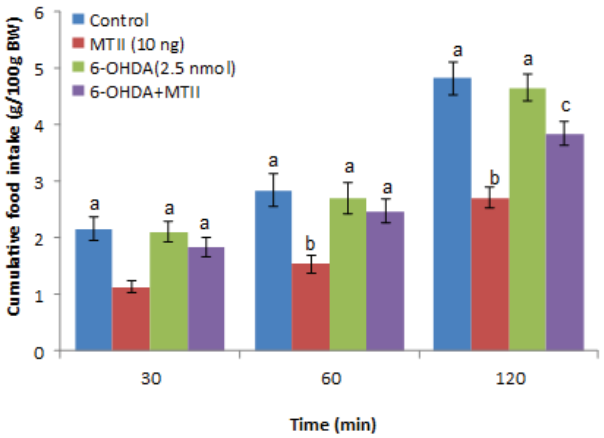


Figure 3.
Effect of intracerebroventricular injection of 6-OHDA (a dopamine depletion) on hypophagia-induced by MTII (MC3/MC4 receptors agonist) in the neonatal layer chickens. Data are expressed as mean ± SEM. Different letters (a, b, and c) indicate significant differences between treatments at each time($p < 0.05$).

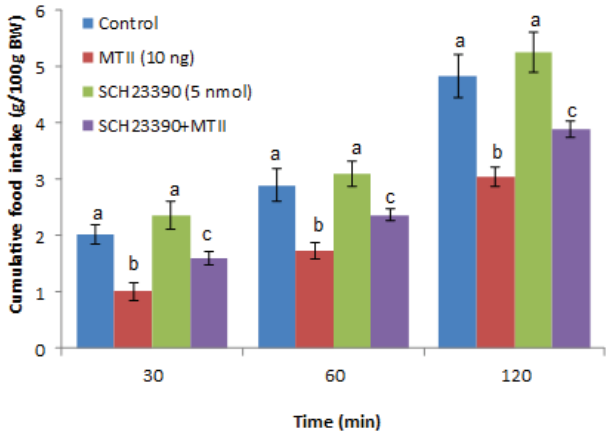


Figure 4.
Effect of intracerebroventricular injection of SCH23390 (D1 receptor antagonist) on hypophagia-induced by MTII (MC3/MC4 receptors agonist) in the neonatal layer chickens. Data are expressed as mean ± SEM. Different letters (a, b, and c) indicate significant differences between treatments at each time($p < 0.05$).

MTII in the chickens at 30, 60, and 120 min following the injection ($p < 0.05$) (Fig. 4).

In experiment No. 5, in comparison with the control group ($p > 0.05$), the MTII (10 ng) ICV injection greatly reduced food intake ($p < 0.05$), whereas the AMI-193 (5 nmol) ICV injection alone has no significant effects on the cumulative food intake. The MTII + AMI-193 co-injection greatly suppressed hypophagia induced by MTII in comparison with the control group ($p < 0.05$) (Fig. 5).

In experiment No. 6, in comparison with the control group ($p > 0.05$), the MTII (10 ng) ICV injection greatly reduced food intake ($p < 0.05$), whereas the NGB2904 (6.4 nmol) ICV injection has no significant effects on the cumulative food intake. Furthermore, the MTII + NGB2904 co-injection did not significantly affect hypophagia induced by MTII in the chickens ($p > 0.05$) (Fig. 6).

In experiment No.7, in comparison with the control group ($p > 0.05$), the MTII (10 ng) ICV injection greatly reduced food intake in the 3-h food-deprived layer chickens ($p < 0.05$), whereas the L-741,742 (6 nmol) ICV injection has no significant effects on the cumulative food intake. Furthermore, the

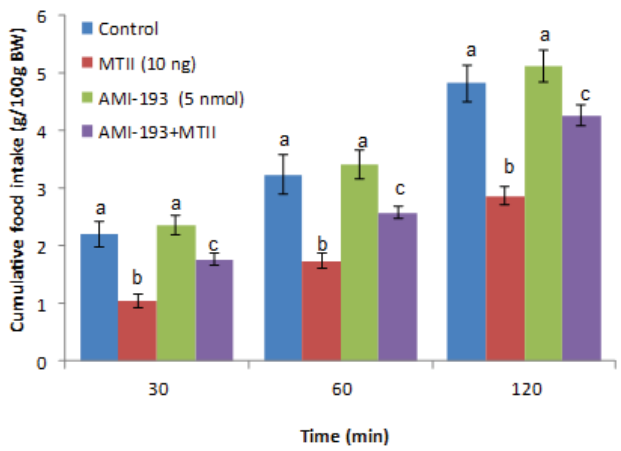


Figure 5.
Effect of intracerebroventricular injection of AMI-193 (D2 receptor antagonist) on hypophagia-induced by MTII (MC3/MC4 receptors agonist) in the neonatal layer chickens. Data are expressed as mean ± SEM. Different letters (a, b, and c) indicate significant differences between treatments at each time($p < 0.05$).

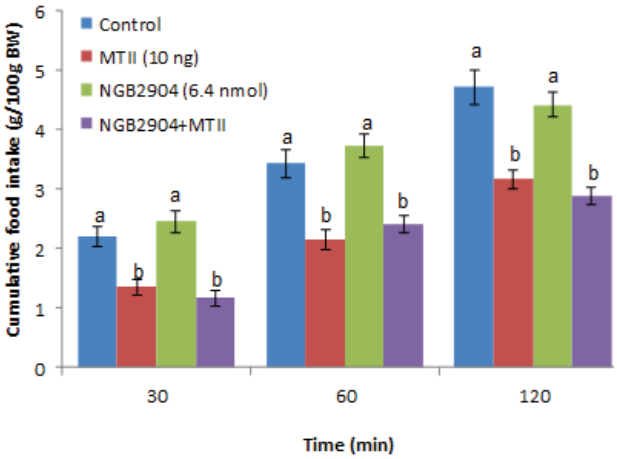


Figure 6.
Effect of intracerebroventricular injection of NGB2904 (D3 receptor antagonist) on hypophagia-induced by MTII (MC3/MC4 receptors agonist) in the neonatal layer chickens. Data are expressed as mean ± SEM. Different letters (a and b) indicate significant differences between treatments at each time($p < 0.05$).

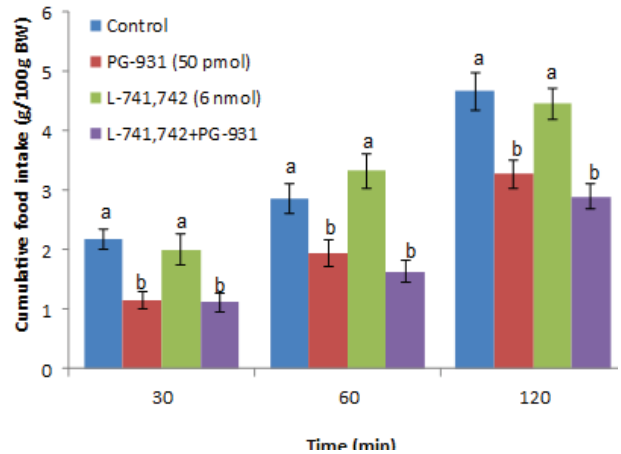


Figure 7.
Effect of intracerebroventricular injection of L-741,742 (D4 receptor antagonist) on hypophagia-induced by MTII (MC3/MC4 receptors agonist) in the neonatal layer chickens. Data are expressed as mean ± SEM. Different letters (a, b, and c) indicate significant differences between treatments at each time($p < 0.05$).

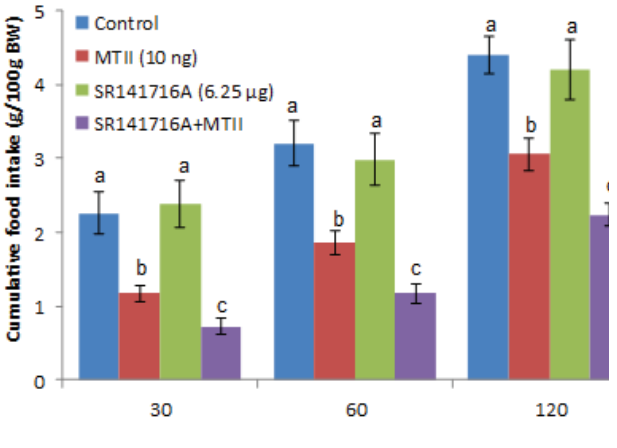


Figure 8.
Effect of intracerebroventricular injection of SR141716A (CB1 receptor antagonist) on hypophagia-induced by MTII (MC3/MC4 receptors agonist) in the neonatal layer chickens. Data are expressed as mean ± SEM. Different letters (a, b, and c) indicate significant differences between treatments at each time($p < 0.05$).

MTII+L-741,742 co-injection did not significantly affect MTII induced hypophagia in the neonatal layer chickens ($p > 0.05$) (Fig. 7).

As shown in Figure 8 (experiment No.8), in comparison with the control group ($p > 0.05$), the MTII (10 ng) ICV injection greatly reduces food intake ($p < 0.05$), whereas the SR141716A (6.25 µg) ICV injection has no significant effects on the cumulative food intake. In addition, the MTII+SR141716A co-injection greatly increased hypophagia-induced by MTII in comparison with the control group ($p < 0.05$) (Fig. 8).

In experiment No.9, in comparison with the control group ($p > 0.05$), MTII (10 ng) ICV injection greatly reduced food intake ($p < 0.05$). Also, the AM630 (5 µg) ICV injection has no significant effects on the cumulative food intake. Furthermore, the MTII + AM630 co-injection did not significantly affect hypophagia induced by MTII in 3-h food-deprived neonatal layer chickens ($p > 0.05$) (Fig. 9).

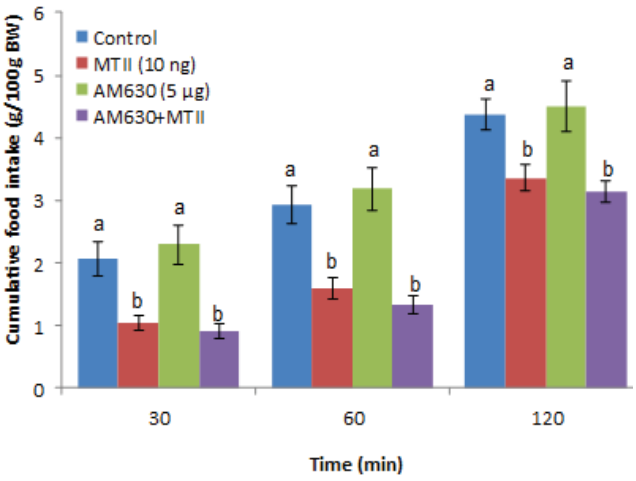


Figure 9.
Effect of intracerebroventricular injection of AM630 (CB2 receptor antagonist) on hypophagia-induced by MTII (MC3/MC4 receptors agonist) in the neonatal layer chickens. Data are expressed as mean ± SEM. Different letters (a and b) indicate significant differences between treatments at each time ($p < 0.05$).

Discussion

This research aimed to see how the melanocortin interacts with the dopaminergic and cannabinoidergic mechanisms to affect food intake in the neonatal layer chickens. As far as we know, this is the first study conducted to show how central melanocortin-induced hypophagia interconnect with these mechanisms in layer chickens. The results demonstrated dose-dependent hypophagia following the MTII (2.5, 5, and 10 ng) ICV injection in the 3-h food-deprived neonatal layer chickens. According to Ahmadi et al. [29, 30], the MTII (2.5, 5, and 10 ng) ICV injection reduces feeding behaviors in neonatal meat-type chickens in a dose-dependent manner. The MC3/4R activation reduces feeding behavior in rodents, while the MC3/4R antagonist ICV injection increases food intake [31]. Although both MC3R and MC4R are expressed in the rat brain, only MC4R appears to be found in the bird's brain [32]. A group of peptides known as melanocortin includes adrenocorticotropic hormone (ACTH) and various types of POMC-derived α-MSH in the pituitary gland.

[33]. POMC and α-MSH contribute to food intake regulation, of which; the latter is a key endogenous ligand for MC3R and MC4R in the arcuate nucleus [34, 35]. Also, the agouti-related protein (AgRP) is a natural antagonist of MC3R and MC4R, which is often co-expressed with neuropeptide Y(NPY), and the α-MSH effects can be suppressed by their co-injection [18]. Despite the identification of melanocortin-related signaling pathways regulating mammals' central food intake, little is known about melanocortin in birds [36]. According to the literature, hypophagia induced by MTII seems to be weakened with a protein kinase A (PKA) inhibitor [37].

The MTII+L-DOPA co-injection increased hypophagia induced by MTII in the chickens. The MTII+6-OHDA co-injection greatly suppressed hypophagia induced by MTII in the chickens. Also, co-injection of MTII+D1 receptors antagonist greatly suppressed hypophagia induced by MTII in the chickens. The co-injection of MTII+D2 receptors antagonist greatly suppressed hypophagia induced by MTII. Food intake reduces through D1 and D2 receptors in rats [13]. Besides, the D1 receptors mediate hypophagia induced by dopamine in the chickens, whereas others (D2-D4) may not be involved in appetite regulation of meat-type chickens [1]. Melanocortin acts through MC4R on mesolimbic dopamine pathways on feeding behavior and motivation to food intake suppressed in rats by the MCR agonist ICV injection into the ventral tegmental area containing dopaminergic neurons [38]. However, this effect is reversed by the SHU9119 (MCR antagonist) ICV injection [38]. The anorexigenic and orexigenic effects of α-MSH and AgRP were blocked completely, respectively, by the nonselective α-flupenthixol (dopamine antagonist), reflecting dopamine signaling pathways are involved in the regulating melanocortin peptides and AgRP effects [39]. The process includes dopaminergic neurons in the ventral tegmental area, responsible for the effects of reward and reinforcement, and MC4R expressing neurons in the nucleus accumbens [39]. MC4R regulates D1 and D2 neurons in the nucleus accumbens and is elicited to consume sweet foods [20]. Activation of dopaminergic neurons in the ventral tegmental area by melanocortin and D1

and D2 neurons of the nucleus accumbens suppresses the dopamine-mediated feeding behavior [21]. In the ventral tegmental area, both MC3R and MC4R are expressed in dopaminergic neurons innervated by POMC-expressing neurons in the arcuate nucleus [21]. Data on the colocalization of dopamine and MCR in neurons for their regulatory role in appetite regulation and food intake support the melanocortin-dopamine interactions [40]. In this regard, Yoon and Baik [41] reported that MC4R and D2 receptors in the hypothalamic area are responsible for food intake and control of energy homeostasis.

Based on the results, co-injection of MTII + CB1 receptors antagonist increased hypophagia induced by MTII while CB2 receptors had little effect on hypophagia induced by MTII in the neonatal layer chickens. Food intake increases through both CB1 and CB2 receptors in the layer chickens, similar to mammals [7, 41, 43], but not in broilers where only the CB2 receptors interact on feeding [8, 9]. The SR141716SA or AM251 ICV injection reduced food intake in normal mice but not in CB1 knockout mice, indicating that the endocannabinoids contribute to appetite by activating CB1 receptors [41]. Also, AM251 has been shown to decrease food intake in food-restricted rats [15]. In the present study, the potential effect of SR141716A (an analog of AM251) was examined on food intake in 3-h food-deprived birds. Based on the evidence, an interaction exists between melanocortin and cannabinoidergic mechanisms on feeding behavior through MC4R and CB1 receptors [44]. SR141716 and α-MSH weakened food intake, and ICV injection of sub-effective doses of SR 141716 and α-MSH weakened baseline food intake synergistically [44]. Both cannabinoid and melanocortin receptors localized in the hypothalamus and administration of THC, SR141716, and MTII lead to c-fos expression, a marker of neural activation [44]. Despite direct cellular mechanisms underlying the interconnection of these systems, it is assumed that they may interact at the signal transduction by increasing cAMP synthesis through an effect on Gi proteins. GPCRs include cannabinoids and melanocortin, and blockade of CB1 receptors and stimulation of MC4R enhance cAMP production [45]. Additionally, it is assumed that synergistic interaction between the cannabinoidergic and melanocortin mechanisms is mediated by the opioidergic system [44]. Also, leptin synoptically interacts with the melanocortinergic neurons, while cannabinoid and NPY systems are modulated by the melanocortin system. Thus, signaling by melanocortin receptor seems to be downstream of leptin receptors and upstream of the endogenous cannabinoid, NPY, and opioid-producing neurons. Given the approximate 300 million years of evolutionary distance between mammals and birds, it is not surprising that the differences in the central food intake and energy expenditure regulation have been identified [9].

In conclusion, these results indicated that melanocortin-induced hypophagia is mediated through D1, D2, and CB1 receptors in the neonatal layer chickens. In a rat model, many studies have been conducted on the central regulation of food intake. It is well established that the central food intake regulation differs in mammals and birds [14]. As a result, it is reasonable to believe that regulatory systems in birds manage these processes [10]. As shown, there has never been a study on the melanocortin interconnection with dopaminergic and cannabinoidergic mechanisms on food intake in birds. Therefore, the authors could not compare the results. This data can be seen as a starting point for learning about the central regulation of feeding behavior in chickens.

Materials & Methods

Animals

A total of 396 one-day-old layer chickens (Hy-Line) were purchased from a local hatchery (Morghak Co., Iran). Birds were kept in stabilizing electrically heated batteries at a temperature of 32 °C ± 1, with a relative humidity of 40-50% and a 23:1 lighting/dark period [22]. They were held as flocks for two days, and then a single cage was allocated per bird randomly. During the study, the birds were fed with a commercial diet comprising 2850 kcal/kg metabolizable energy and 21% crude protein, with unlimited access to diet and freshwater (Chineh Co., Iran). The 3-h food-deprived was applied before the injections, while the birds had unlimited access to water. ICV injections were performed at five days of age. Animal handling and experimental practices were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, National Institutes of Health, USA (publication No. 85-23, revised 1996) and existing Iranian government animal welfare regulations, which were approved by the Institutional Animal Ethics Committee (IAEC), Faculty of Veterinary Medicine, University of Tehran.

Experimental medications

The used medications, including MTII (MC3 and MC4 receptors), SCH 23390 (D1 receptor antagonist), AMI-193 (D2 receptor antagonist), NGB2904 (D3 receptor antagonist), L-741,742 (D4 receptor antagonist), L-DOPA (precursor of dopamine), 6-OHDA (6-hydroxy dopamine), SR141716A (CB1 receptor antagonist), AM630 (CB2 receptor antagonist), and Evans blue were purchased from Sigma-Aldrich (USA) and Tocris Co (UK). All of the medications were first dissolved in absolute dimethyl sulfoxide (DMSO) and then diluted with 0.85% saline containing Evans blue with a ratio of 1/250 (0.4% DMSO). The resulting mixture containing Evans blue-contained DMSO/saline mixture was utilized as a control group.

ICV injection protocol

Birds were randomly divided into nine study groups, each with four sub-groups (n = 44). The chickens were weighed and divided into study groups concerning their body weight before each experiment, ensuring that the mean body weight between treatment groups was as standardized as possible. The chickens

were ICV injected once in each of the experiments by applying a microsyringe (Hamilton, Switzerland) with no anesthesia, as defined by Davis et al. [23] and Furuse et al. [24]. According to Van Tienhoven and Juhasz [25], the head of the chickens was held with an acrylic device with a 45º bill holder and a calvarium parallel to the table surface. The right lateral ventricle's skull was pierced using an orifice plate. When a microsyringe was inserted into the ventricle through the orifice plate, the injected needle tip was just 4 mm under the skull skin [26]. All injections were performed in a volume of 10 µL [27]. The control group received control solution (10 µL) [27]. In newly hatched chickens, this method causes no physiological stress [28]. The chickens were sacrificed by decapitation at the end of trials to demonstrate injection accuracy confirmed by Evans blue and sliced frozen brain tissues. Although birds in each of the groups were given injections, only the data for those who had dye in their lateral ventricle were analyzed (11 chickens per group). All experiments were conducted from 08:00 a.m. to 1:30 p.m.

Feeding experiments

To examine the potential effect of specific dopaminergic (D1, D2, D3, and D4) and cannabinoidergic (CB1 and CB2) receptors on the melanocortin-induced feeding behavior in the 3-h food-deprived newly hatched chickens, nine experiments were conducted. In experiment No.1, the control solution and MTII (MC3 and MC4 receptors; 2.5, 5, and 10 ng) were injected into chickens. In experiment No.2, control solution, L-DOPA (125 nmol), MTII (10 ng), and a combination of them were injected intracerebroventricularly. In experiment No.3, the 3-h food-deprived birds were intracerebroventricularly injected with a control solution, 6-OHDA (150 nmol), MTII (10 ng), and 6-OHDA + MTII co-injection. In experiment No.4, the 3-h food-deprived layer chickens were intracerebroventricularly injected with control solution, SCH23390 (5 nmol), MTII (10 ng), and received the SCH23390+MTII co-injection. In experiment No.5, the control solution, AMI-193 (5 nmol), MTII (10 ng), and a combination of them were intracerebroventricularly injected into the birds. In experiment No.6, chickens were intracerebroventricularly injected with control solution, NGB2904 (6.4 nmol), MTII (10 ng), and NGB2904 + MTII. In experiment No.7, control solution, L-741,742 (6 nmol), MTII (10 ng), and L-741,742 + MTII were injected. In experiment No.8, the 3-h food-deprived birds were intracerebroventricularly injected with control solution, SR141716A (6.25 µg), MTII (10 ng), and SR141716A+MTII. In experiment No.9, control solution, AM630 (5 µg), MTII (10 ng), and AM630 + MTII were injected. Immediately following the injection, food was provided to the birds, and cumulative food intake (g) was measured at 30, 60, and 120 min following the injection. Food intake (g) was calculated as percent of body weight (g/100g BW) to minimize the effect of body weight on the amount of food intake. The doses of medications were determined according to the previous studies [4, 10, 29, 30].

Statistical analysis

In this study, nine experiments were designed. Each experiment included four groups (I-IV). In all groups, a sole injection was done. Also, the result of each experiment was apart from the other experiments. Cumulative food intake (as a percent of body weight) from each experiment was analyzed by repeated measure two-way analysis of variance (ANOVA). All analyses were conducted using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Means were compared by Tukey Kramer test ($p < 0.05$), and data was presented as mean ± SEM (standard error of the mean).

Research involving human participants and/or animals

This manuscript does not contain any studies with human subjects performed by any of the authors. According to the Guide for the Care and Use of Laboratory Animals, all experiments were executed and were approved by the institutional animal ethics committee.

Authors' Contributions

All authors provided critical feedback and helped shape the research, analysis and manuscript.

Acknowledgements

The authors thank the central laboratory (Dr. Rastegar Lab.) of the Faculty of Veterinary Medicine, the University of Tehran for cooperation. This research is conducted as a part of the PhD thesis of the first author.

Competing Interests

Authors have no potential conflicts of interest.

References

1. Zendehdel M, Ghashghayi E, Hassanpour S, Baghbanzadeh A, Jonaidi H. Interaction between opioidergic and dopaminergic systems on food intake in neonatal layer type chickens. Int J Peptide Res Ther. 2016; 22:83-92.

2. Shojaei M, Yousefi A, Zendehdel M, Khodadadi M. Food intake regulation in birds: the role of neurotransmitters and hormones.Iran J Vet Med. 2020; 14:99–115.

3. Fernández-Ruiz J, Hernández M, Ramos JA. Cannabinoid–dopamine interaction in the pathophysiology and treatment of CNS disorders. CNS Neurosci Ther. 2010; 16(3):e72-e91.

4. Alizadeh A, Zendehdel M, Bababpour V, Charkhkar S, Hassanpour S. Role of a cannabinoidergic system on food intake in neonatal layer-type chicken. Vet Res Commun. 2015; 39:151–157.

5. Storr MA, Sharkey KA. The endocannabinoid system and gut-brain signalling. Current Opinion Pharmacol. 2007; 7:575–582.

6. Sierra S, Luquin N, Rico AJ, Gómez-Bautista V, Roda E, Dopeso-Reyes IG, Vázquez A, Martínez-Pinilla E, Labandeira-García JL, Franco R, Lanciego JL. Detection of cannabinoid receptors CB1 and CB2 within basal ganglia output neurons in macaques: changes following experimental parkinsonism. Brain Struct Funct. 2015;220(5):2721-38.

7. Alizadeh A, Zendehdel M, Babapour V, Charkhkar S, Hassanpour S. Role of cannabinoidergic system on food intake in neonatal layer-type chicken. Vet Res Commun. 2015; 39:151–157.

8. Emadi L, Jonaidi H, Hosseini Amir Abad E. The role of central CB2 cannabinoid receptors on food intake in neonatal chicks. J Comp Physiol A. 2011; 197:1143-1147.

9. Novoseletsky N, Nussinovitch A, Friedman-Einat M. Attenuation of food intake in chicks by an inverse agonist of cannabinoid receptor 1 administered by either injection or ingestion in hydrocolloid carriers. Gen Comp Endocrinol. 2011; 170:522-527.

10. Hassanpour S, Zendehdel M, Babapour V, Charkhkar S. Endocannabinoid and nitric oxide interaction mediates food intake in neonatal chicken. Br Poult Sci. 2015; 56(4):443-451.

11. : Zendehdel M, Ghashghayi E, Hassanpour S, Baghbanzadeh A, Jonaidi, H. Interaction between opioidergic and dopaminergic systems on food intake in neonatal layer type chicken. International Journal of Peptide Research and Therapeutics. 2016; 22(1): 83-92.

12. Zanganeh F, Panahi N, Zendehdel M, Asghari A. Interconnection between Adrenergic and Dopaminergic Systems in Feeding Behavior in Neonatal Chicks. Archives of Razi Institute. 2021; 76(2): 345-358.

13. Volkow ND, Wang GJ, Baler RD. Reward, dopamine and the control of food intake: implications for obesity. Trends in Cog Sci. 2011;15(1):37-46.

14. Zendehdel M, Ebrahimi-Yeganeh A, Hassanpour S, Koohi MK. Interaction of the dopaminergic and Nociceptin/Orphanin FQ on central feed intake regulation in chicken. Br Poult Sci. 2019; 60(3):317-322.

15. : Rahmani B, Ghashghayi E, Zendehdel M, Khodadadi M, Hamidi B. (2021). The Crosstalk Between Brain Mediators Regulating Food Intake Behavior in Birds: A Review. International Journal of Peptide Research and Therapeutics. 2021; 27: 2349–2370.

16. Alvaro JD, Taylor JR, Duman RS. Molecular and behavioral interactions between central melanocortins and cocaine. J Pharmacol Exper Ther. 2003; 304:391–399.

17. Schneeberger M, Gomis R, Claret M. Hypothalamic and brainstem neuronal circuits controlling homeostatic energy balance. J Endocrinol. 2014; 220: T25–T46.

18. Strader AD, Schiöth HB, Buntin JD. The role of the melanocortin system and the melanocortin-4 receptor in ring dove (Streptopelia risoria) feeding behavior. Brain Res. 2003; 960:112-121.

19. Cheer JF, Wassum KM, Sombers LA, et al. Phasic dopamine release evoked by abused substances requires cannabinoid receptor activation. Journal of Neuroscience. 2007; 27:791–795.

20. Derkach KV, Romanova IV, Shpakov AO. Functional interaction between the dopamine and melanocortin systems of the brain. Neurosci Behav Physiol. 2018; 48 (2):213-219.

21. Cui H, Lutter M. The expression of MC4Rs in D1R neurons regulates food intake and locomotor sensitization to cocaine. Genes Brain Behav. 2013;12(6):658-65.

22. Olanrewaju HA, Purswell J, Collier SD, Branton SL. Effects of light ingress through ventilation fan apertures on selected blood variables of male broilers. Int J Poult Sci. 2017; 16: 288-295.

23. Davis JL, Masuoka DT, Gerbrandt LK, Cherkin A. Autoradiographic distribution of L- proline in chicks after intracerebral injection. Physiol Behav. 1979;22: 693-695.

24. Furuse M, Matsumoto M, Saito N, Sugahara K, Hasegava S. The central corticotropin-releasing factor and glucagon-like peptide -1 in food intake of the neonatal chick. Eur J Pharmacol. 1997; 339: 211-214.

25. Van Tienhoven A, Juhasz LP. The chicken telencephalon, diencephalon and mesencephalon in sterotaxic coordinates. J Comp Neurol. 1962; 118:185-197.

26. Jonaidi H, Noori Z. Neuropeptide Y-induced feeding is dependent on GABAA receptors in neonatal chicks. J Comp Physiol A. 2012; 198: 827-832.

27. Furuse M, Ando R, Bungo T, Ao R, ShimoJO M, Masuda Y. Intracerebroventricular injection of orexins does not stimulate food intake in neonatal chicks. Br Poult Sci. 1999; 40: 698-700.

28. Saito ES, Kaiya H, Tachibana T, Denbow DM, Kangawa K, Furuse M. Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. Regul Peptides. 2005; 125:201-208.

29. Ahmadi F, Zendehdel M, Babapour V, Panahi N. CRF1/CRF2 and MC3/MC4 receptors affect glutamate- induced food intake in neonatal meat-type chicken. Br J Poult Sci. 2019; 21(1):1-10.

30. Ahmadi F, Zendehdel M, Babapour V, Panahi N, Hassanpour S, Khodadadi M. Modulatory function of NMDA glutamate receptor on MC3/MC4 receptors agonist-induced hypophagia in neonatal meat-type chicken. Vet Res Commun. 2017; 41(4):241-248.

31. Ahmadi F, Zendehdel M, Babapour V, Panahi N. CRF1/CRF2 and MC3/MC4 receptors affect glutamate- induced food intake in neonatal meat-type chicken. Br J Poult Sci. 2019; 21(1):1-10.

32. Takeuchi S, Takahashi S. Melanocortin receptor genes in the chicken-tissue distributions. Gen Comp Endocrinol. 1998; 112:220-231.

33. Campos CA, Ritter RC. NMDA-type Glutamate Receptors Participate in Reduction of Food Intake Following Hindbrain Melanocortin Receptor Activation. American Journal of Physiology-Regulatory, Integrative Comp Physiol. 2015; 308(1): R1-9.

34. Takeuchi S, Takahashi S. A possible involvement of melanocortin 3 receptor in the regulation of adrenal gland function in the chicken. Biochimica et Biophys Acta. 1999; 1448: 512-518.

35. Takeuchi S, Teshigawara K, Takahashi S. Molecular cloning and characterization of the chicken proopiomelanocortin (POMC) gene. Biochimica et Biophys Acta. 1999; 1450: 452-459.

36. Campos CA, Shiina H, Ritter RC. Central Vagal Afferent Endings Mediate Reduction of Food Intake by Melanocortin-3/4 Receptor Agonist. J Neurosci. 1994; 34(38):12636-12645.

37. Carlos A, Campos C, Ritter RC. NMDA-type glutamate receptors participate in reduction of food intake following hindbrain melanocortin receptor activation. American Journal of Physiology Regulatory Integrative Comp Physiol. 2015; 308:R1-R9.

38. Roseberry AG. Altered feeding and body weight following melanocortin administration to the ventral tegmental area in adult rats," Psychopharmacol. (Berl.). 2013; 226(1): 25–34.

39. Pandit R, van der Zwaal EM., Luijendijk MC, et al. Central melanocortins regulate the motivation for sucrose reward," PLoS One. 2015; 10(3):e0121768.

40. Cui H, Mason BL, Lee C, et al. Melanocortin 4 receptor signaling in dopamine 1 receptor neurons is required for procedural memory learning. Physiol Behav. 2012; 106(2): 201–210.

41. Yoon YR, Baik JH. Melanocortin 4 receptor and dopamine D2 receptor expression in brain areas involved in food intake. Endocrinol Metabol. 2015; 30: 576-583.

42. Di Marzo V GS, Wang L, Liu J, Batkai S, Jarai Z, Fezza F, Miura GI, Palmiter RD, Sugiura T, Kunos G. Leptin regulated endocannabinoids are involved in maintaining food intake. Nature. 2001; 410: 822-825.

43. Wiley JL, Marusich JA, Zhang Y, Fulp A, Maitra R, Thomas BF, Mahadevan A. Structural analogs of pyrazole and sulfonamide cannabinoids: Effects on acute food intake in mice. Eur J Pharmacol. 2012; 695: 62-70.

44. Verty ANA, McFarlane JR, McGregor IS, Mallet PE. Evidence for an interaction between CB1 cannabinoid and melanocortin MCR-4 receptors in regulating food intake. Endocrinol. 2004; 145(7):3224–3231.

45. Daniels D, Patten CS, Roth JD, Yee DK, Fluharty SJ. Melanocortin receptor signaling through mitogen-activated protein kinase in vitro and in rat hypothalamus. Brain Res. 2003; 986: 1–11.

46. Hen G, Yosefi S, Ronin A, Einat P, Rosenblum CI, Denver RJ, Friedman-Einat M. Monitoring leptin activity using the chicken leptin receptor. J Endocrinol. 2008; 197: 325-333.

47. Farkašová H, Hron T, Pačes J, Pajer P, Elleder D. Identification of a GC-rich leptin gene in chicken. Agri Gene. 2016; 1:88–92.

48. Khodadadi M, Zendehdel M, Baghbanzadeh A, Babapour V. Consequence of dopamine D2 receptor blockade on the hyperphagic effect induced by cannabinoid CB1 and CB2 receptors in layers. Br Poult Sci. 2017 Oct;58(5):585-593.

49. Yoefvand S, Hamidi F. The role of ventromedial hypothalamus receptors in the central regulation of food intake. Int J Pept Res Ther. 2021; 27(1): 689-702

How to cite this article
Bameri M, Zendehdel M, Vazir B, Asghari A, Panahi N. Evidence for an interaction between cannabinoidergic and dopaminergic systems with melanocortin MC3/ MC4 receptors in regulating food intake of neonatal chick. Iran J Vet Sci Technol. 2021;13(2): 37-46.
DOI: https://doi.org/10.22067/ijvst.2021.69380.1028
URL: https://ijvst.um.ac.ir/article_40556.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Jun- 20
Accepted after revision: 2021- Oct- 24
Published online: 2021- Dec- 20

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2021.70946.1055

Molecular identification and phylogenetic analysis of *Pulex irritans* in different regions of Iran

Shahin Seidy, Mousa Tavassoli, Farnaz Malekifard

Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, West Azerbaijan, Iran.

ABSTRACT

The present study was conducted to perform a molecular comparison of *Pulex irritans* based on the mitochondrial genome in four different climatic regions including the Caspian Sea region, a mountainous region, Persian Gulf region, and the Central Desert region, and based on nuclear ribosome genome in the west and northwestern Iran. A total of 1937 adult flea samples were collected including 1019 *P. irritans* (52.61%) and 918 *Ctenocephalides canis* (47.39%) from various hosts including humans (14.1%), sheep (22%), goats (33.5%), dogs (25.6%) and houses (6.7%) between April 2018 and May 2019. The samples collected from different hosts had similar morphological characteristics. However, there were slight differences based on mitochondrial markers and nuclear ribosomal markers in the study populations. The results from the phylogenetic tree based on three nuclear ribosome and mitochondrial markers showed that despite the slight differences in this sequence of different hosts and cities, all samples from different regions are in the same phylogeny. The results of ribosomal and mitochondrial genome analysis showed that these pieces are useful for demonstrating intraspecific similarity, and differentiation at species level and genus of *P. irritans*.

Keywords

Flea, Iran, ITS1, ITS2, Cox1, Pulex irritans

Number of Figures: 8
Number of Tables: 2
Number of References:: 30
Number of Pages: 12

Abbreviations

COX1: Cytochrome oxidase 1
PCR: Polymerase chain reaction
ITS1: Internal transcribed spacer I
ITS2: Internal transcribed spacerII
mtDNA: Mitochondrial DNA
rDNA: ribosomal DNA
MBST: Molecular Biological System Transfer
µl: microliter

Corresponding author: *Moussa Tavassoli*
Email: *mtavassoli@urmia.ac.ir*
Tel: *+98 (44)1277-1926*

Introduction

Pulex irritans belongs to *Siphonaptera* as a relatively-small order of wingless holometabolous insects, known as ectoparasites in their adult stage [1].

Certain flea species can transmit pathogens such as *Yersinia pestis*, *Rickettsia typhi,* and *Bartonella henselae* through their bites, feces and saliva. Biting can also cause severe itching and skin infections [2, 3, 4]. *P. irritans* can cause serious health complications and transmit diseases in many parts of the world [5]. Global warming, international trade, travel, and population growth influence the epidemiology of these infections [6, 7, 8].

Cladistic analysis using morphological features including head, chest, and abdomen have been conducted to classify various fleas, nevertheless, accurately identifying certain flea species is difficult owing to variations in their morphological characteristics. Molecular markers are therefore used to accurately identify different flea species and investigate their phylogenetic relationships [9, 10, 11, 12].

Molecular markers can be used to effectively characterize and specify an insect owing to their high stability and conserved nature; for instance, ribosomal DNA (rDNA) with its subunits such as 28s, 18s, 5.8s, ITS1 and ITS2 is commonly used to identify insect species [9, 10, 11]. As the largest protein-encoding gene in the mitochondria of metazoan organisms, the cytochrome oxidase subunit I (COXI) gene has been extensively used to perform phylogenetic research and identify species and their differences [13, 14].

Accurately identifying flea species is crucial given that they constitute a vector of dangerous pathogens and thus a serious threat to public health. This study employed ITS1, ITS2, and COX1 as molecular markers to investigate the molecular characteristics of different populations of P. irritans and their phylogenetic relationships in four areas in Iran.

Results

After morphological examinations using reliable identification keys for Iranian fleas, 1019 (52.61%) out of 1937 samples were identified as *P. irritans* (Table 1). Furthermore, the amplicons obtained from PCR for ITS1, ITS2 (rDNA), and COX1 (mtDNA) were approximately 1000 bp, 500 bp and 700 bp, respectively. The Clustal Omega based comparison of the ITS1 sequence in *P. irritans* showed a similarity of 99.59% and a molecular diversity of 0.41% between the sequences in ten different regions (Figure 1). A 100% similarity was observed among the sequences of Sanandaj (MN684797.1), Khorramabad (MN684787.1), Urmia (MN684784.1), Bahar (MN684777.1), and Kermanshah(MN 684776.1). These sequences were also similar (99.79%) to those of Hamadan (MN684779.1), Kuhdasht (MN684790.1), Kamyaran (MN684786.1), Gilangharb(MN684775.1), and Mahabad (MN684778.1) with four nucleotide differences. Four nucleotide polymorphisms were observed at positions 152, 165, 193 and 279 within the ITS1 spacer in the ten populations. Sequencing the ITS1 fragment showed three 99-bp replicas in the first half of the sequence beginning at positions 146, 246, and 352 and ending at position 431. Comparing the ITS2 gene sequences in the ten regions showed a 100% similarity between the sequences (Figure 2). Comparing the COX1 sequence of the nuclear genome (mtDNA) in the four different geographical zones (accession ID: MN173748-761) found all the species to belong to *P. irritans* and showed a 99.86% similarity between the sequences (Figure 3).

Multiple alignments of amino acids in COX1 showed a 99.86% similarity and only one difference at position 54 of the methionine isolate and other isoleucine isolates in Hamadan (Figure 4). Two subclades of the phylogenetic tree based on the similarities between COX1 sequences in the present study and registered sequences in GenBank comprised subclade A1, including *P. irritans* of the present and previous studies conducted in Iran and that isolated in China, Turkey, Jerusalem, Spain and Croatia. Subclade A2 included *Ctenocephalides felis* in Iran and *C. canis* in Turkey. Subclades A3 and A4 included *C. orientis* and *C. canis,* respectively. Iranian subclade B also included *Nosopsyllus fasciatus* and *Xenopsylla cheopis* (Figure 5).

Three subclades of the phylogenetic tree based on the similarities between ITS1 sequences in the present study and registered sequences in GenBank comprised subclade A, including the sequences of the present and previous studies conducted in Iran. Subclades B and C included *C. canis* and *N. fasciatus,* respectively (Figure 6).

Four subclades of the phylogenetic tree based on the similarities between ITS2 sequences in the present study and registered sequences in GenBank comprised subclade C, including the sequences of the present study. Subclades A and B included *C. canis* and *X. cheopis,* respectively . Subclade D also included *N. fasciatus* (Figure 7).

The morphological characteristics of *P. irritans* were consistent with the molecular findings. Analyzing the partial COX1 gene can help identify *P. irritans* species and assess their intraspecific similarity. ITS1 and ITS2 sequences could also be used as useful

Abbreviations-Cont'd

µm: micromolar
mM: millimolar

Seydi et al., IJVST 2021; Vol.13, No.2
DOI: 10.22067/ijvst.2021.70946.1055

Table 1.
Nucleotide sequence and specificity of the primers used.

Isolation source	Location	Longitude, latitude	Total	host					Accession number		
				Sheep	Goat	Human	Dog	Home	COX1	ITS1	ITS2
Kermanshah	Ker	34.1397° N, 45.9206° E	86 (8/4%)	41(47/6)	19(22)	4(4/6)	16(18/6)	6(6/9)	MN173752.1	MN684755.1	MN684792.1
	Ghi	34.3277° N, 47.0778° E	84 (8/4%)	30(35/7)	17(20/2)	15(17/8	7(8/3)	15(17/8)	MN173749.1	MN684776.1	MN684782.1
Kurdestan	San	35.3219° N, 46.9862° E	92(9%)	22(23/9)	25(27/1)	20(21/7)	21(22/8)	4(4/3)	MN173760.1	MN68797.1	MN684801.1
	Kam	34.7956° N, 46.9368° E	62(6%)	26(41/9)	3(4/8)	9(14/5)	24(38/7)	-	MN173754.1	MN684796.1	MN684781.1
West Azerbaijan	Urm	37.5498° N, 45.0786° E	139 (13/64%)	58(41/7)	45(32/3)	6(4/3)	30(21/5)	-	MN173761.1	MN684784.1	MN684780.1
	Mah	36.7684° N, 45.7337° E	63(6/1%)	4(6/3)	30(47/6)	13(20/6)	14(22/2)	2(3/1)	MN173758.1	MN684778.1	MN684803.1
Hamadan	Ham	34.9083° N, 48.4393° E	79(7/75%)	15(18/9)	35(44/3)	3(3/7)	26(32/9)	-	MN173753.1	MN684779.1	MN684800.1
	Bah	34.9083° N, 48.4393° E	101(9/91%)	19(18/8)	50(49/5)	2(1/9)	29(28/7)	1(0/9)	MN173751.1	MN684777.1	MN684798.1
Lorestan	Kho	33.4647° N, 48.3390° E	98(9/61%)	8(8/1)	33(33/6)	8(8/1)	44(44/8)	5(5/1)	MN173756.1	MN684787.1	MN684794.1
	Kuh	33.5275° N, 47.6111°E	93(9/12%)	2(2/1)	45(48/3)	3(3/2)	40(43)	3(3/2)	MN173757.1	MN684790.1	MN684793.1
Khozestan	Ahv	31.3183° N, 48.6706° E	21(2%)	-	5(23/8)	10(47/6)	3(14/2)	3(14/2)	MN173750.1	-	-
Mazandaran	Sar	36.5659° N, 53.0586° E	31(3%)	-	15(48/3)	9(29)	2(6/4)	5(16/1)	MN173759.1	-	-
Kerman	Kerm	30.2839° N, 57.0834° E	15(1/47%)	3(20)	-	2(13/3)	5(33/3)	10(66/6)	MN173748.1	-	-
North Khorasan	Esf	37.4710° N, 57.1013° E	55(5/39%)	-	-	40(72/7)	-	15(27/2)	MN173755.1	-	-

Sampling sites/regions: West (Ker: Kermanshah, Ghi: Gilan-e Gharb, Kam:Kamiyaran, San: Sanandaj, Ham: Hamedan, Bah: Bahar, Kuh: Kuhdasht, Kho: Khorramabad), South (Ahv: Ahvaz, Kem: Kerman, (North (Sar: Sari), North East (Esf: Esfarayen), and Northwest (Urm: Urmia, Mah: Mahabad) of Iran.

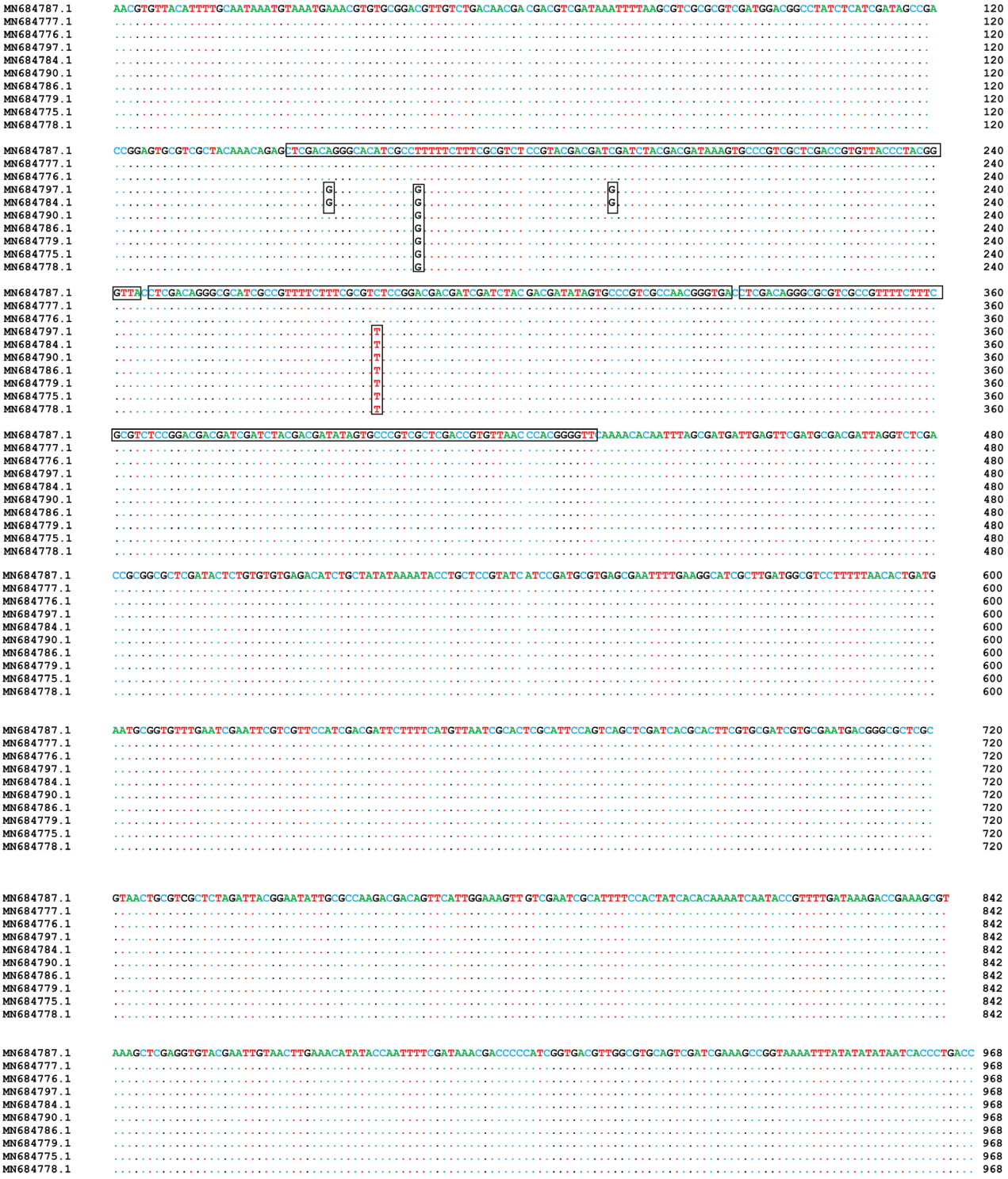


Figure 1.
Alignments of partial ribosomal DNA (ITS1) sequences in *Pulex irritans* isolated from sheep, human, goats, and dogs in the west and northwest of Iran (Kho(sheep), Bah(sheep), Ker(human), San(goat), Urm(sheep), Kuh(human), Kam(human), Ham(sheep), Ghi(goat), and Mah(dog) with accession nos. MN684787.1, MN684777.1, MN 684776.1, MN684797.1, MN684784.1, MN684790.1, MN684786.1, MN684779.1, MN684775.1, MN684778.1) Nucleotides in horizontal boxes display repeated units, and vertical boxes indicates the polymorphic site. Bah: Bahar; Ghi: Gilan-e Gharb; Ham: Hamedan; Kam: Kamiyaran; Ker: Kermanshah; Kho:Khorramabad; Kuh: Kuhdasht; Mah: Mahabad; San:Sanandaj; Urm: Urmia.

markers for species-level differentiation and intra-specific similarity determination. The COX1 gene is, however, more efficient than the ITS1 and ITS2 genes in detecting genus, species and intra-species similarities. The diversity at the genus level and even between members of the same genus caused by high levels of replacement in the Internal transcribed spacer counteracted their beneficial effects. Given this diversity, the sequences of the heterogenic versions of ITS fragments are determined in cloning plasmids and then specifically, which requires laborious and costly studies[15].

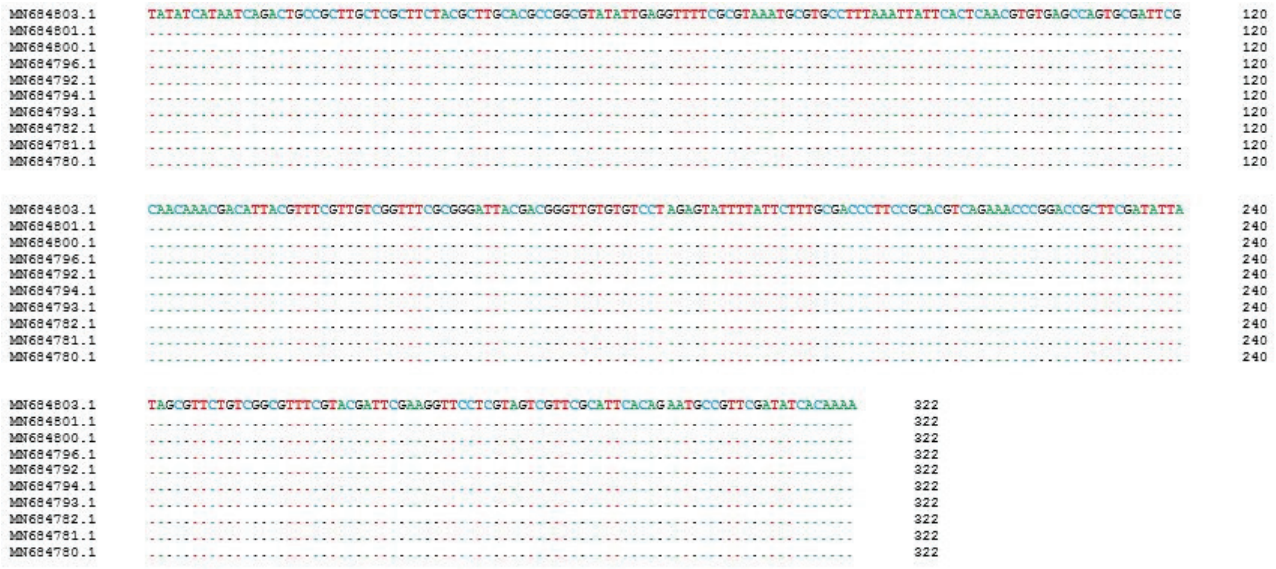


Figure 2.
Alignments of partial ribosomal DNA (ITS2) sequences in Pulex irritans isolated from sheep, human, goats, and dogs in the west and northwest of Iran (Mah(human), San(human), Ham(sheep), Bah(human), Ghi(goat), Kho(human), Kuh(sheep), Ker(dog), Kam(dog), and Urm(dog) with accession nos. MN684803.1, MN684801.1, MN 684800.1, MN684796.1, MN684792.1, MN684794.1, MN684793.1, MN684792.1, MN684781.1, MN684780.1). Bah: Bahar; Ghi: Gilan-e Gharb; Ham: Hamedan; Kam: Kamiyaran; Ker: Kermanshah; Kho:Khorramabad; Kuh: Kuhdasht; San:Sanandaj; Urm: Urmia.

Discussion

Molecular methods have enabled the identification of different ectoparasite species with high morphological similarities [16]. The present study employed phylogenetic and molecular approaches to compare *P. irritans* in four different geographical zones of Iran. The morphological characteristics of *P. irritans* were found to be consistent with those obtained in previous studies conducted in Iran to determine the flea fauna in different hosts and cities. [17, 4, 18].

The host specificity can affect the intraspecific genetic diversity given the high levels of intraspecific genetic variations in general parasite species, which cause their infestation of broad ranges of hosts [19]. Research suggests no specific hosts for fleas, as they prefer diverse types of the host, which demonstrates intraspecific genetic diversity. Hornok et al. (2018) reported diversity in synanthropic flea species such as *C. canis* and *P. irritans* by investigating mitochondrial sequences [20]. The present morphological data showed no differences between the *P. irritans* specimens collected from different geographical zones. The results of the morphological investigation were also consistent with those of the molecular exploration. The present study reported a 100% similarity between the nucleotide sequence of COX 1 gene in the *P. irritans* and the sequence of this gene in the *P. irritans* in similar studies conducted in Iran. [MF380389.1, MF380390.1, MF380391.1 (NCBI)]. Similarities to the sequences in Spain (LT797470.1) and China (MF000666.1) were also reported as 99.85% and 99.55%, respectively. Furthermore, the present findings were consistent with those obtained by Seyyedzadeh et al. (2018), who reported no differences between the isolates of *C. canis* in different areas of West Azerbaijan, Iran [21] and with those obtained by Zurita et al. (2019), who observed no significant differences in morphological data between *P. irritans* in Spain and Argentina. In addition, Zurita et al. (2019) reported a significant intraspecific similarity between the two populations based on mitochondrial genes [22]. Hornok et al. (2018) observed no morphological differences between human and wild carnivorous *P. irritans* specimens in Hungary and Croatia [20], which is consistent with the present results. In contrast, Krasnov et al. (2015) found morphological differences between the flea species isolated from different hosts in different geographic areas to suggest high levels of genetic diversity [23].

Evidence suggests ITS1 and ITS2 constitute appropriate molecular markers for analyzing phylogenetic relationships in fleas at their species level [24, 11]. The present research observed a 99.59% similarity between the nucleotide sequences of the ITS1 spacer, in five provinces in Iran. Analyzing the nucleotide sequence of the ITS1 spacer, also showed three 99-bp replicas in its first half, which is consistent with the results of Ghawami et al. (2018), who compared the

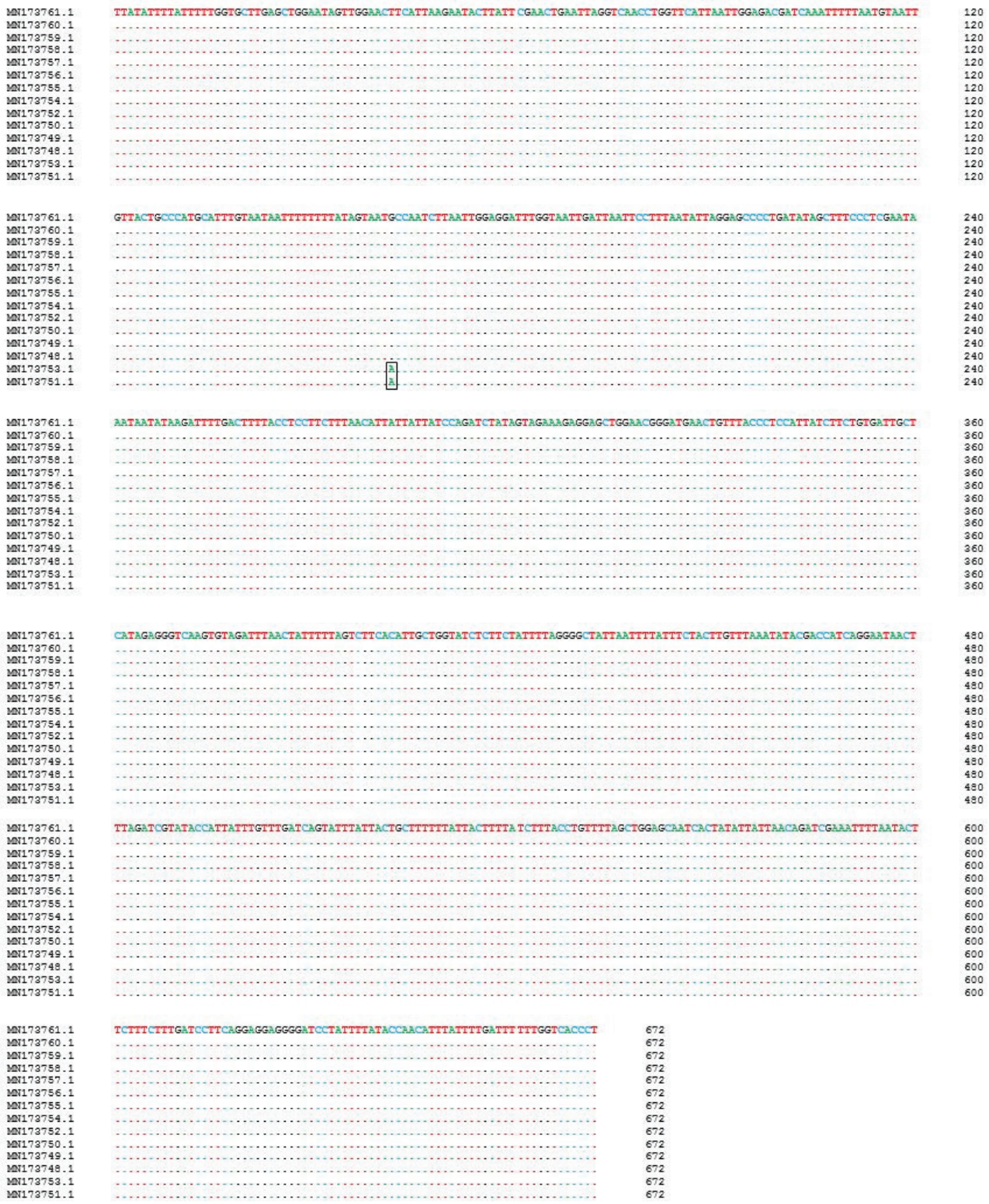


Figure 3.
Alignments of partial mitochondrial DNA (COX1) sequences of Pulex irritans among populations from this study (Urm(human), San(sheep), Sar(sheep), Mah(goat), Kuh(sheep), Kho(human), Esf(human), Kam(sheep), Ghi(sheep), Ahv(goat), Ker(human), Kerm (sheep), Ham (human), and Bah(human) with accession nos. MN173761.1, MN173760.1, MN 173759, MN173758.1, MN173757.1, MN173756.1, MN173755.1, MN173754.1, MN173752.1, MN173750.1, MN173749.1, MN173748.1, MN173753.1, MN173751.1). The vertical boxes indicate the polymorphic sites. Bah: Bahar; Ghi: Gilan-e Gharb; Ham: Hamedan; Kam: Kamiyaran; Ker: Kermanshah; Kho:Khorramabad; Kuh: Kuhdasht; San:Sanandaj; Urm: Urmia.

nucleotide sequence of the ITS1 spacer, in *P. irritans* in two geographic areas and reported only one nucleotide difference with a 99.85% similarity [25]. In line with the present results, those obtained by Vobis et al. (2004) suggested a relatively-constant nucleotide sequence of the ITS1 spacer, in different *C. felis* populations [11].

A 100% similarity was observed between the ITS2 nucleotide sequence of different samples. Zurita et al.

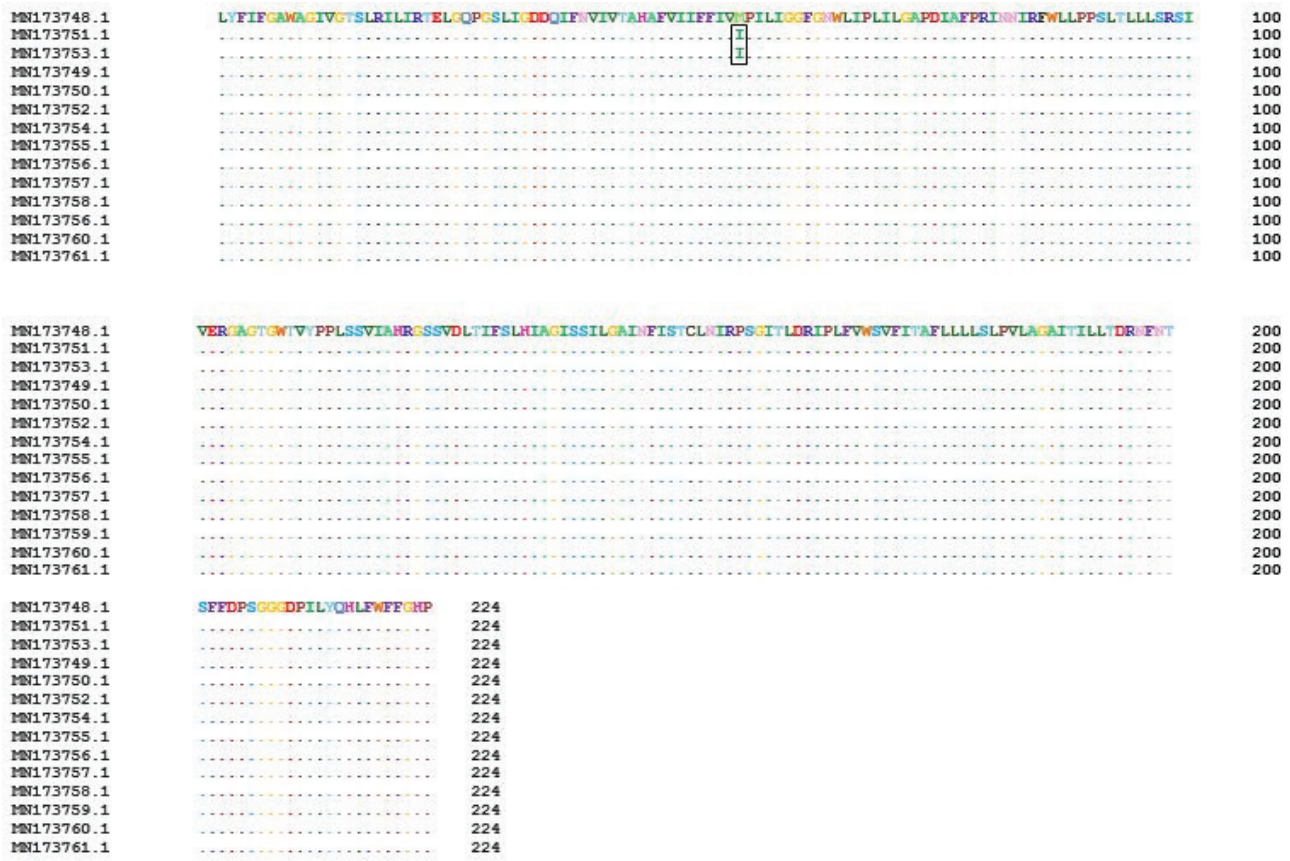


Figure 4.
Sequence alignment of COX1 amino acids for Pulex irritans in different geographical areas of Iran (Kerm (sheep), Bah (human), Ham (human), Ker(human), Ahv(goat), Ghi(sheep), Kam(sheep), Esf(human), Kho(human), Kuh(sheep), Mah(goat), Sar(sheep), San(sheep), Urm(human), with accession nos MN173748.1, MN173751.1, MN173753.1, MN173749.1, MN173750.1, MN173752.1, MN173754.1, MN173755.1, MN173756.1, MN173757.1, MN173758.1, MN 173759.1, MN173760.1, MN173761.1). The vertical boxes indicate the polymorphic sites. Ahv: Ahvaz; Bah: Bahar; ESF: Esfarayen; Ghi: Gilan-e Gharb; Ham: Hamedan; Kam: Kamiyaran; Ker: Kermanshah; Kerm: Kerman; Kho:Khorramabad; Kuh: Kuhdasht; San:Sanandaj; Urm: Urmia.

(2019) reported an intraspecific variation of 90.1%-100% in four *C. canis* clones based on the ITS2 spacer,, which is consistent with the present results. Vobis et al. (2004) also used the ITS2 sequence to identify different flea species and determine their intra-species differences [11]. *P. irritans* and *C. canis* belonging to the *Pulicidae* family constitute the main flea genera infesting human and cattle in the west and northwest of Iran. No significant differences were observed among the samples collected from different hosts. Insignificant differences were found between the study populations in terms of mitochondrial markers and the nuclear ribosome. The results of plotting the phylogenetic tree showed all the samples collected from different regions in the same branch despite their negligible sequence differences. Analyzing mitochondrial genome also showed that this sequence is more useful than the nuclear ribosomal genome in illustrating intra-species similarity and differentiation at genus and species levels.

Materials & Methods

Location

The Iranian plateau is located in the northern hemisphere at 32.4279 °N, 53.6880 °E. The four study geographical zones in Iran included region 1: Caspian Sea (temperature: 8.00-26.00 ˚C, annual precipitation: 400-1500 mm), region 2: Mountainous area (temperature: -5.00-29.00 ˚C, annual precipitation: 200-500 mm), region 3: Persian Gulf (temperature: 12.60-35.00 ˚C, annual precipitation: 200-300 mm) and region 4: The Central Desert (temperature: -4.00-44.00 ˚C, annual rainfall: below 100 mm). According to meteorological data, Iran is climatically divided into three zones, i.e. warm-dry, cold, temperate-humid, and warm-humid [26]. Four different geographical zones in this country from which adult fleas were collected included Kermanshah, Kordestan, Hamadan, Lorestan, West Azerbaijan, Khuzestan, Khorasan, Mazandaran, and Kerman (Figure 8).

Sampling

A total of 1937 flea samples were directly isolated from the host body and houses using light traps and human-baited traps. They were transferred in 70% ethanol to the parasitology laboratory of the Faculty of Veterinary Medicine. They were then cleaned with distilled water several times, immersed in 5% potassium hydroxide for 24 hours, re-washed with distilled water, and dehydrated with a graded series of alcohol (50%-96%(. The fleas were ultimately identified by observing the slides with an optical microscope according to diagnostic keys [27, 28].

Morphological identification

All the *P. irritans* samples collected from different provinces and hosts were morphologically characterized by the following

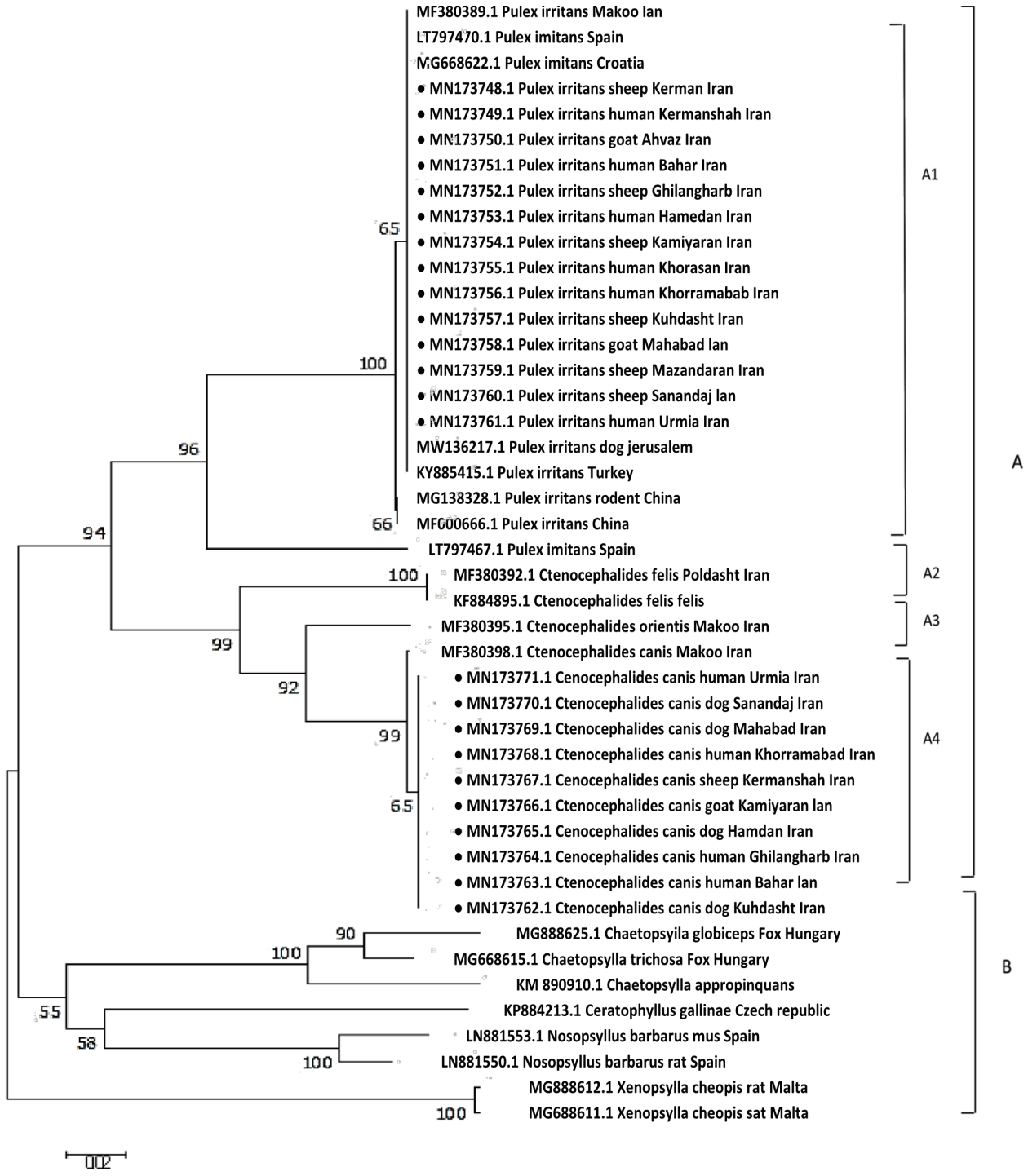


Figure 5.
The phylogenetic tree of *Pulex irritans* based on COX1. The molecular phylogenetic analysis was performed and the evolutionary history was inferred by using the Maximum Likelihood method. The log likelihood (-1898.1614) of the phylogenetic tree was the highest. The percentage of the tree including a cluster of the relevant taxa is shown beside the branches whose length was measured as the number of substitutions per site on this scale drawing of the tree. The analysis involved 44 nucleotide sequences. All the positions containing gaps and missing data were eliminated. The final dataset contained 448 positions. Evolutionary analyses were conducted in MEGA6.

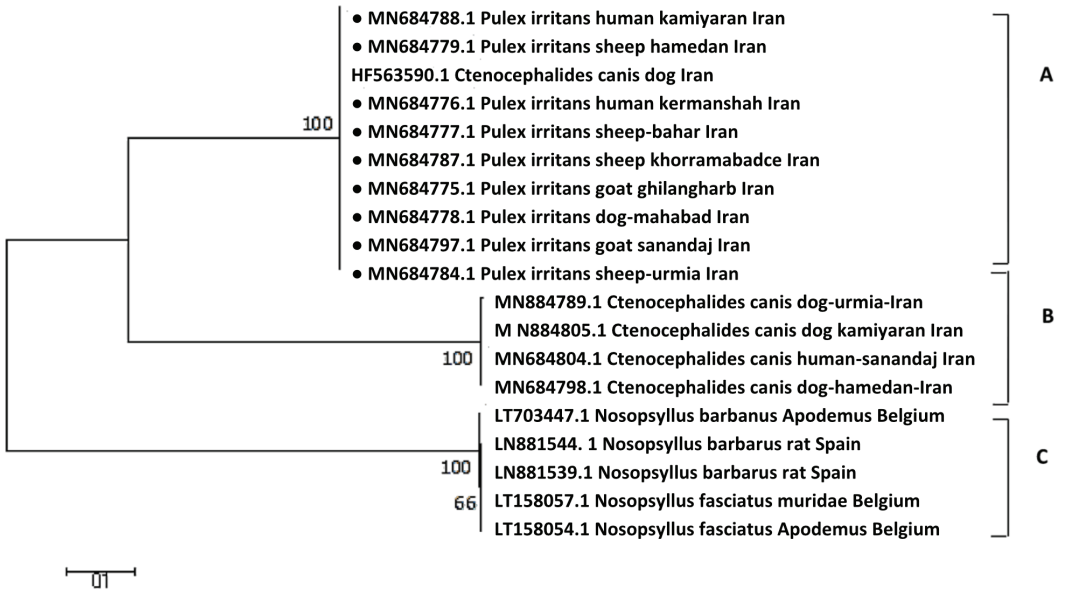


Figure 6.
The phylogenetic tree of *Pulex irritans* based on ITS1. The molecular phylogenetic analysis was performed and the evolutionary history obtained using maximum likelihood. The log likelihood (-1703.2418) of the phylogenetic tree was the highest. The percentage of the tree including a cluster of the relevant taxa is shown beside the branches whose length was measured as the number of substitutions per site on this scale drawing of the tree. The analysis involved 19 nucleotide sequences. All the positions containing gaps and missing data were eliminated. The final dataset contained 436 positions. Evolutionary analyses were conducted in MEGA6.

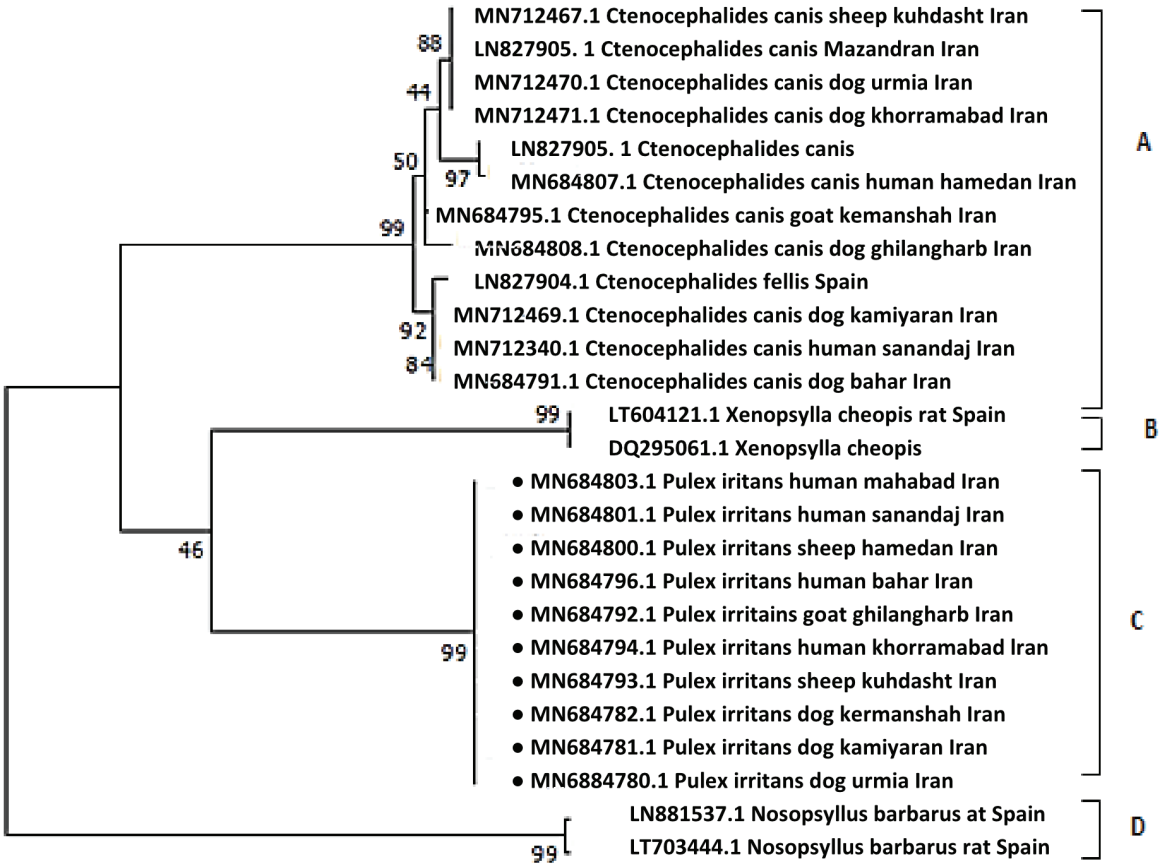


Figure 7.
The phylogenetic tree of *Pulex irritans* based on ITS2. The molecular phylogenetic analysis was performed and the evolutionary history obtained using maximum likelihood. The log likelihood (-1272.22468) of the phylogenetic tree was the highest. The percentage of the tree including a cluster of the relevant taxa is shown beside the branches whose length was measured as the number of substitutions per site on this scale drawing of the tree. The analysis involved 26 nucleotide sequences. All the positions containing gaps and missing data were eliminated. The final dataset contained 291 positions. Evolutionary analyses were conducted in MEGA6.

Figure 8.
Provinces of the study in the four geographical areas included Kermanshah, Kordestan, Hamadan, Lorestan, West Azerbaijan, Khuzestan, Khorasan, Mazandaran and Kerman.

features: the round-headed anterior margin with a pair of eyes and no protrusion, absence of genal and pronotal ctenidia, asymmetrical club of antenna, strong setae under the eyes, row of small spines mass on the inner surface of the posterior side of the coxa in females 7-10 and in males 8-12, and no pleural rod on the mesopleural segment [27]. Comparing the samples collected from different provinces and hosts based on morphological characteristics identified 52.61% to belong to *P. irritans* species despite their negligible differences.

Molecular analysis

The samples were individually crushed with a sterile scalpel, poured into a sterile microtube and their total DNA was extracted using DNA extraction kits (MBST, Tehran, Iran) according to the manufacturer's instructions. The extracted DNA samples were stored in sterile microtubes at -20 °C until use. DNA was extracted from the samples isolated from different hosts in different areas. The specific primers used by Vobis et al (2004). were employed to identify *P. irritans* and 1000-bp ITS1 and 500-bp ITS2 fragments in the rDNA of *P. irritans.* Primers used by Folmer et al. (1994) were used to amplify the mitochondrial COX1 gene fleas *P. irritans* (Table 2). The result of this proliferation was observed as a 700 bp band [29]. To determine molecular differences between the species isolated from different hosts and areas, PCR was performed in a total volume of 50 µl, containing a 5-µl DNA template, a5-µl 10x PCR buffer, 1-µl Taq Polymerase, 1 µl of each primer (20 µM), 1 µl of dNTP (100 µM) and 4 µl of MgCl2 (50 mM). All these materials were procured from SinaClon, Iran.

The thermal cycle of the PCR on fragments ITS1 and ITS2 was as follows: incubation at 94 °C for 5 minutes to denature double-stranded DNA followed by 30 cycles of one-minute denaturation cycles at 94 °C, annealing at 54 °C for 1 minute, extension at 72 °C for 1 minute and an additional extension at 72 °C for 5 minutes. The COX1 gene also underwent replication at 95 °C for 5 minutes followed by 35 cycles of denaturation cycles at 95 °C for 1 minute, annealing at 55 °C for 45 seconds, extension at 72 °C for 45 seconds, and final extension at 72 °C for 10 minutes. The PCR products were electrophoresed on 1.5% agarose gel, stained with a safe stain, visualized under UV light and then purified for sequencing. After purifying the PCR products, 34 samples, including 10 positive samples for ITS1, 10 positive for ITS2, and 14 positive for COX1 sequence, collected from different hosts were transferred to Takapouzist Company for sequencing.

Data analysis

A total of 34 PCR products were transferred to Takapouzist for sequencing. An accession number was obtained by online recording the sequencing results on NCBI by the host and geographical area. The data were analyzed using blasting sequences on NCBI and plotting the phylogenetic tree in Mega 6 using maximum likelihood and bootstrapping (1000 replicates). The clustering method proposed by Zurita et al.(2019) was also conducted. The required sequences were ultimately aligned and compared using EMBOSS Needle and Clustal Omega to evaluate the similarity percentage of the nucleotide sequences of mitochondrial (mtDNA) and ribosomal genomes (rDNA) in different provinces and hosts.

Table 2.
Nucleotide sequence and specificity of the primers used.

Gene	Primer nucleotide sequence	Annealing temperature (°C)	Fragment length (base pair; bp)	Reference
ITS1	For: GTA CAC ACC GCC CGT GCG TAC T Rev: GCT GCG TTC TTC ATC GAC CC	54 °C	1000 bp	[29]
ITS2	For: GGG TCG ATG AAG AAC GCA GC Rev: GCG CAC ATG CTA GAC TCC GTGGTT CAA G	54 °C	500 bp	[29]
COX1	For: GGT CAA CAA ATC ATA AAGATA TTG G Rev: GAA GGG TCA AAG AAT GAT GT	55 °C	700 bp	[30]

Authors' Contributions

Conceived and designed the experiments and revised the manuscript draft: S.S., M.T., performed the experiments, analysed the data and drafted the manuscript: S.S. All authors approved the final version of the manuscript.

Acknowledgements

The authors would like to express their gratitude to the authorities of the Faculty of Veterinary Medicine and Research Council of Urmia University for their approval and financial support of this research.

Competing Interests

The authors declare that there is no conflict of interest.

References

1. Lewis RE. Resume of the Siphonaptera (Insecta) of the world. J Med Entom. 1998; 1; 35(4): 377-389.

2. Azizi MH, Azizi F. A history of the human plague in Iran. Arch Iran Med. 2010; 13(6): 563-569.

3. Esamaeili S, Azadmanesh K, Naddaf SR, Rajerison M, Carniel E, Mostafavi E. Serologic survey of plague in animals, Western Iran. Emerg Infec Dis. 2013; 19(9):1549.

4. Tavassoli M, Ahmadi A, Imani A, Ahmadiara E, Javadi S, Hadian M. Survey of flea infestation in dogs in different geographical regions of Iran. Korean J parasitol. 2010; 48(2):145.

5. Bitam I, Dittmar K, Parola P, Whiting MF, Raoult D. Fleas and flea-borne diseases. In J Infec Dis. 2010;14(8): 667-676.

6. Leulmi H, Socolovschi C, Laudisoit A, Houemenou G, Davoust B, Bitam I, Raoult D, Parola P. Detection of Rickettsia felis, Rickettsia typhi, Bartonella species and Yersinia pestis in fleas (Siphonaptera) from Africa. PLoS Negl Trop Dis. 2014; 8(10): 3152.

7. Ratovonjato J, Rajerison M, Rahelinirina S, Boyer S. Yersinia pestis in Pulex irritans fleas during plague outbreak, Madagascar. Emerg Infect Dis. 2014; 20(8):e1414.

8. Stenseth NC, Atshabar BB, Begon M, Belmain SR, Bertherat E, Carniel E, Gage KL, Leirs H, Rahalison L. Plague: past, present, and future. PLoS Med. 2008; 5(1):e3152.

9. Azarm A, Dalimi A, Mohebali M, Mohammadiha A, Zarei Z. Morphological and molecular characterization of Ctenocephalides spp isolated from dogs in north of Iran. J Entomol. 2016; 4(4):713-717.

10. Gamerschlag S, Mehlhorn H, Heukelbach J, Feldmeier H, D'Haese J. Repetitive sequences in the ITS1 region of the ribosomal DNA of Tunga penetrans and other flea species (Insecta, Siphonaptera). Parasito Res. 2008; 102(2): 193-199.

11. Vobis M, D'Haese J, Mehlhorn H, Mencke N, Blagburn BL, Bond R, Denholm I, Dryden MW, Payne P, Rust MK, Schroeder I. Molecular phylogeny of isolates of Ctenocephalides felis and related species based on analysis of ITS1, ITS2 and mitochondrial 16S rDNA sequences and random binding primers. Parasitol Res. 2004; 94(3): 219-226

12. Zurita A, Callejon R, Garcia-sanchez ÁM, Urdapilleta M, Lareschi M, Cutillas C. Origin, evolution, phylogeny and taxonomy of Pulex irritans. Med Vet Entomol. 2019; 33(2): 296-311.

13. Bowen BW, Clark AM, Abreu-Grobois FA, Chaves A, Reichart HA, Ferl RJ. Global phylogeography of the ridley sea turtles (Lepidochelys spp.) as inferred from mitochondrial DNA sequences. Genetica. 1997; 101(3): 179-189.

14. Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann Entomol Soc Am. 1994; 87(6): 651-701.

15. Mclain DK, Wesson DM, Oliver Jr JH, Collins FH. Variation in ribosomal DNA internal transcribed spacers 1 among eastern populations of Ixodes scapularis (Acari: Ixodidae). J Med Entomol. 1995; 32(3):353-560.

16. Lv J, Wu S, Zhang Y, Chen Y, Feng C, Yuan X, Jia G, Deng J, Wang C, Wang Q, Mei L, Lin X. Assessment of four DNA fragments (COI, 16S rDNA, ITS2, 12S rDNA) for species identification of the Ixodida (Acari: Ixodida). Parasit Vectors. 2014 Mar 3; 7:93.

17. Bahrami S, Kharrati L, Makki M. The First Case Report of Human Infestation with Ctenocephalides canis in Behbahan (Khuzestan Province). J of Ardabil Univ of Med Sci. 2016; 16(3): 317-322.

18. Yakhchali M, Hosseine A. Prevalence and ectoparasites fauna of sheep and goats flocks in Urmia suburb, Iran. Vet Arhiv. 2006; 76(5): 431-442.

19. Van der Mescht L, Matthee S, Matthee CA. Comparative phylogeography between two generalist flea species reveal a complex interaction between parasite life history and host vicariance: parasite-host association matters. BMC Evol Biol. 2015; 15(1):1-5.

20. Hornok S, Beck R, Farkas R, Grima A, Otranto D, Kontschan J, Takacs N, Horváth G, Szoke K, Szekeres S, Majoros G. High mitochondrial sequence divergence in synanthropic flea species (Insecta: Siphonaptera) from Europe and the Mediterranean. Parasites vectors. 2018; 11(1):1-1.

21. Seyyed-Zadeh SJ, Bozorg-Omid F, Telmadarraiy Z, Terenius O, Chavshin AR. Evidence for the presence of Ctenocephalides orientis in livestock dwellings in northwest Iran. Med Vet Entomol. 2018; 32(3): 383-387.

22. Zurita A, Callejon R, De Rojas M, Halajian A, Cutillas C. Ctenocephalides felis and Ctenocephalides canis: introgressive hybridization?. Syst Entomol. 2016; 41(3): 567-579.

23. Krasnov BR, Shenbrot GI, Khokhlova IS. Historical biogeography of fleas: the former Bering Land Bridge and phylogenetic dissimilarity between the Nearctic and Palearctic assemblages. Parasitol Res. 2015; 114(5): 1677-1686.

24. Marrugal A, Callejón R, de Rojas M, Halajian A, Cutillas C. Morphological, biometrical, and molecular characterization of Ctenocephalides felis and Ctenocephalides canis isolated from dogs from different geographical regions. Parasitol Res. 2013; 112(6): 2289-2298.

25. Ghavami MB, Mirzadeh H, Mohammadi J, Fazaeli A. Molecular survey of ITS1 spacer and Rickettsia infection in human flea, Pulex irritans. Parasitol Res. 2018; 117(5):1433-1442.

26. Skerman KD, Shahlapoor AA, Eslami AH, Eliazian M. Observations on the incidence, epidemiology, control and economic importance of gastro-intestinal parasites of sheep and goats in Iran. Arch Razi Inst. 1970; 22(1): 187-196.

27. Asmar M, Piazak N, Karimi Y. An illustrated key for flea of Iran: Pasteur Institute of Iran, Tehran (Persian), 1979; 2-15.

28. Shayan A, Rafinejad J. Arthropod parasites of rodents in Khorram Abbad district, Lorestan Provincen of Iran. Iranian J Public Health. 2006; 70-6.

29. Lawrence AL, Hii SF, Jirsova D, Panakova L. Integrated morphological and molecular identification of cat fleas (Ctenocephalides felis) and dog fleas (Ctenocephalides canis) vectoring Rickettsia felisin central Europe. Vet Parasitol. 2015; 10(3-4): 215–23.

30. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoeket R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Ma Biol Biotechnol. 1994; 3(5): 294–299.

How to cite this article

Seidi Sh, Tavassoli M, Malekifard F. Molecular identification and phylogenetic analysis of Pulex irritans in different regions of Iran Iran J Vet Sci Technol. 2021; 13(2): 46-57.
DOI: https://doi.org/10.22067/ijvst.2021.70946.1055
URL: https://ijvst.um.ac.ir/article_40770.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Apr- 09
Accepted after revision: 2021- Oct- 19
Published online: 2021- Dec- 30

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2021.69518.1030

The basolateral amygdala has a critical role in food-matched visual-cue memory and post-ingestion food preferences in rats

Mahnaz Zamyad[a], Mehdi Abbasnejad[a], Saeed Esmaeili-Mahani[a], Vahid Sheibani[b], Maryam Raoof[c]

[a] Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran.
[b] Laboratory of Molecular Neuroscience, Kerman Neuroscience Research Center (KNRC), Kerman University of Medical Sciences, Kerman, Iran.
[c] Department of Orofacial Pain and Dysfunction, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and Vrije Universiteit Amsterdam, Amsterdam, The Netherlands.

ABSTRACT

The Basolateral Amygdala (BLA) has been shown to have an important role in food-related learning behaviors. Using a novel approach, we have evaluated the role of BLA in food preference and Food memory related to visual cues in rats. Thirty-two adult male Wistar rats, weighing 200–250 g, were used for the experiments. Electric lesion of BLA was produced by passing 1.5 mA of current for 7 s. Food-related behaviors and preferences were evaluated by using an automated apparatus. Geometric visual cues were also constructed. Food-deprived rats were presented with different diets in 6 consecutive trial performances. The number of visits, time consumed on each food zone and port, distance traveled in each visit, and the total amount of food eaten was evaluated. The changes in hippocampal c-Fos expression were determined by immunoblotting. The control sham group showed a high and low preference for biscuit and white flour, respectively. BLA lesion rats exhibited a shifted preference curve. In the sham group, a more significant amount of food consumption was associated with an increased number of references to each zone and port, along with more time spent there. Furthermore, a decrease in hippocampal c-Fos expression was observed in the BLA- lesion animals. Taken together, the basolateral amygdala has a significant role in rats' food-matched visual-cue memory and high-calorie/ sweetness preferences.

Keywords

Food preferences, Learning and memory, Visual-cue, Basolateral amygdala, Lesion, c-Fos, Rats

Number of Figures: 6
Number of Tables: 2
Number of References:: 37
Number of Pages: 10

Abbreviations

BLA: Basolateral amygdala
OFC: Orbitofrontal cortex

Corresponding author: *Mehdi Abbasnejad*
Email: *mabbas@uk.ac.ir*
Tel: *+98 (91)3731-4160*

Introduction

Animal's approach-behaviors to stimuli are related to the previously paired rewarding, escape, or avoidance experiences [1]. Post-ingestion consequences result in automatic selection or rejection of food, and through such experiences, the animals learn about the different characteristics of food [2].

The amygdala, especially its basolateral [3] and central [4] parts are involved in controlling feeding behaviors such as food preferences. Gustatory-related projections from parabrachial nuclei, insular cortex, and olfactory piriform cortex project to the basolateral amygdala (BLA) [5]. The orbitofrontal cortex (OFC) projections are required to the BLA for encoding the value of a reward [6]. BLA play important role in taste preference and olfactory classical conditioning. . Lesions of the basolateral amygdala have been shown to be associated with taste-related disorder behaviors [7]. Furthermore, the BLA has a crucial role in experiencing the hedonic impact of the food outcomes [8, 9]. The BLA is also involved in the processing of sensory features of food, including smell, taste, buccal quick sense, and post-ingestion effects [10].

It has been demonstrated that the rats prefer to drink sweetened solutions over tap water, while radiation exposure caused a decrease in the consumption of sweetened solutions (taste aversion) [11, 12]. It has been demonstrated that animals can learn and memorize preferences regarding the characteristics of food. For instance, food-deprived rats prefer the flavor of a nutritious diet a few days after deprivation [1]. Furthermore, preferences for odors can be easily created or changed when the odor is matched with positive or negative reinforcement [13].

Studies have shown that both pure taste sensory information from the oral cavity, and post-ingestion factors are involved in c-Fos induction [14]. There is a positive relationship between the amount of food consumed and the expression of the c-fos protein. Several studies have shown that some nutrients, especially lipids and carbohydrates, induce c-Fos protein expression [15]. Also, it has been shown that the c-Fos protein expression is dramatically increased in the hippocampus of food preference trained rats [16].

It seems that when animals are given more opportunities for food selection, they prefer the most healthy (or rather, least unhealthy) option [17]. The calorific value and the nutritional composition of food are also the determinant factors influencing the food preference of animals [18]. It seems that animals can remember information about their food location. Moreover, place conditioning is a common, and potentially useful, procedure to assess the positive or negative motivational effects of exposure to various food stimuli [19, 20]. Nevertheless, it is unclear whether the BLA influences the food-matched visual-cue memory and post-ingestion food preference. Therefore, to elucidate the above, the present research has been designed using a new examination protocol.

Results

The amount of food consumed

Sham rats showed significantly increased preferences for biscuit ($p < 0.001$) and wholemeal + sugar ($p < 0.05$) than animals with a BLA lesion. However, there were no statistically significant differences in the preferences for white flour and wholemeal within the sham group. The preference order of BLA lesion groups was significantly ($p < 0.001$) different from that of the sham ($p < 0.001$).

There were significant differences in the amount of wholemeal ($p < 0.01$), wholemeal + sugar ($p < 0.001$), and white flour ($p < 0.001$) consumed between the groups. The order of consumed food (in grams) in the sham group was a biscuit, wholemeal + sugar, wholemeal and white flour. The BLA lesion group has shown this order of preferences: wholemeal+ sugar, white flour, wholemeal and, biscuit, respectively (Figure 1A).

Traveled distance

The analysis of the distance traveled in wholemeal, wholemeal + sugar, white flour and biscuit meal-related zones revealed significant differences among the various food zones in the BLA lesion and sham groups ($p < 0.001$) (Figure 1B).

Number of visits

There was a significant difference between the sham and the BLA lesion group regarding the number of visits to the food zones and ports (Figure 2, A and B).

Time spent

As shown in Figures 3 A and B, the time spent in the biscuit port and zone was significantly higher than that of the other foods in the sham group ($p < 0.01$, $p < 0.001$). However, the rats with a BLA lesion spent approximately equal time in white flour, biscuit, and wholemeal + sugar ports. Compared to the sham groups, the BLA lesion group significantly more time spent in white flour ports ($p < 0.001$) and zones ($p < 0.01$). Nevertheless, they spent significantly less time in biscuit ports ($p < 0.05$) and zones ($p < 0.001$) in comparison with the sham rats (Figures 3A, and 3B).

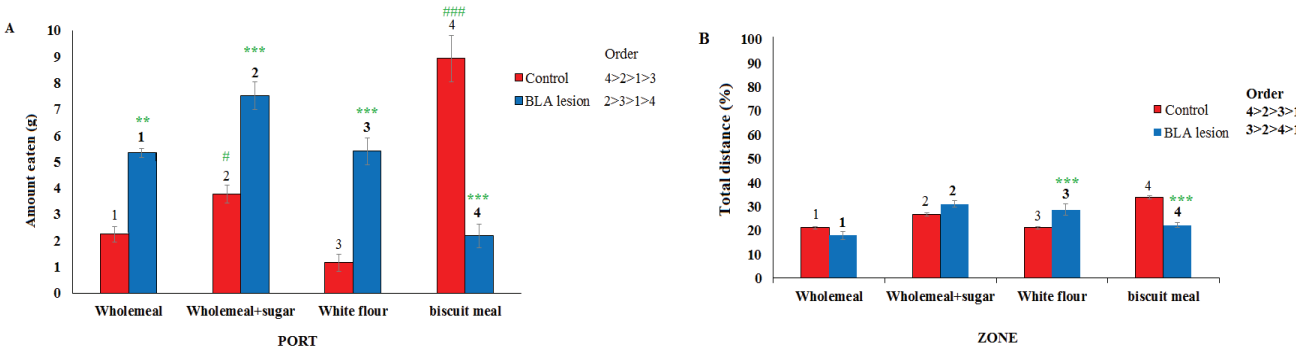
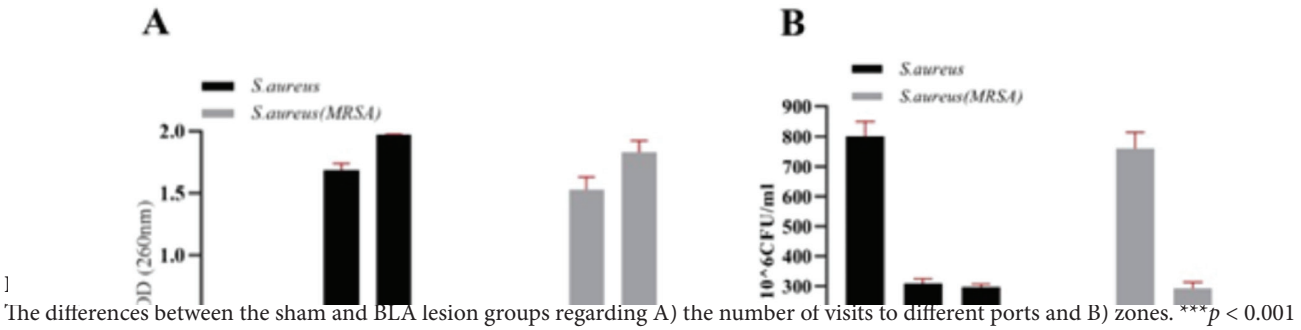


Figure 1.
The differences in food consumptions (in grams) between the sham and BLA lesion groups. A) The differences between the sham and BLA lesion groups regarding the amount of eaten food.**p <0.01, ***p <0.001 indicate signficant differences compared with the sham group, and #p <0.05, ###p <0.001 indicate signficant differences compared with the wholemeal and white flour in the sham group, and biscuit meal in the BLA-lesion (n=16). B) The differences between the sham and BLA lesion groups regarding the distance traveled in various food zones. ***p < 0.001 in comparison with the sham group (n=16).



]
The differences between the sham and BLA lesion groups regarding A) the number of visits to different ports and B) zones. ***p < 0.001 compared with the same food in sham group (n=16).

The relationships between the amount of food eaten with the visits to, as well as the time spent, in the related zones

There was a positive correlation between the amount of food eaten with the visits to (Figure 4A), as well as the time spent (Figure 4B), in the related zones in sham rats. Figures 4C and D indicate the relationship between the food intake of a subject about the number of visits made (r = 0.45) and the spent time (r = 0.37) in the BLA lesion group. A positive correlation was found in the sham group regarding the amount of food eaten in conjunction with the frequency and length of visits, but not in the BLA lesion group (r = 0.75; r = 0.79, p < 0.001) (n = 16).

Food memory related to visual cues Table.1 shows behavioral data in various zones with both empty and filled containers. Using the visual cues, the sham rats spent significantly more time in biscuit zones (p < 0.001) than in any other. They also made more visits to the biscuit zones compared to the others (p < 0.001).

The number of references, and the time spent in each zone, there were no significant differences between the empty and filled containers. The order of

Table 1.
Acquisition of the spatial memory (comparing the data from 5th and 6th trials).

Groups	Parameter	Zone	Whole meal(g)	Whole meal+sugar(g)	White flour(g)	Biscuit meal(g)
Control	Number of port visits	Filled container	9 ± 3	12 ± 1	4 ± 0	15 ± 3***
		Empty container	8 ± 0	14 ± 5	3 ± 1	16 ± 2***
	Spent time in the zone (s)	Filled container	116 ± 4	230 ± 2	17 ± 1	237 ± 2***
		Empty container	125 ± 2	213 ± 4	14 ± 1	230 ± 1***
BLA lesion	Number of port visits	Filled container	71 ± 1	117 ± 1	89 ± 1	72 ± 3
		Empty container	52 ± 2	51 ± 1###	40 ± 2###	38 ± 1#
	Spent time in the zone (s)	Filled container	1288 ± 4	1424 ± 3	1302 ± 2	2030 ± 1
		Empty container	754 ± 2	563 ± 2#	537 ± 1#	372 ± 1###

Data are presented as mean ± SEM. ***p < 0.001 indicates signficant difference compared with the other meals in the control group; #p < 0.05 and ###p < 0.001 indicate signficant differences compared with the data from the same zone with different container conditions (n=16).

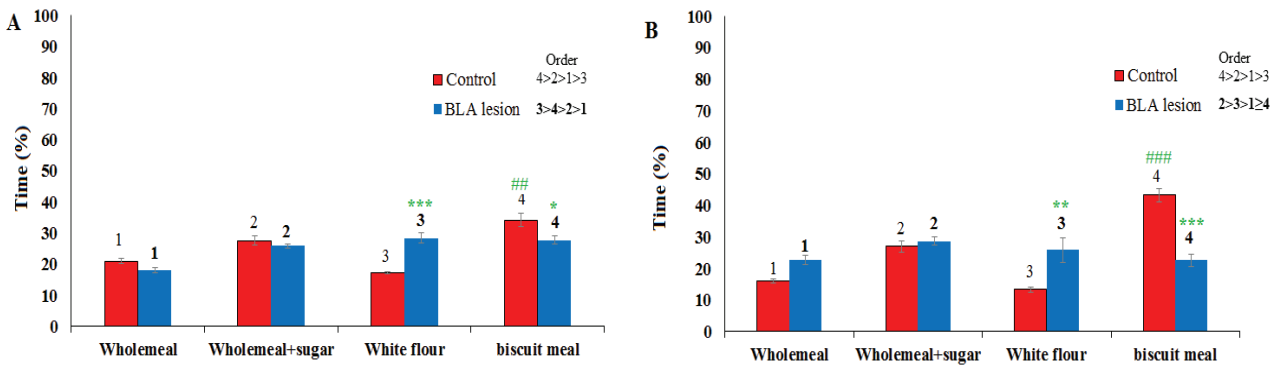


Figure 3.
The differences between the sham and BLA lesion groups regarding A) the time spent in different food ports and B) zones. *p < 0.05, **p < 0.01 and ***p < 0.001 indicate signficant differences compared with the same meal in the sham group, and ##p < 0.01, ###p < 0.001 indicate signficant differences compared with the wholemeal and white flour in the sham group (n=16).

food preferences in the fifth trial was biscuit, wholemeal + sugar, wholemeal and white flour, respectively. Comparing the ordering results of the 5th and 6th trials indicated that the animals had the ability of spatial memory.

The rats with a BLA lesion, however, showed significant differences with regards to the number of references and the time spent in each zone, according to whether it was filled or empty (p < 0.05, p < 0.001).

The effects of basolateral amygdala lesions on hippocampal c-Fos expression

The data showed significant differences in hippocampal c-Fos protein levels in different experimental groups by Immunoblot analysis. As shown in Figure 5, c-Fos expression was significantly increased in the sham group due to the food preference training (p < 0.01), which was reversed by BLA lesion. However, in BLA lesion rats, the c-Fos level was less than those in control animals (p < 0.001).

Discussion

Here we used four validated meal options according to the study done by Barnett et al., 1953 [21]. For the present study, we have developed a new apparatus and protocol. Considering the amount of food con-

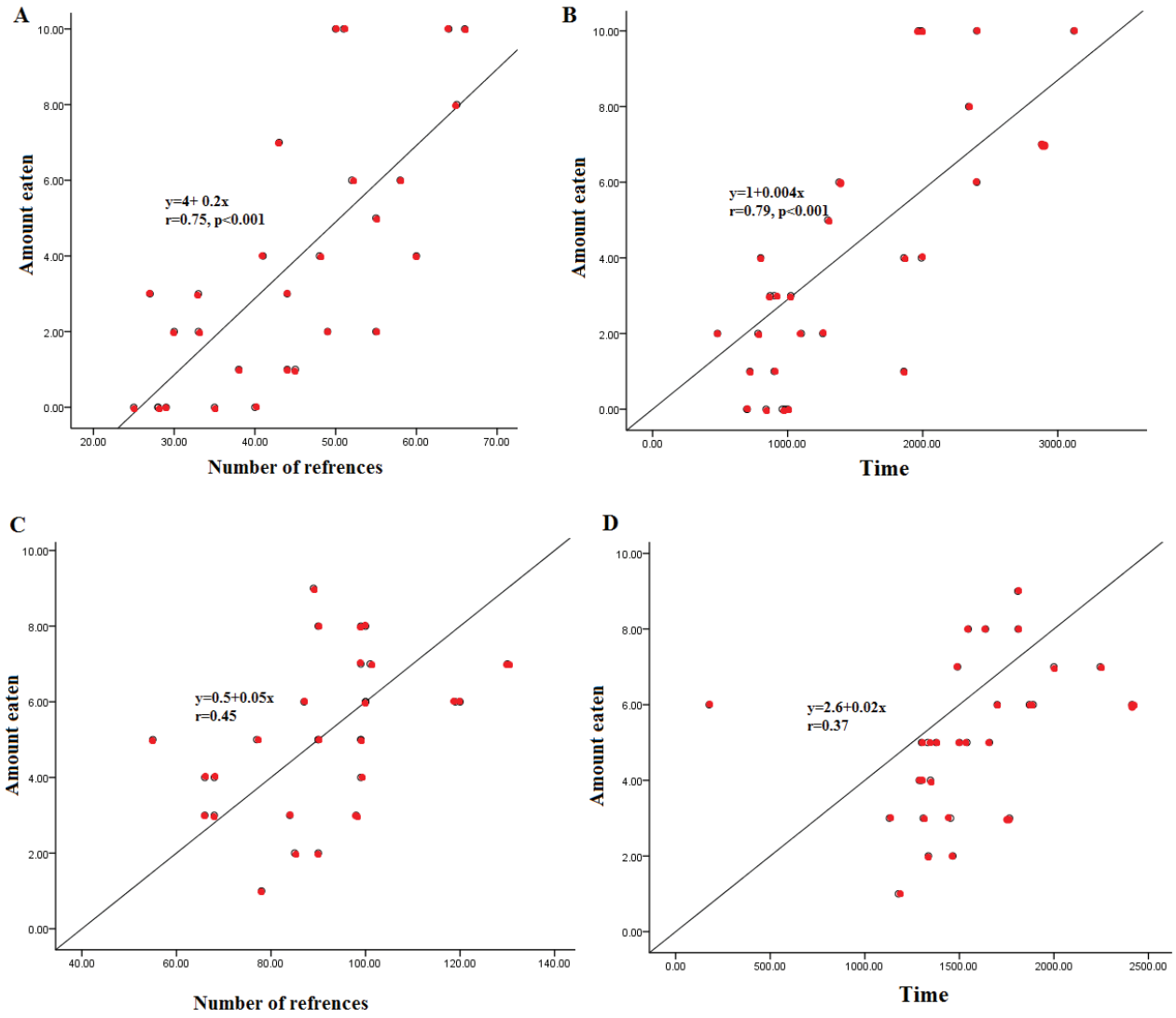


Figure 4.
The linear regression analysis of the relationships between the amount of food eaten and visits to and time spent in the related zones in the sham (A, B) and BLA lesion groups (C, D). *Significant regression, $p < 0.001$ (n=16).

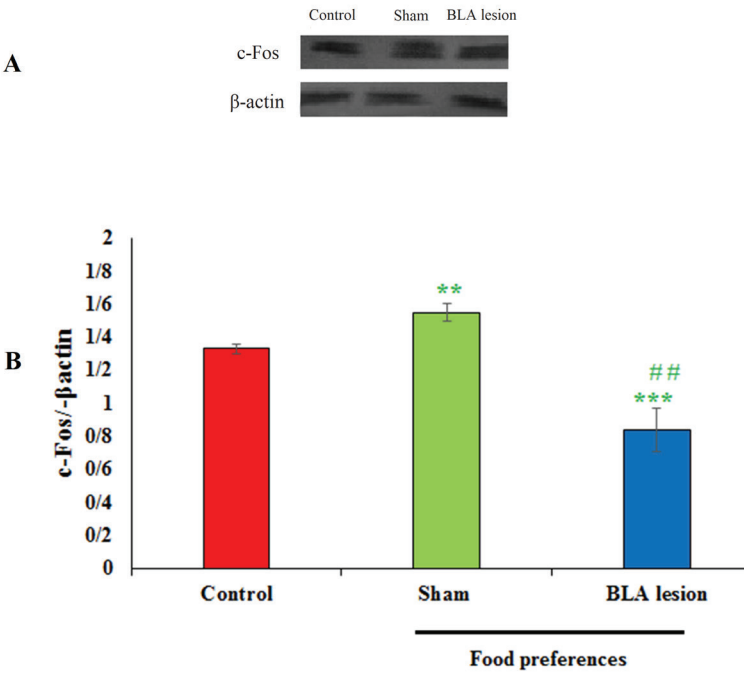


Figure 5.
A) Effects of BLA lesion and food preferences on c-Fos expression in the hippocampus. B) Statistical comparison of c-Fos experssion in the hippocampus between groups.
$^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences compared with the control group (n = 16). $^{\#\#}p < 0.01$ indicate significant differences compared with the sham group.

sumed, Barnett et al., showed the following food preference order found in rats: biscuit, wholemeal + sugar, wholemeal and white flour. Our study confirmed the validity and reliability of these food preferences and protocols in rats. Furthermore, in the present study, we provided enough time for the animals to distinguish the post-ingestion consequences of food. Interference effects of post-ingestion products on eating certain foods or medication on food preferences have been extensively demonstrated [22].

In this study, a variety of food consumption variables including the number of references to the zones (whole surface of the square just in front of the food container) and ports (the entrance of the food containers), time spent, and distance traveled in each zone have been assessed. We noticed an association between the order of time spent in different zones and ports and the order of food preferences in rats. Illustratively, the order of the amount of time the rat spent in different zones was as follows: biscuit zone and port, wholemeal + sugar, wholemeal and white flour; the same as the previously discovered ordering of food preferences.

It has already been reported that rats have a preference for sweet taste [23]. Rats, like humans, have a natural bias towards consuming high-calorie food [18]. Moreover, the phenomenon of animals preferring high-fat foods has been considered a natural behavior. The high palatability and hedonic preference for fatty and sweet foods have already been reported. [24]. There are multiple causative factors including, texture, flavor, taste, and post-ingestive effect for high palatability of fat [25]. According to table 1, the biscuit has the highest amount of fat, protein, and calorie. It is also a high-carbohydrate meal. These specific features may help explain why biscuit was the first preference made by the rats. An increased appetite for high-calorie foods happens due to information received by the brain from the gastrointestinal tract through sensory nerves and chemical receptors [26].

On the other hand, the animals presented long-term memory formation driven by the visual cues associated with specific meals and locations. It has already been demonstrated that rats could learn to associate a specific stimulus, with a matched visual cue [27].

However, the present study is unique in that a food preference memory by use of matched visual cues has been used. The similar results of the 5th and 6th trials describe a positive post-ingestion consequence of foods, while the different data out of the two trials indicate negative post-ingestion effects. Indeed, the animals can learn to determine the suitability and preference of food according to the post-ingestion experiences [2, 28], and several types of mechanisms are involved in this regard [29].

In the present study, the preferred meal of the sham rats was biscuits. However, wholemeal + sugar, rather than a biscuit, ranks as the first choice for rats with BLA lesions. It suggests that the food preference is influenced by the carbohydrate-mediated post-ingestive effect [30] and the BLA may have a role in this regard. The rodent's appetite for fat seems to be stronger than that for carbohydrates.

BLA lesions have been reported to be associated with taste disorder-related behaviors and with changes in a variety of taste and odor-related learning paradigms, including conditioned taste preference, taste-potentiated odor aversion, and conditioned taste aversion [3]. Since the BLA has a very crucial role in the induction of food-related memory, post-ingestive consequences of eating [28], and food behavior control [31], here we considered and evaluated all the mentioned conditions in rats with BLA lesions. Almost all of the data out of food consumption, time spent in, and visits to the zones and the ports, revealed that the rats with a BLA lesion had different food preferences than the sham animals. Besides, an induced BLA lesion has led to a change in the order of food preference in the 5th and 6th trials. The results indicate that the BLA is a critical region involved in Food memory related to visual cues.

In the present study, the following food preferences training, the amount of hippocampal c-Fos protein was increased. It is in line with the previously reported study that showed that in the dorsal and ventral hippocampus, the expression of c-Fos is increased in rats trained on socially transmitted food preference [13]. The BLA is strongly correlated with food-related learning and memory [3]. It also has connections with the hippocampus [32]. These connections seem to be necessary for visual cue discrimination and spatial memory formation. A lesion in the BLA negatively affects both reward-related learning and conditional learning and memory [33]. Also, c-Fos protein is induced by a wide range of stimuli and is a reliable indicator for neural activity such as food-related learning behaviors [13] and increases after learning and memory induction. In the present study, c-Fos expression decreased after BLA lesion. The reduction in the expression of c-Fos may be due to the role of the basolateral amygdala in food-related learning and memory; however, this issue needs further investigation.

Changes in the food preferences of the BLA lesion-affected rats have already been reported by Edmund et al. in 1973 [34]. However, our study employed both an innovative apparatus and protocol that produced more data related to feeding behaviors. Moreover, in the present study, we provided enough time for the animals to experience post-ingestion con-

sequences. We also assessed visual cue-conditioning with food-dependent memory. The amygdala, especially the basolateral nucleus (BLA), has been involved in visual processing [35]. This area has connections with visual association areas, including primary and secondary, and supplementary visual cortices.

The present study sought to fill the gap in the related literature by examining the relationships among BLA function, changes in rat's food preference, and visual cue-dependent memory. We propose that besides the amount of food consumption, the number of visits to the food zones or ports, the time spent and traveled distance in the zones might also be valuable to assess food preference in rats. Moreover, we found that the results of food-matched visual-cue-dependent memory assessment are negatively affected by a lesion in the BLA of rats.

Materials & Methods

Subjects

In this study, a total of 32 Wistar male rats aged two months (adult young), weighing 200–250g, were used. The rats (n=16) [36] were randomly assigned into two groups and evaluated for their food behaviors: the sham-lesion control group was given no treatment, and the BLA lesion group received electrical stimulation and stereotaxic surgery. The rats were kept in a temperature-controlled room at 23±1°C, with a standard 12 hours light/dark cycle. The animals received food and water ad libitum. All experimental procedures were approved by the Animal Research Ethics Committee of Shahid Bahonar University, Kerman, Iran (IR.UK.VETMED.REC.1398.018).

Food type

Four different diets have been considered according to a previous study [21] as follows: wholemeal, wholemeal + sugar, white flour, and biscuit (hard-baked) for testing food-related behaviors. To prevent a familiar effect, the ordinary laboratory rat food was powdered or mixed (50 percent) with the ingredients mentioned above (Table 2). According to Kasper and Johnson [18], rats present approach bias towards high-calorie and high-fat foods. Each of the ingredients was chopped into pieces no larger than 0.5 cm.

Surgery

The rats were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg) (Stoelting Co., USA), and mounted on a stereotaxic instrument. The skulls were exposed and two holes were drilled into the skull over the BLA at stereotaxic coordinates: AP = –2.28, ML = ±5, and DV = 8.6 mm from bregma. A bipolar electrode (Teflon-coated stainless steel, 0.125 mm diameter, Advent Co., UK) was positioned in the BLA, and an electrical lesion was produced by passing 1.5 mA of current for 7 s [37]. Animals were allowed one week to recover from surgery. At the end of the experiments, the correct location of the lesions was verified histologically.

Apparatus

An automatic device made of black Plexiglas (60 cm long × 60 cm wide × 30 cm high), was used. The floor was imaginarily divided into nine identical squares. As shown in Figure 6A, the device was equipped with water and food storage containers and an electronic sensor to provide information on the animal's locations, through container weight changes (Figure 6A).
The data of the four middle areas that include the ports (zones 2, 4, 6, and 8) presented areas to the animal's preference for the container. Four corner squares (1, 3, 7, and 9squer) were provided for resting (rats like rest in corners). The central square (square 5) was used as an animal release site. The device was supported by special software that assessed the number of visits to each zone, port, the location of the rats, the time spent, and distance traveled in each zone and port, food consumption per visit, as well as the total food consumption.

Experimental design

One day before the test phase, following recovery from the surgery, rats were habituated to the test environment. Each rat was allowed to freely explore the chamber that was free of food, for 15 min. If an animal spent more time in a specific area or didn't show exploratory

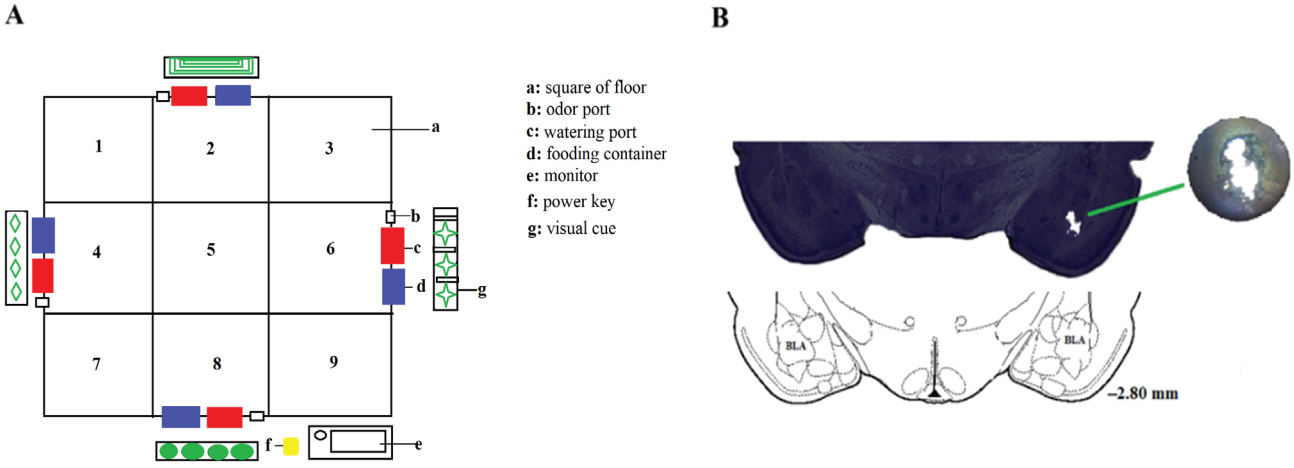


Figure 6.
A) Different parts of the preference meter device. B) The region of damage in BLA-lesioned rats.

behavior, it would be excluded from the experiment.

The test stage includes 6 trials in total with 15 min inter-trial intervals. The trials were as follows: In the first trial, container A was provided with 10 g wholemeal. Rats were placed in the central zone with dark cylindrical-shaped Plexiglas (to prevent their inclination to stray sideways). They were allowed to have access to this food for a 12-h period. The feeding behavior-related data was assessed and analyzed with the help of the aforementioned software.

The second trial began after the whole meal was removed from container A and when the apparatus had been thoroughly cleaned. Then, wholemeal + sugar was put in container B, and as with the previous trial, animals were released in the central square, and the feeding behavior was evaluated for 12 hours. The third and fourth trials were then conducted in the same manner, with white flour (container C) and biscuit (container D).

In the fifth trial, each of the various diets found in containers A-D were made simultaneously accessible. Dedicated visual cues were also included. The visual cues helped the animal to remember the taste memory. This trial was designed the animal's food preference is influenced by previous experience with food consumption. For this trial, latency to the first container is also important. During the final trial, the food containers were empty.

With the help of the software, the following criteria were evaluated for each of the trials: the number of visits to each food zone and port, distance traveled and the time spent in each zone and port, the food consumption per visit, and total food consumption.

Histology confirmation of lesion

Histological examination of the lesion was performed, just after decapitation, the rats' brain was removed and stored in 10% formalin for at least three days. Also, serial transverse sections (30 µm) were cut, and the lesion sites were determined according to a rat brain atlas (Figure 6B).

Western blot analysis

After preparing the RIPA buffer (10 mM Tris–HCl: pH 7.4, 1 Mm ethylenediaminetetraacetic acid, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% NP-40 and protease inhibitors, 0.1% Na-deoxycholate, and 1 mM sodium orthovanadate) added to the hippocampal tissue of the brain of rats for Lysis. 40 µg of protein per sample were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinyl difluoride membrane. Then for two hours, blots were blocked with 3% nonfat milk in 0.1% tween-tris-buffered saline, followed by overnight (at 4 °C) incubation with c-Fos primary antibody. The primary antibody was detected with goat anti-rabbit IgG antibody peroxidase-conjugated secondary antibody. The antibody-antigen complexes were detected by the ECL system and exposed to Lumi-Film chemiluminescent detection film (Roche, Germany). To assess the intensity of the blotting bands, the Lab Works analyzing software was used. We used β-actin as the loading control. The expression values were presented as c-Fos / β-actin ratio for each sample.

Statistical analysis

Behavioral data are presented as mean ± SEM and were evaluated using two-way ANOVA with statistical significance set at $p < 0.05$, followed by *Tukey's* post-hoc correction for multiple comparisons, where applicable. Regression coefficient r was used for calculating the dependency between food consumption and the number of visits, as well as the time spent in each zone.

Authors' Contributions

MA: designed the study protocol, MZ: collected the data, SEM: carried out the statistical analyses, MA, VS, and MR drafted the manuscript.

Acknowledgements

This work was supported by Shahid Bahonar University of Kerman, Kerman, Iran. The authors would also like to thank Mr. Baroaby Robinson who took burden of proofreading of the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

Table 2.
The material and calorie of each diet.

Food stuff	Description	Cal/10g.
Wholemeal	Wholemeal*+ pellet	33.30
Wholemeal + sugar	Wholemeal+ sugar*+ pellet	33.76
White flour	White flour *+ pellet	30.10
Biscuit meal	Biscuit meal* + pellet	3830

*Results of analyses carried out by Cereals Research Station:

Food stuff	Total fat/10g	Protein/10g	Carbohydrates/10g	Sugar/10g
Wholemeal	0.34 g	1.3 g	4.1 g	0.6 g
Wholemeal + sugar	0.34 g	1.3 g	7.5 g	5.6 g
White flour	0.1 g	1 g	7.6 g	0.03 g
Biscuit meal	0.85 g	3.2 g	6.8 g	2.24 g

References

1. Velimirov A, Lueck L, Shiel R, Plöger A, Leifert C. Preference of laboratory rats for food based on wheat grown under organic versus conventional production conditions. NJAS: Wageningen Journal of Life Sciences. 2011; 58 (3-4): 85-88.

2. Benoit SC, Davis JF, Davidson TL. Learned and cognitive controls of food intake. Brain Res. 2010 Sep 2;1350:71-6.

3. Wang Y, Fontanini A, Katz DB. Temporary basolateral amygdala lesions disrupt acquisition of socially transmitted food preferences in rats. Learn Mem. 2006 Nov-Dec;13(6):794-800.

4. Kemble E.D, Schwartzbaum J. Reactivity to taste properties of solutions following amygdaloid lesions. Physiology & Behavior. 1969; 4(6): 981-985.

5. Paré D. Role of the basolateral amygdala in memory consolidation. Progress in neurobiology. 2003; 70(5): 409-420.

6. Malvaez M, Shieh C, Murphy MD, Greenfield VY, Wassum KM. Distinct cortical-amygdala projections drive reward value encoding and retrieval. Nat Neurosci. 2019 May;22(5):762-769.

7. Box BM, Mogenson GJ. Alterations in ingestive behaviors after bilateral lesions of the amygdala in the rat. Physiol Behav. 1975 Dec;15(6):679-88.

8. Vicario CM. Inborn mechanisms of food preference and avoidance: the role of polymorphisms in neuromodulatory systems. Front Mol Neurosci. 2013 Jul 5; 6:16.

9. Peciña S, Smith KS. Hedonic and motivational roles of opioids in food reward: implications for overeating disorders. Pharmacol Biochem Behav. 2010 Nov;97(1):34-46.

10. 10. Wilbur L. The Effects of Color on Food Preference. Department of Communication, University of Utah. 2013.

11. 11. Garcia J, Kimeldorf DJ, Koelling RA. Conditioned aversion to saccharin resulting from exposure to gamma radiation. Science. 1955; 122(3160): 157-158.

12. Garcia J, Kimeldorf DJ, Hunt E. Spatial avoidance in the rat as a result of exposure to ionizing radiation. The British journal of radiology. 1957; 30(354): 318-321.

13. Countryman RA, Kaban NL, Colombo PJ. Hippocampal c-fos is necessary for long-term memory of a socially transmitted food preference. Neurobiology of learning and memory. 2005; 84(3):175-183.

14. Kapitzke D, Vetter I, Cabot PJ. Endogenous opioid analgesia in peripheral tissues and the clinical implications for pain control. Therapeutics and clinical risk management. 2005; 1(4): 279.

15. Zittel TT, Glatzle J, Kreis ME, Starlinger M, Eichner M, Raybould HE, Becker HD, Jehle EC. C-fos protein expression in the nucleus of the solitary tract correlates with cholecystokinin dose injected and food intake in rats. Brain Res. 1999 Oct 30;846(1):1-11.

16. Mitra A, Lenglos C, Martin J, Mbende N, Gagné A, Timofeeva E. Sucrose modifies c-fos mRNA expression in the brain of rats maintained on feeding schedules. Neuroscience. 2011 Sep 29;192:459-74.

17. Birch LL. Development of food preferences. Annu Rev Nutr. 1999;19:41-62.

18. Kasper JM, Johnson SB, Hommel JD. Fat Preference: a novel model of eating behavior in rats. J Vis Exp. 2014 Jun 27;(88):e51575.

19. Astur RS, Carew AW, Deaton BE. Conditioned place preferences in humans using virtual reality. Behav Brain Res. 2014 ;267:173-7.

20. Cunningham CL, Gremel CM, Groblewski PA. Drug-induced conditioned place preference and aversion in mice. Nat Protoc. 2006;1(4):1662-70.

21. Barnett SA, Spencer MM. Experiments on the food preferences of wild rats (Rattus norvegicus Berkenhout). J Hyg (Lond). 1953;51(1):16-34.

22. Nascimento ED, Lima COR, Araújo NCDM. Palatability: from formation to possible influence on weight mass. Adv Obes Weight Manag Control. 2018;8(2):134–141.

23. De Jonghe BC, Hajnal A, Covasa M. Conditioned preference for sweet stimuli in OLETF rat: effects of food deprivation. Am J Physiol Regul Integr Comp Physiol. 2007 ;292(5):R1819-27.

24. Imaizumi M, Sawano S, Takeda M, Fushiki T. Grooming behavior in mice induced by stimuli of corn oil in oral cavity. Physiol Behav. 2000 Nov 1-15;71(3-4):409-14.

25. Suzuki A, Yamane T, Imaizumi M, Fushiki T. Integration of orosensory and postingestive stimuli for the control of excessive fat intake in mice. Nutrition. 2003 Jan;19(1):36-40.

26. Manabe Y, Matsumura S, Fushiki T. Preference for High-Fat Food in Animals. In: Montmayeur JP, le Coutre J, editors. Fat Detection: Taste, Texture, and Post Ingestive Effects. Boca Raton (FL): CRC Press/Taylor & Francis; 2010. Chapter 10.

27. Zoccolan D. Invariant visual object recognition and shape processing in rats. Behav Brain Res. 2015 May 15;285:10-33.

28. Sclafani A. How food preferences are learned: laboratory animal models. Proc Nutr Soc. 1995 Jul;54(2):419-27.

29. Benoit SC, Tracy AL, Air EL, Kinzig K, Seeley RJ, Davidson TL. The role of the hypothalamic melanocortin system in behavioral appetitive processes. Pharmacol Biochem Behav.

2001; 69(3-4):603-9.

30. Sclafani A. Carbohydrate taste, appetite, and obesity: an overview. Neurosci Biobehav Rev. 1987 Summer;11(2):131-53.

31. Sun X, Kroemer NB, Veldhuizen MG, Babbs AE, de Araujo IE, Gitelman DR, Sherwin RS, Sinha R, Small DM. Basolateral amygdala response to food cues in the absence of hunger is associated with weight gain susceptibility. J Neurosci. 2015 ; 35(20):7964-76.

32. Yang Y, Wang JZ. From Structure to Behavior in Basolateral Amygdala-Hippocampus Circuits. Front Neural Circuits. 2017;11:86.

33. Chesworth R, Corbit L. The Contribution of the Amygdala to Reward-Related Learning and Extinction. The Amygdala: Where Emotions Shape Perception. Learning and Memories. 2017; 305-325.

34. Rolls ET, Rolls BJ. Altered food preferences after lesions in the basolateral region of the amygdala in the rat. J Comp Physiol Psychol. 1973; 83(2):248-59.

35. Holland PC, Hatfield T, Gallagher M. Rats with basolateral amygdala lesions show normal increases in conditioned stimulus processing but reduced conditioned potentiation of eating. Behav Neurosci. 2001;115(4):945-50.

36. Bake T, Hellgren KT, Dickson SL. Acute ghrelin changes food preference from a high-fat diet to chow during binge-like eating in rodents. J Neuroendocrinol. 2017.

37. Ranjbar H, Radahmadi M, Alaei H, Reisi P, Karimi S. The effect of basolateral amygdala nucleus lesion on memory under acute,mid and chronic stress in male rats. Turk J Med Sci. 2016;46(6):1915-1925.

How to cite this article
Zamyad M, Abbasnejad M, Esmaeili-Mahani S, Sheibani V, Raoof M. The basolateral amygdala has a critical role in food-matched visual-cue memory and post-ingestion food preferences in rats. Iran J Vet Sci Technol. 2021; 13(2): 58- 67
DOI: https://doi.org/10.22067/ijvst.2021.69518.1030
URL: https://ijvst.um.ac.ir/article_40769.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Oct- 06
Accepted after revision: 2021- Nov- 16
Published online: 2021- Dec- 30

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2021.72518.1079

The identification of single strand DNA aptamers which specifically bind to platelets using cell-SELEX technique

Fatemeh Alemi[a], Mojtaba Sankian[b], Alireza Haghparast[a], Mohammad Reza Bassami[a, c], Gholamreza Hashemi Tabar[a, c]

[a] Division of Biotechnology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[b] Immunology Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.
[c] Research Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran.

ABSTRACT

Aptamers are oligonucleotides that can be easily synthesized and bind to their targets with high affinity and specificity. Several aptamers specific to soluble factors of coagulation cascade have been produced, however, aptamers specific to platelet cell membrane molecules have not been reported yet. We aimed to discover DNA aptamers that specifically bind to human platelets. The cell-SELEX method was used for aptamer discovery. Synthetic 79 nucleotides length single-strand oligonucleotides were used as a library. Ultra-pure platelets were prepared using differential centrifugation steps and magnetic-bead-assisted removal of contaminating cells. The FITC-labeled forward primer was used for amplification of the selected oligonucleotides by PCR, and Lambda exonuclease was used for digestion of the lagging strand. After 12 rounds of cell-SELEX, selected oligos were amplified and cloned to pTG19-T vector, transfected into *E. coli* (TOP10) and sequenced. Sequences of aptamers from 200 individual positive colonies were aligned and seven clusters were identified. Representative aptamers were amplified and their affinity, specificity, and digestibility of their targets were evaluated. Interferences of the aptamers to two platelet function tests were also investigated. Affinity (K_D) of the representative aptamers were between 109 and 340 nM. Trypsin exposure of the platelets completely abolished the binding of the 7 aptamers to the targets. The binding of the four aptamers fully protected their target molecules from digestion. No one of the aptamers changed the parameters of the platelet function tests. Seven aptamers specific to platelets were identified and characterized. These aptamers may have potentially diverse applications in the diagnosis or treatment of platelet disorders.

Keywords

Cell-SELEX; platelet; DNA aptamer; Platelet-specific aptamer.

Number of Figures: 9
Number of Tables: 1
Number of References:: 52
Number of Pages: 16

Abbreviations

SELEX: Systematic evolution of ligands by exponential enrichment

K_D: Dissociation constant
PCR: Polymerase chain reaction
SEM: Standard error of the mean

Corresponding author:
Gholamreza Hashemi Tabar
Email: Hashemit@um.ac.ir
Tel: +98 9153138954

Introduction

Nucleic acid aptamers are promising alternatives to antibodies in life science and because of their unique properties, have gained increasing attention from scientists. They are small (25-100 bp), single-stranded, synthetic nucleic acids [1] which can be folded into their 3D conformation capable of binding to certain targets with extremely high affinity and specificity. They can be easily synthesized, modified, or conjugated by chemical reactions. The aptamer can be denatured and renatured frequently without loss of its activity. They are more than 15-20 times smaller than antibodies, therefore, can easily diffuse across cell membranes or penetrate areas that are not accessible for antibodies [2]. Aptamers also possess little or no immunogenicity and low systemic toxicity in vivo [3].

Systematic evolution of ligands by exponential enrichment (SELEX) technology for selection and enrichment of aptamers has been explained in detail elsewhere [4,5]. Briefly, a single-stranded nucleic acid pool is prepared and incubated with the target molecules, to recover oligonucleotide variants with desired binding affinity to the targets. Bound oligonucleotides are isolated from their targets and amplified using conventional polymerase chain reaction (PCR). The selection and amplification cycles are sequentially repeated and the ligand-specific signal intensity is determined after each round of selection until the gradual increases in the signal intensity reach the plateau state (no further increase in the signal intensity between two or three successive rounds) [5].

Initially, aptamers have been produced solely against small molecules in a pure state [6]. Therefore, production of aptamers specific to cell membrane-associated molecules had been feasible only when the molecules had been prepared in the pure form, however, the purification process is usually associated with denaturing and disturbing the native conformation of the molecule, and aptamers produced using a denatured form of the molecule, probably, will not be able to recognize and bind to their targets that are in native forms [5,7].

The first complex matrix that was used as a target for aptamer discovery was bacterial ribosomes [8]. Morris et al produced high-affinity aptamers against red blood cells in 1998 [9]. Since then, many efforts have been made to develop high-affinity aptamers against eukaryotic or prokaryotic cells [10]. Cell-SELEX, as performing SELEX using a homogeneous population of eukaryotic or prokaryotic cells, has been introduced for the simultaneous development of numerous aptamers against cell-associated molecules. Recently, Cell-SELEX technology has been increasingly used in biomarker discovery [11,12]. Prior knowledge of the target molecule is not required for the development of a target-specific aptamer through cell-SELEX. The cell membrane surface has a countless number of molecules, therefore at the end of a successful cell-SELEX procedure, numerous aptamers are generated for many different targets.

Aptamers have been used as drug delivery agents with tunable release capacity or antidote-assisted prevention of drug-related side-effects [13]. They are also used for biomarker discovery or purification of target molecules [14,15]. They have found also applications in diagnostic approaches, e.g., ELISA [16,17], or biosensor design [18]. Aptamer attachment may also inhibit the biological activities of the target molecule or may prevent it from enzymatic digestion [19,20].

Platelets are small, anucleate, disc-shaped cells in blood with dimensions ranging 2-4 micrometers. They actively contribute to blood hemostasis, inflammation, host defense, tumor growth, and metastasis. After red blood cells, platelets are the second abundant cells in blood with normal platelet counts ranging from 1.5-4.5 x 10^5 cells per microliter. Any abnormalities in platelets can lead to various bleeding disorders [21]. Platelets have receptors and adhesion molecules on their surface to interact with immune cells and also with circulating pathogens [22]. Blood platelets are usually brought into close contact and have frequent interactions with vascular walls, red blood cells, leukocytes, other platelets, plasma contents, and even with foreign bodies penetrated the circulation (e.g., infectious agents or toxins). Platelet membrane-associated molecules play a major role in the reciprocal interactions. Several aptamers have been produced specifically to soluble factors of the coagulation cascade [23], however, to the best of our

Abbreviations-Cont'd

LTA: Light transmission aggregometry
PFA-100: Platelet function assay 100
MAR: Maximal aggregation rate
WBC: White blood cell
RBC: Red blood cell
PGE1: Prostaglandin E1
ASA: Acetylsalicylic acid, Aspirin
P2Y12: An ADP receptor
COX: Cyclooxygenase
PT-VWD: Platelet type von Willebrand factor
GPI: Glycoprotein I
ADP: Adenosine diphosphate
EDTA: Ethylene diamine tetra acetic acid
ACD: Acid citrate dextrose
PRP: Platelet rich plasma
PPP: Platelet poor plasma
RT: Room temperature
LPA: Linear polyacrylamide
CEPI: Collagen-Epinephrin
CAPD: Collagen-ADP

knowledge, aptamers specific to platelet membrane molecules have not been produced yet. Our goal was to produce DNA aptamers that bind specifically to platelet membrane-associated molecules.

Results

Quality and purity of cells

Through the cell preparation step, platelets of 3x10 ml blood samples (per day) from three fixed donors(O+) were concentrated in one ml. Our mission was to obtain highly pure target cells to apply in the aptamer discovery process and all conditions were adjusted to achieve the goal. Magnetic beads equipped with RBC- and WBC-specific antibodies swept all unwanted cells from the suspension, including activated platelets complexed with the cells. During the 13 working days (2-4 weeks intervals), a total of 39 blood samples were drawn and platelet density was measured in the initial samples and the final concentrates. Platelet recovery rates were ranged between 62-68% (mean ± SEM: 65.69 ± 0.56 %). Inclusion of the inhibitors in the anticoagulant solution efficiently prevented platelet activation during the purification process confirmed by flow cytometric analysis of the activation and apoptotic markers in the collected cells using anti-CD62p-PE and Annexin-V-FITC (figure 1). Concomitantly, a highly pure suspension of white/red blood cells was prepared for counter selection. The viability of the cells assayed by the Trypan blue exclusion method was 0-2%.

Visualization of the PCR product: Rapid and effective way for a demonstration of the efficacy of the PCR reagents and the entire process is a visualization of the products using agarose gel electrophoresis. PCR amplified samples from eight rounds of SELEX were resolved on 3% agarose gel with TBE (0.5x) as running buffer and DNA safe-stain as coloring agent (Figure 2B).

Single-strand preparation: Oligonucleotides collected from each round of SELEX were amplified through PCR assay using FITC-labeled sense primer and 5'phosphorylated antisense primer. For the next round of SELEX single-stranded FITC-labeled oligonucleotides are required, though, the phosphorylated strand was digested using Lambda exonuclease enzyme. For demonstrating the reliability of the enzyme and for optimizing reaction conditions, double-strand PCR products were exposed to the enzyme for 0, 10, 20, 30, and 40 minutes at 37 °C obeying the supplier's instructions. Agarose gel electrophoresis (3% and 10%) for resolving the single-strand preparation was not successful and was associated with a fully smeared electrophoretic band (data not shown). We took the advantage of native polyacrylamide gel (10%, TBE as running buffer, and Methylene blue as coloring agent) electrophoresis for visualization of the rescued strand (figure 2A).

Monitoring the efficiency of enrichment process

Totally 13 rounds of SELEX were applied and ssDNA samples were collected for flow cytometric evaluation of the enrichment process. Figure 3 shows the efficiency of the enrichment process during the progression of the SELEX toward the 13th round.

Characterization of the selected aptamers: Plasmids originating from the 200 positive colonies were

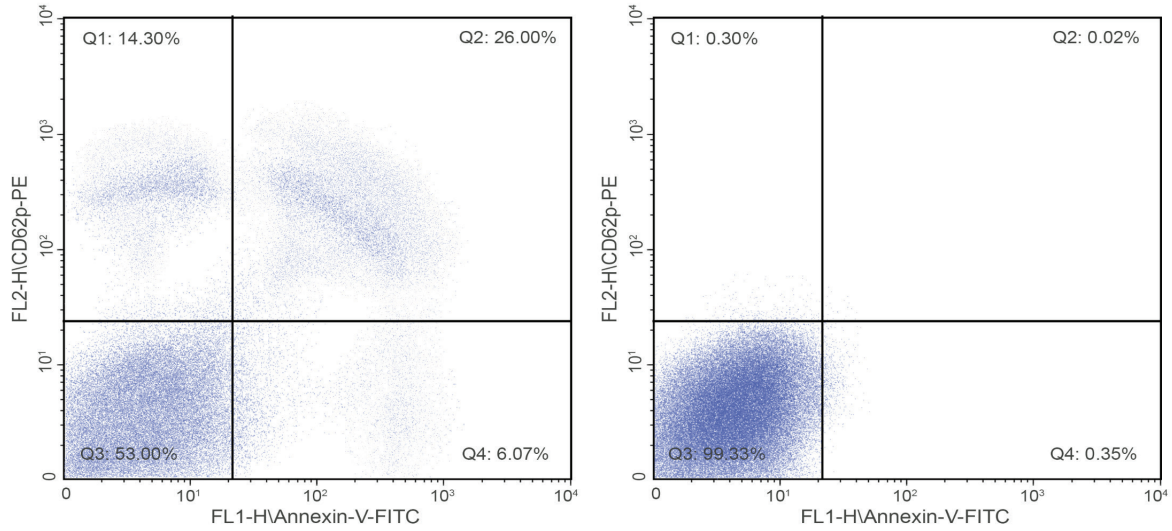


Figure 1.
Evaluation of platelet activation and apoptosis markers in highly purified platelets. A. Dot plot diagram of collagen-treated platelets (as a positive control) showing high frequencies of activated and apoptotic cells stained by anti-CD62p-PE and Annexin-V-FITC. B. Dot plot diagram of purified platelets showing very low frequencies of cells stained by the two fluorochromes. Quadrants were drawn using isotype controls.

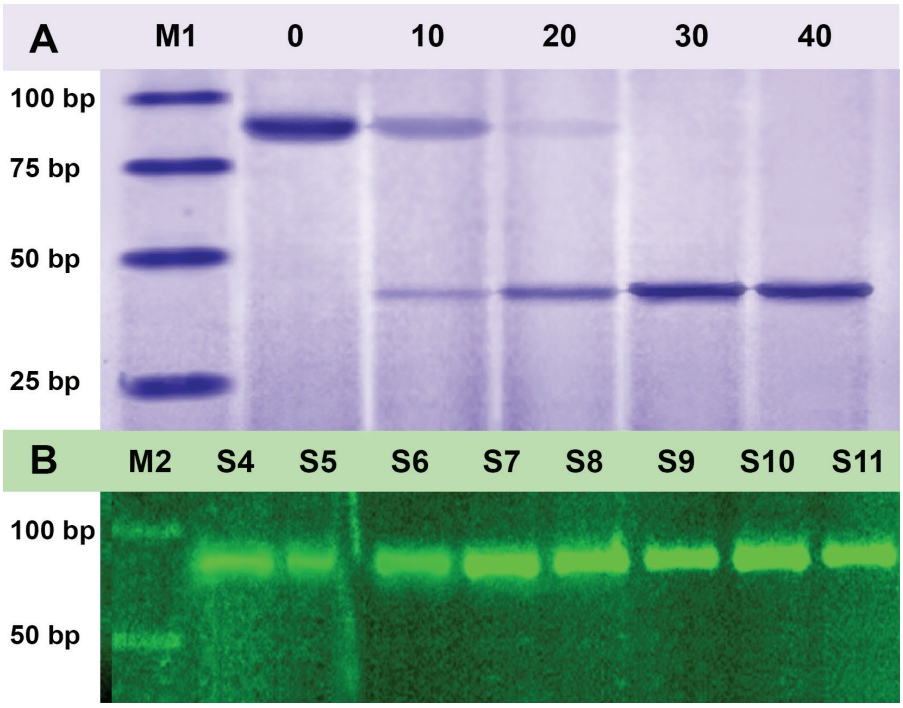


Figure 2.
Results of Native-PAGE and Agarose gel electrophoresis. A) native polyacrylamide gel electrophoresis of double-strand PCR products exposed to Lambda exonuclease enzyme for different time points (0, 10, 20, 30 and 40 minutes). M1: 25 bp DNA Ladder. Digestion of phosphorylated strands of double-strand PCR products was complete after 30 minutes exposure to the enzyme. B) Agarose gel electrophoresis of PCR products from 8 rounds of SELEX. Oligonucleotides collected from each round of SELEX were amplified by PCR. Five µL from the PCR product was mixed with 1 µl loading buffer and resolved by 3% agarose gel electrophoresis (100V). DNA safe stain was used for visualization. M2: 50 bp DNA ladder. S: SELEX.

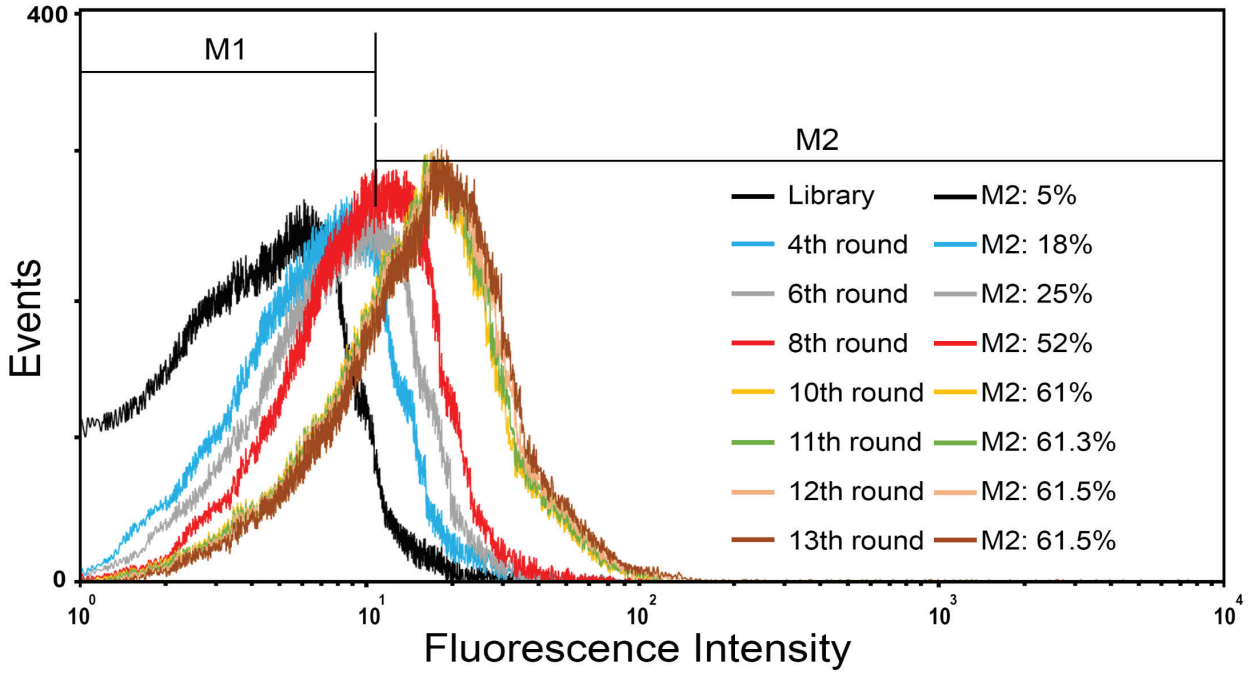


Figure 3.
Frequency of positive cells stained with FITC-labeled aptamers. PCR products from eight rounds of SELEX were incubated with fixed numbers of pure platelets and analyzed. M1 and M2 regions were drawn using control library and adjusted to contain 95% and 5% of the cells, respectively. The same regions were used for segregation of the positive cells stained by outputs of the later rounds. As the histograms show, gradual increases in the frequency of positive cells were observed during the SELEX progression and the frequency reached from M2: 5% in the first round to M2: 61.5% in the 12th round and not changed during the 13th round.

sequenced, aptamer sequences aligned and divided into 7 clusters (Table 1) based on the sequence similarity. Sequence variation within each cluster was presented in figure 4. 2D structure of the seven representative aptamers was presented in Figure 5. Figure 6 shows the binding affinity and calculated K_D values of the aptamers.

Effects of Aptamers on platelet function in vitro

Seven aptamers at three concentrations were incubated with platelets and subjected to aggregometry by the LTA method in the presence of collagen or epinephrine. Slop and MAR was determined and compared (figure 9A/9B). No one of the seven aptamers had noticeable effects on slope or MAR of the platelets by the LTA method (paired-samples *t*-test: $p > 0.05$). PFA-100 test results were also not significant compared to the controls (figure 9C/9D).

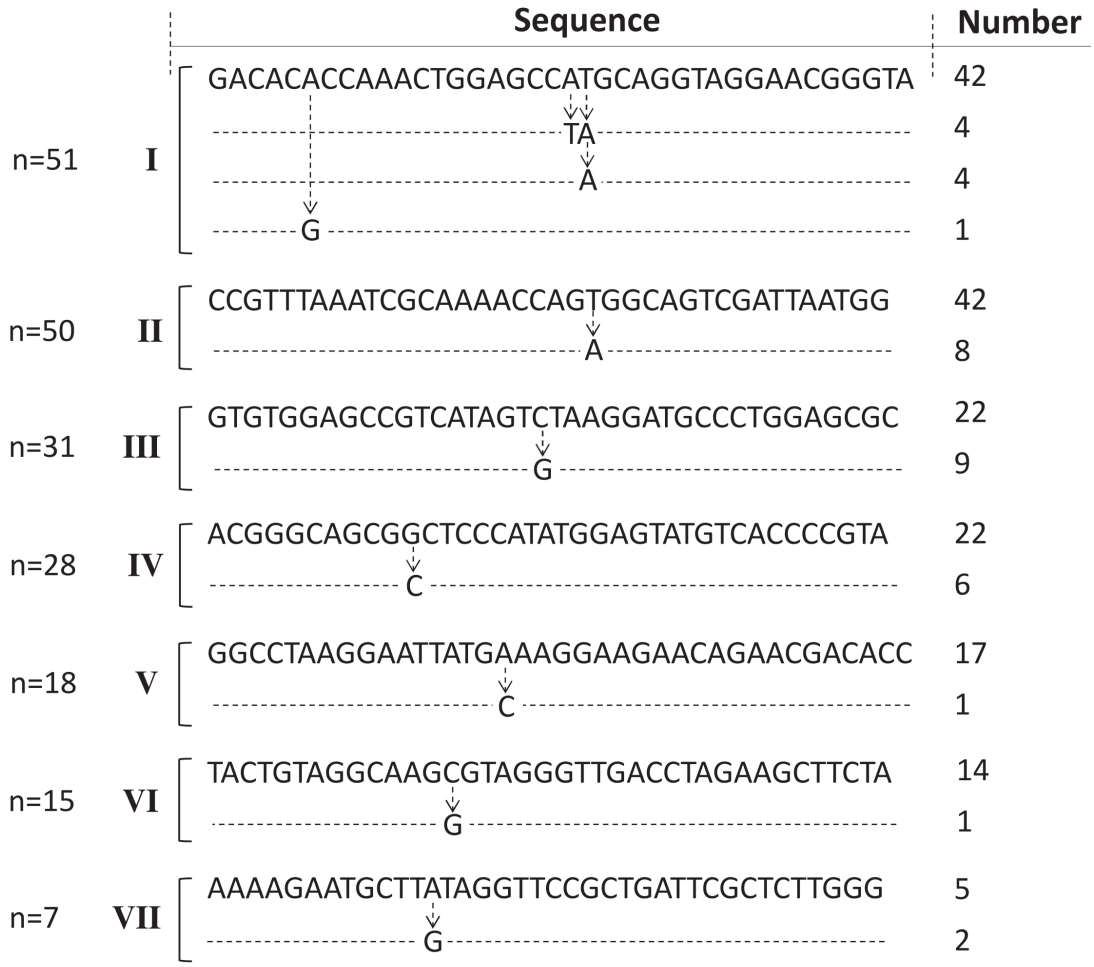


Figure 4.
Nucleotide variation within each cluster. Total number of oligos in each cluster was presented in the left. Number of sequenced oligos in each variant was provided in the right. Roman numerals (I-VII) show cluster numbers.

Table 1.
Nucleotide sequences of the seven aptamers (without fixed regions).

No.	Name	Sequence
1	APT1	GACACACCAAACTGGAGCCATGCAGGTAGGAACGGGTA
2	APT2	CCGTTTAAATCGCAAAACCAGTGGCAGTCGATTAATGG
3	APT3	GTGTGGAGCCGTCATAGTCTAAGGATGCCCTGGAGCGC
4	APT4	ACGGGCAGCGGCTCCCATATGGAGTATGTCACCCCGTA
5	APT5	GGCCTAAGGAATTATGAAAGGAAGAACAGAACGACACC
6	APT6	TACTGTAGGCAAGCGTAGGGTTGACCTAGAAGCTTCTA
7	APT7	AAAAGAATGCTTATAGGTTCCGCTGATTCGCTCTTGGG

Figure 5.
Configurations of the representative aptamers (graphed by RNAstructure 6.2 software). Ionic strength was set to the physiological conditions (144 mM Na^+) and temperature was set to 21 °C.

Discussion

Our goal through the current study was the discovery of high affinity and specificity DNA aptamers against human platelets and the cell-SELEX method introduced by Sefah [5] was used for the experimental process. Ultra-highly pure and intact cells were needed for a successful cell-SELEX procedure; however, platelets are very susceptible to physical and chemical stimuli and are highly prone to activation. In the circulation, platelets are prohibited from unnecessary activation, however, upon draining, platelets come

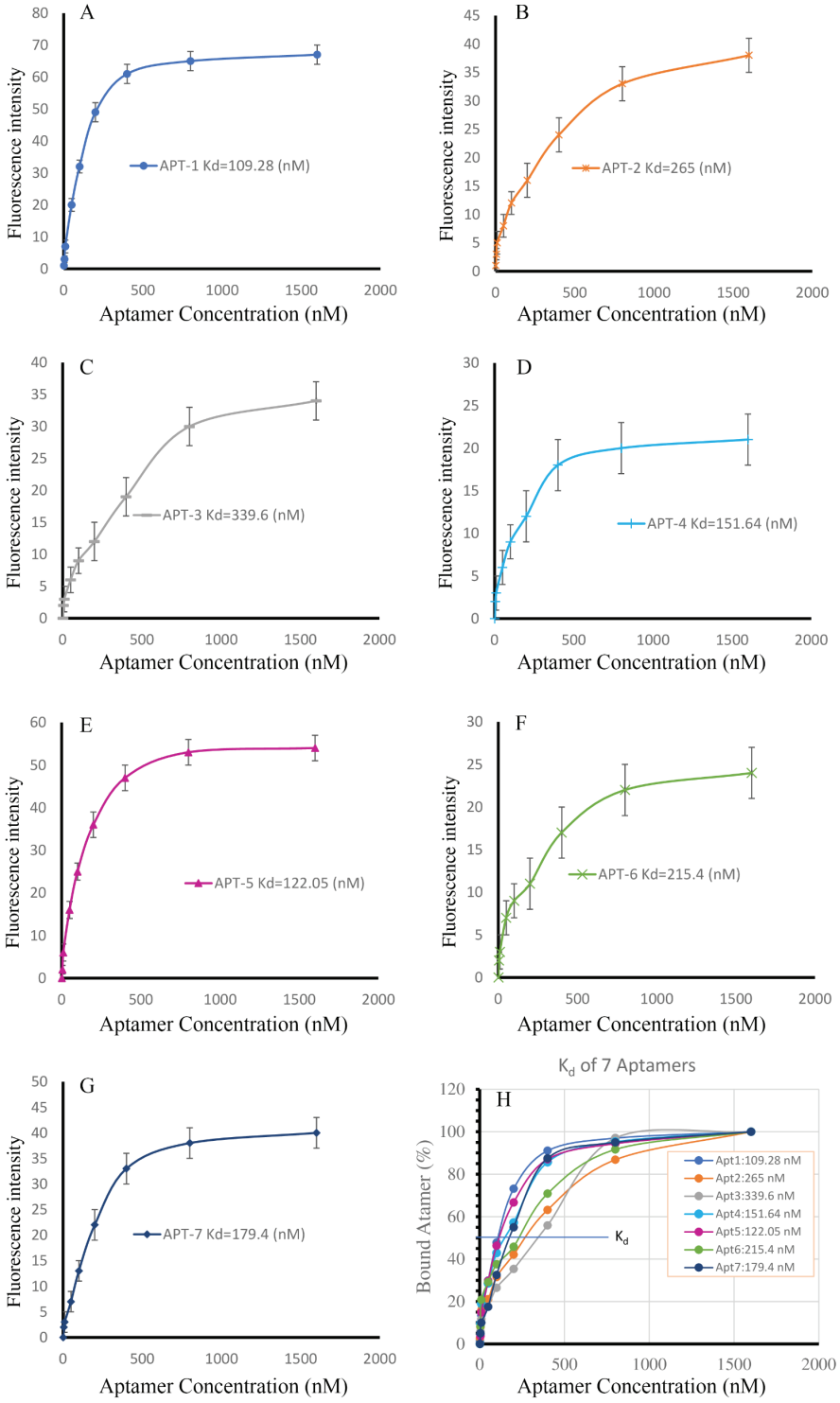


Figure 6.
Binding affinity of seven aptamers to platelets and their K_D values. A to G presented geometric mean fluorescence intensity of each concentration of the aptamer that bound to fixed number of platelets in vertical axis. Error bars presented SD values calculated using triplicate experiments. The highest levels of fluorescence (plateau tail of the curve) are different between aptamers, probably, due to the different densities of the target molecules on the platelet surface. H shows K_D values in a comparable manner between aptamers. Vertical axis presents fluorescence intensity of each experiment relative to the maximum fluorescence from the same aptamer, expressed as percent. K_D values were estimated using one-site saturation equation (see text).

into close contact with foreign surfaces and are fairly activated. Upon activation, cell structure and morphology undergo tremendous changes and the cells adhere to other cells and surfaces leading to the cell aggregation and release of a huge number of chemicals. Therefore, the preparation of highly pure intact platelets requires the implementation of optimized protocols and unique protecting materials. The Amisten [24] method with some modifications was selected and used for cell purification. The method had been optimized by Amisten for mRNA detection by microarray analysis and had four main phases: blood collection, PRP preparation, leukocyte removal by filtration, and magnetic bead-assisted depletion of contaminating cells. We modified the method for achieving the highest platelet recovery rate and purity.

The highest platelet recovery rate had been reported by Wrzyszcz [25] which was 53.3 ± 13.1 (%), while the recovery rate in the current study was estimated to be 65.69 ± 0.56 (%). The first step in PRP preparation is the precipitation of the red and white blood cells by a low-speed centrifugation step, while, a substan-

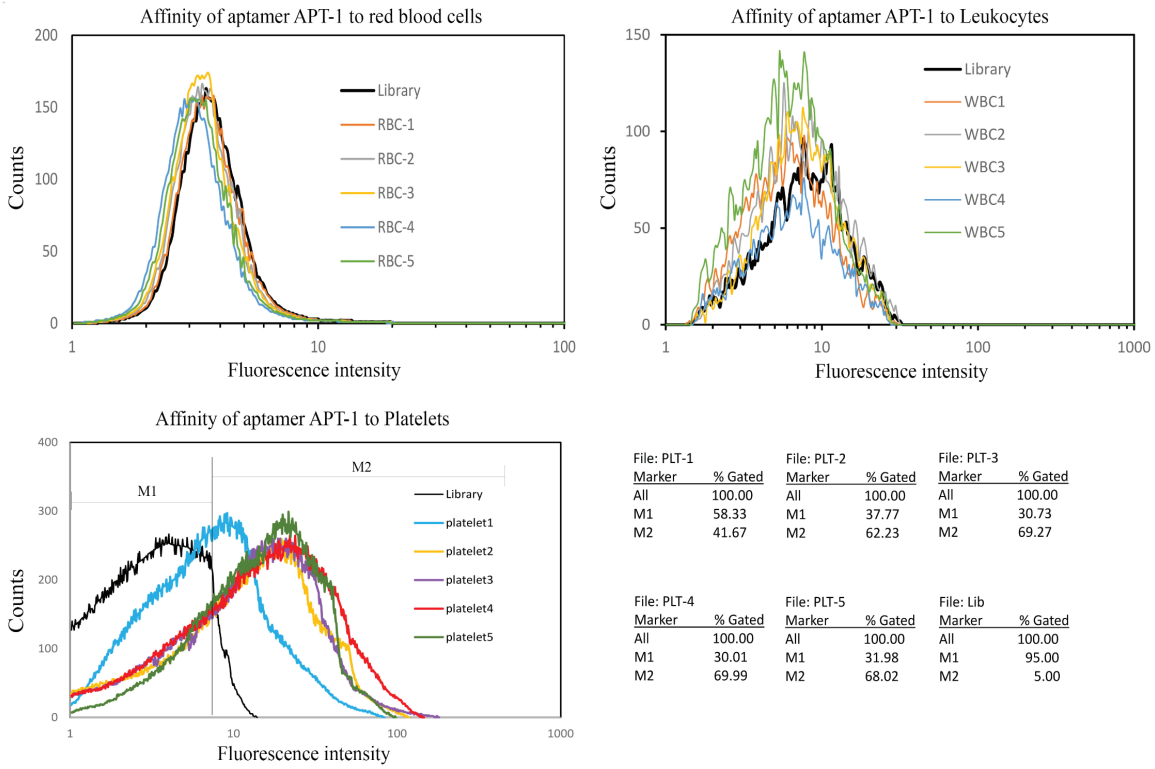


Figure 7.
Affinity of aptamer APT-1 to pure cells from five donors. For selectivity assessment pure RBC, WBC and platelets from five donors were incubated with aptamer APT-1 and fluorescence signals from cell-bound aptamers were analyzed by flow cytometry. Bold black line represents control library. Fluorescence intensity of APT-1 aptamer bound to RBCs or WBCs (five color lines) were exactly equal to the control library, therefore, APT-1 aptamer to not have any affinity to the cells. In contrast, platelets from the five donors all captured APT-1 aptamers and resultant fluorescence signal intensities were obviously higher than control library. M2 regions for platelet1-5 contain 41.67%, 62.23%, 69.27%, 69.99%, 68.02% of cells while the initial library was set to contain only 5% of cells in the region.

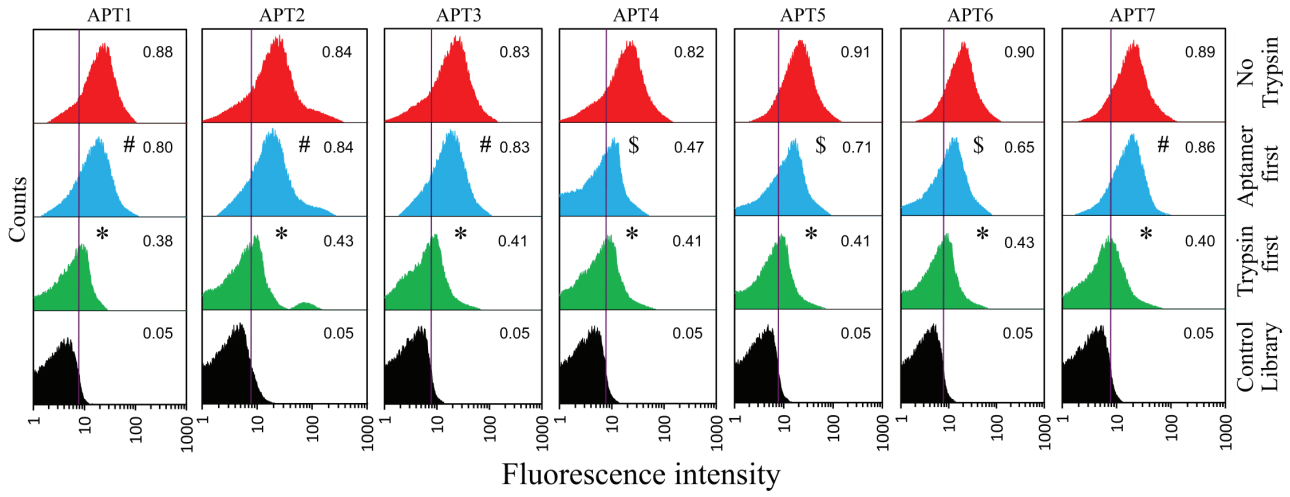


Figure 8.
Digestibility of ligands before and after aptamer binding. Platelets were treated with trypsin before (green histograms) or after (blue histograms) incubation with aptamers APT1-APT7. Black histograms represent control library (5% positive). Red histograms show platelets not treated with trypsin but exposed to the seven aptamers. Asterisk signs (*) mark all green histograms displaying a significant reduction in the fluorescence intensity (shift to the left compared to the red histograms) that means ligands have been digested by the enzyme activity. Hash signs (#) mean that aptamer binding completely protected ligand against trypsin digestion. Dollar signs ($) means partial protection of ligand after aptamer binding.

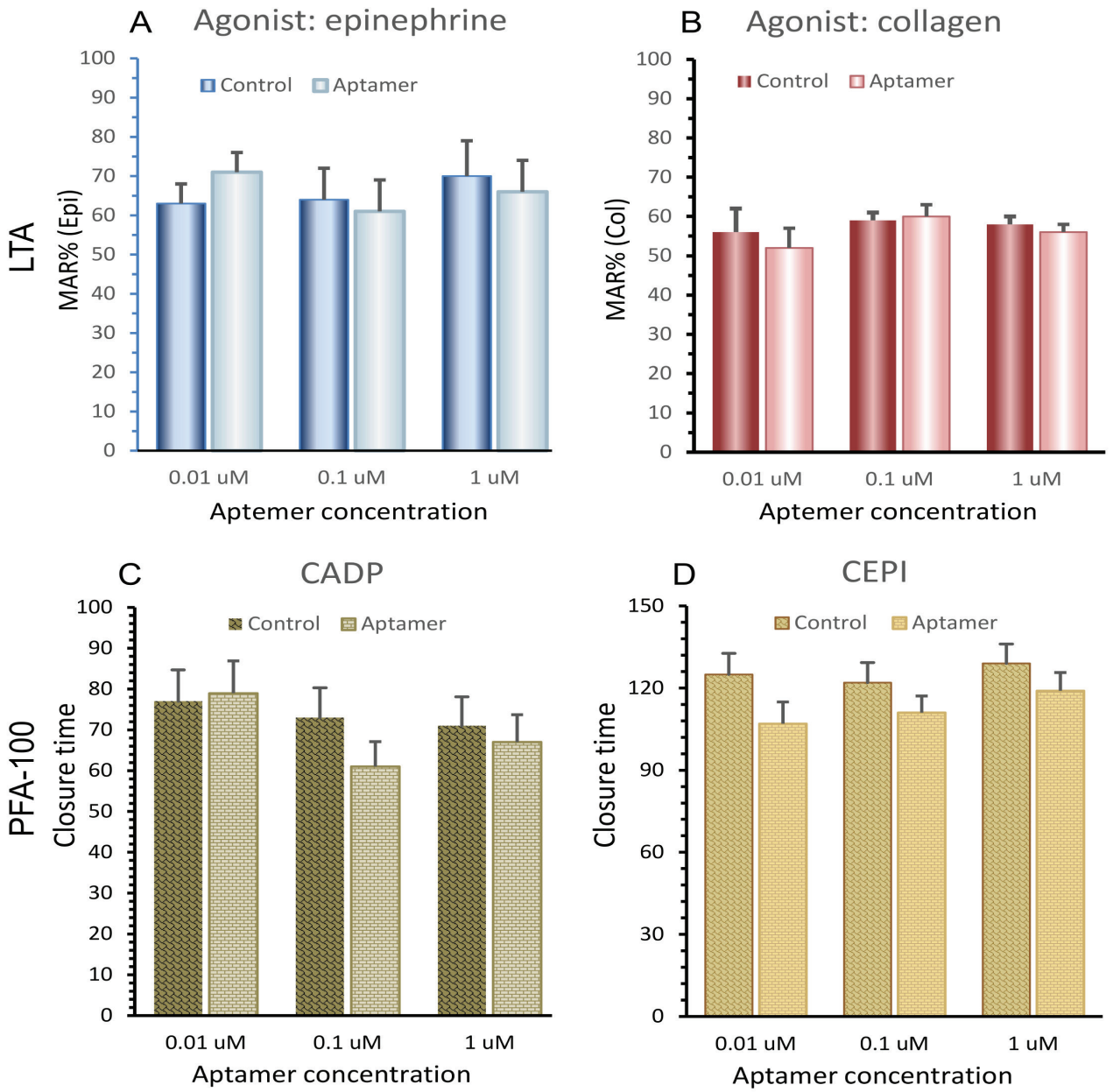


Figure 9.
Aptamer effects on two conventional platelet function tests: LTA and PFA-100. A&B: Aptamer effects on the results of light transmission aggregometry (LTA). C&D: Aptamer effects on the platelet function assay with PFA-100 instrument. Three concentrations (0.01, 0.1, 1 µM) of aptamers were prepared using routine samples (platelet-rich plasma in LTA assay and citrated blood in PFA-100 test) and assayed. Control samples prepared using initial library (instead of select aptamer). Epinephrine (A) or Collagen (B) were used as agonists in LTA assay. Cartridge in the PFA-100 tests equipped with Collagen + ADP (C) or Collagen + Epinephrine (D). Results of LTA assay expressed as mean "maximal aggregation rate" (MAR: %) and results of PFA-100 test expressed as mean "closure time" (second). All seven aptamers were analyzed, however, results of APT1 only presented here. Paired samples t-test was used for statistical comparisons. In collection, probably, due to the high variability of the data obtained from these tests, statistical comparisons did not demonstrate any significant differences between groups. Calculated coefficient of variations (CV) for LTA (COL/EPI) and PFA-100 (CADP/CEPI) assays were 15%/18% and 6.5%/7.9%, respectively.

tial amount of the platelets is also coprecipitated along with the cells. Platelet poor plasma, PPP, was prepared and added to the precipitated cells and gently mixed by upside-down method, and centrifuged again for the recovery of the coprecipitated platelets.

A filtration step has been proposed by Amisten to remove WBCs, however, platelet activation was seen during the process, and subsequently, the filtration step was omitted and the entire process was optimized to achieve the highest purity and intactness. In addition, leukocyte removal filters did not remove erythrocytes, a drawback that has also been reported

by Wrzyszcz [25].

Platelet activation is associated with a major change of the molecular composition of the cell membrane and triggers platelet adherence to the adjacent cells and surfaces. For achieving the highest cell recovery rate and keeping intactness of the cells, PGE1 and ASA were added to the anticoagulant solution and working buffers. ASA covalently binds to Ser530 in the cyclooxygenase, COX, molecule and hinders the access of arachidonic acid to the catalytic site of the enzyme; therefore, prohibiting thromboxane synthesis. Prevention of thromboxane generation by ASA prevents fibrinogen receptor activation and impairs platelet-platelet interactions [26]. PGE1 virtually antagonizes P2Y1 receptor activation on the platelet membrane. PGE1 binds to its receptor on the platelet and inhibits phospholipase C enzyme activation through increased production of cAMP that eventually inhibits mobilization of intracellular calcium and cell aggregation [27].

Through the cell preparation step, experimental conditions for differential centrifugation were optimized and magnetic bead-assisted removal of the contaminating cells was applied. Magnetic beads equipped with anti-CD45 (a common leukocyte marker) or anti-CD235a (a common erythrocyte antigen) were used for further purification of platelets and in the same manner, magnetic beads equipped with anti-CD41 (a common platelet marker) was used for further purification of WBC and RBC suspension. Finally, ultra-pure cells were applied for the cell-SELEX procedure.

Aptamers are usually discovered and amplified by a sophisticated method named SELEX (abbreviations for Systematic Evolution of Ligands by Exponential enrichment) [28]. Through the SELEX procedure, a chemically synthesized library of random oligonucleotides (DNA or RNA or their modified forms; 40-100 nucleotides length, single-stranded) is exposed to the target molecules. Few oligonucleotides bind to the targets and Ligand-target complexes are then separated from unbound forms. Target-specific ligands are isolated and amplified for further processing. Initially, vigorously purified molecules were being used as aptamer targets; however, the SELEX procedure was gradually extended by the researcher to favor discovering aptamers against cells and complex matrices. The procedure in which cells are used as the target for aptamer binding was named cell-SELEX [9,29]. The cell-SELEX method introduced by Sefah was selected and followed for the current research.

Platelets, a disc-shaped anucleate cell in the blood, or thrombocytes are produced from bone marrow resident progenitor cells, named megakaryocytes, and 10^{11} cells per day are continuously being released into the circulation to maintain their normal count in the blood (150000-450000 cells per microliter) [30]. They are short-lived cells (lifespan: 7-10 days) with numerous fundamental functions, including, direct involvement in blood clotting, tissue repair/wound healing, angiogenesis, inflammation, cell proliferation, tumor progression, and metastasis [31]. Like other cells, the platelet cell membrane is made up of two layers of phospholipid and more than 50 classes of membrane-bound receptors mediate platelet interactions with the vessel wall, other cells, and soluble factors. Any congenital or acquired disorders of the receptors are associated with defects in platelet procoagulant activity and manifest as hemorrhagic diseases. A clear example of the disease is platelet-type von Willebrand disease, PT-VWD, an inherited platelet disorder caused by the enhanced affinity of membranous GPI-bα to soluble VWF [32]. Platelets are also actively involved in atherosclerotic cardiovascular disease. Atherosclerotic plaque rupture exposes damaged endothelium and facilitates platelet interaction with exposed vascular surface leading to platelet adhesion, activation, and thrombus formation. Platelet regulatory drugs (or anti-platelet drugs) are frequently administered in many clinical conditions and the drugs either antagonize platelet membrane receptors or inactivate key member(s) of the intracellular signaling pathways. For instance, aspirin inactivates the COX enzyme and clopidogrel antagonizes ADP receptor P2Y12 on platelets. These drugs have often a narrow therapeutic window with no antidotes if the intervention was associated with side effects. In addition, 'one dose for all strategy' was common with the use of antiplatelet agents, therefore, an increased risk of serious bleeding is always being expected with the interventions [33]. Moreover, increased resistance or enhanced susceptibility of patients to the drugs are common, while no criteria for predicting the situation exist [34]. Aptamer technology has facilitated the development of a new generation of therapeutic agents with target specificity and reversibility even after administration. For instance, Oney [35] and Nimjee [13], both developed RNA aptamers that bind VWF with high affinity and inhibit platelet adhesion and aggregation. Antidote molecules were also designed to quickly reverse aptamer function, a new important invention promising creation of safer and regulable drugs. Aptamers are oligonucleotides (DNA or RNA) composed of less than 80 nucleotides and bind with high affinity and specificity to the target molecules (ligands). Two or more aptamers can be combined and used for complex and unique purposes [36]. Aptamers immobilized on a solid surface can be used for the detection of target molecules [17].

Through the current study, we used a randomly

synthesized oligonucleotide library of single-stranded DNA molecules of 79 nucleotides. The sense primer was FITC labeled and antisense primer was phosphorylated in the 5' end. Polymerase chain reaction, PCR, was used for amplification of the strands, and lambda exonuclease was used for digestion of the phosphorylated strand. Purified human platelets were used as aptamer targets and non-specific oligos were absorbed to RBC and WBC from the same donor. Flow cytometry was used for the evaluation of the ligand enrichment process. The cell-SELEX procedure was progressed according to the Sefah [5] method and platelet-specific ligands were gradually enriched, which was demonstrated by increased fluorescence signals detected by flow cytometry. The frequency of the platelets stained with FITC-labeled aptamer reached the highest rate in the 11th cycle of the cell-SELEX and remained nearly unchanged during the 12th and 13th cycles. To the best of our knowledge, the production of platelet-specific aptamers has not been reported yet. The number of SELEX cycles required before cloning of the selected aptamers did not adhere to an especial rule. The number of cycles for developing aptamers against prion proteins by Bibby [37] was eight. Mehennaoui [18] operated 19 cycles of SELEX for producing aptamers against dexamethasone. Chen [38] used seven cycles of SELEX for producing aptamers against hepatic stellate cells. In our study, PCR products from the 12th round of SELEX were amplified using unlabeled primers and cloned. Positive colonies (200 colonies) were selected and propagated in liquid media. Plasmids were extracted and sequenced. Sequenced aptamers were aligned and seven representative aptamers were further evaluated for affinity determination and elucidation of the aptamers' effects on platelet function tests. The affinity of the aptamers to platelets ranged between 109 and 339 nM, which were comparable to the previous reports. The affinity of DNA aptamers discovered by other researchers is also in nanomolar levels. For instance, Affinity of DNA aptamers have been developed by Forier [14], Duan [39], Tang [40], Wu [16], Berg [41], Zhou [42], Spiga [43], Baig [44], Moon [45], Moosavian [46], Mozioglu [47] and Oney [35] were reported to be 1.2 nM, 32.24 nM, 669-998 nM, 23 nM, 137 nM, 4-12 nM, 200 nM, 210-1000 nM, 3.49 nM, 133-191 nM, 5.09 nM and less than 20 nM, respectively, (range:1.2-1000 nM) . Regarding the nature of target molecules on platelets, binding of all 7 aptamers to their targets was abolished after trypsin treatment of the cells, demonstrating the protein nature of the molecules. In addition, the pre-binding of three aptamers to their targets on platelets partially protected the targets from digestion by Trypsin. No one of the seven aptamers could change the results of the platelet function tests PFA-100 and LTA, considerably. The explanation for the failure is the high variability of the data obtained from the two tests. It seems that a new method with low variation and high reproducibility is needed for the evaluation of aptamer effects on platelet functions.

We isolated 200 colonies harboring aptamer-containing plasmids and evaluated seven representative aptamers targeting protein molecules on the platelets. Our results showed the discovery of platelet-specific aptamers through the cell-SELEX method was feasible and the affinity of the aptamers was in an acceptable range and favorable for application in research. Purification of the target molecules using aptamer-affinity column and further identification of the targets is our goal for a future study. These aptamers can also be used for the detection of human platelets in forensic medicine.

Materials & Methods

Oligonucleotides

HPLC purified, Single-stranded random oligonucleotide library consisted of 38 random nucleotides in the middle portion and two 21 and 20 constant sequences in the 5' and 3' ends, respectively (5'-GCCTGTTGTGAGCCTCCTAAC [N38] CATGCTTATTCTTGT-CTCCC-3') [48]. Pairs of primers (labeled/unlabeled) were ordered to "TAG Copenhagen, Denmark" (forward primer: 5'-GCCTGTTGTGAGCCTCCTAAC-3', reverse primer: 5'-GGGAGACAAGAATAAGCATG-3'). The forward primer was FITC-labeled and the reverse strand was 5'-phosphorylated. Unlabeled primers were used for cloning and sequencing experiments.

Buffers and antibodies

The "Dynabeads® M-280 Sheep anti-Mouse IgG" magnetic beads were from ThermoFisher (Cat. No:11202D). Anti-CD45, anti-CD-235a and anti-CD41 antibodies (ab8216/ab212432/ab11024 produced in mice) were from Abcam. Acid Citrate-Dextrose (ACD; C3821), Prostaglandin E1 (PGE1; P5515), Acetylsalicylic acid (ASA; A5376), and EDTA (ED2P) all were from Sigma-Aldrich. Annexin-V-FITC and anti-CD62p-PE were from Becton Dickinson (Cat. No. 556570 & 555524). Platelet washing buffer [49] was composed of NaCl (129 mM), KCl (2.8 mM), $NaHCO_3$ (8.9 mM), KH_2PO_4 (0.8 mM), $MgCl_2$ (0.8 mM), EGTA (2 mM), Glucose (5.6 mM), HEPES (10 mM), BSA (0.35%), pH:7.4, prepared in 1000 ml DW (final volume). The binding buffer was composed of the same ingredients except for EGTA that was replaced by $CaCl_2$ (1 mM). EGTA, as an anticoagulant, was included in the platelet washing buffer because it chelates Ca^{2+} ions with high affinity (compared to the EDTA) and prevents platelet activation. EGTA was replaced by $CaCl_2$ in a binding buffer because Ca^{2+} ions are necessary and facilitate interactions between oligomers and platelet-surface molecules.

Preparation of cells

Freshly prepared ultra-pure blood platelets were used as targets. Experimental conditions were optimized for preserving the intactness of the cells [24]. Blood was drawn using an 18-gauge needle through the intravenous cannula. Anticoagulant was made up of 18 ml ACD, 12 µl PGE1 (1mM), 120 µl ASA and 480

µl EDTA (0.5 M). Hematologic parameters were measured and platelet-rich plasma (PRP) was prepared by repeated centrifugation of the anticoagulated blood at 200 g.

Immunomagnetic separation

Magnetic Dynabeads were equipped with capture antibodies according to the manufacturer's recommendations. Anti-CD45 and anti-CD235a capture antibodies were used for WBC and RBC depletion of PRP preparation [50]. The magnetic field was used for precipitation of the bead-bound cells and floating cells (platelets) were separated and precipitated. Highly pure platelets were counted and resuspended in the washing buffer (Tyrode-Hepes buffer with EGTA). P-selectin (CD62p) is a platelet activation marker. Annexin-V binds to phosphatidylserine residue that is exposed on the surface of apoptotic cells. FITC-labeled Annexin-V and PE-labeled mouse anti-human CD62p antibodies were used for flow cytometric detection of the two markers in purified platelets [51].

Preparation of cells for counter selection

White and red blood cells (WBC/RBC) were prepared and used for counter selection. Anticoagulated blood was precipitated and a complete buffy coat section plus a small fraction of the underlying red cells were removed and resuspended in platelet-poor plasma (PPP) derived from the same blood sample. Magnetic Dynabeads equipped with anti-CD41 antibody were used for depletion of the platelets from the suspension. The viability of the WBCs was determined using Trypan blue exclusion method.

Cell-SELEX protocol

Cell-SELEX was performed obeying the procedure introduced by Sefah [5]. For folding into its 3D configuration, 5 nmol single-stranded oligonucleotide library was resolved in 1ml binding buffer, incubated at 95°C for 15 min, and cooled immediately. Ultra-pure platelets ($5x10^6$ cells) were washed 3 times with washing buffer and resuspended in 1 ml binding buffer. Both library and target cells were adjusted to room temperature (18°C) and mixed and then incubated for one hour at RT. Unbound oligos were separated by centrifugation at 200 g for 15 minutes followed by three additional washing steps. Bound oligos were eluted from the platelets by incubating diluted cells at 95°C for 30 min with gentle agitation followed by centrifugation at 800 g for 15 min. Eluted oligonucleotides from the first round of SELEX were purified and amplified through a preparatory PCR and single-stranded DNA was prepared by Lambda exonuclease and aliquoted as "control library".

PCR conditions

Eluted oligonucleotides (1 µl: 18pg), 10x PCR buffer (5 µl), primers (2 + 2 µl: 0.4 µM each), dNTP (2 µl: 0.4 mM) and $MgCl_2$ (0.5 µl: 0.5 mM) were mixed and then 0.3 µl (1.5 Unit) Taq DNA-polymerase was added. Finally, 37.5 µl DDW was added to reach the final volume (50 µl). PCR conditions were optimized and set to 5 min at 94 °C for initial denaturation, followed by 35 cycles of 60 sec at 94 °C for denaturation, 60 sec at 54 °C for annealing and 90 sec at 72 °C for extension and an additional 10 min at 72 °C was applied for final extension step. Agarose gel (3%) electrophoresis with TBE (0.5x) buffer as mobile phase and "DNA safe stain" (Sinaclon, Cat. No: EP5082) as the nucleic acid stain was used for visualization of the PCR products.

Single-strand preparation

PCR products from amplification of eluted oligonucleotides are double-strand, one strand is FITC-labeled and the other strand is 5' end phosphorylated. For application in the next round of SELEX, single-strand FITC-labeled oligonucleotides should be separated from complementary sequences in isolated form. Lambda exonuclease (ThermoScientific; #EN0561) is the unique enzyme that can break phosphorylated strands down while leaving the other strand intact. The reaction is simple and strand-specific and only digest phosphorylated strand. Reaction conditions were optimized and the reliability of the enzyme was confirmed through native polyacrylamide gel electrophoresis before application for the main study.

Desalting and extraction of oligonucleotides

PCR assay is susceptible to even a trace amount of impurities of salts in the DNA samples and desalting is a necessary step for successful amplification of the target sequence. Oligonucleotides (in the current study) are short DNA strands with 79 bp length and successful isolation of the short DNA sequences from samples requires the inclusion of some accessory materials, usually known as carriers. Linear polyacrylamide is a newly introduced carrier that is chemically inert and does not interfere with subsequent procedures. In addition, LPA can be produced with high purity in any laboratory setting. Isopropyl alcohol and ethyl alcohol are two DNA precipitating agents that show their advantages, while the required volume of isopropanol for DNA precipitation is half of the ethanol. There is no need for a cold incubation period (isopropanol, unlike ETOH, also works well at room temperature), however, ETOH works better for desalting. We, initially, concentrated DNA strands from diluted samples using isopropanol and then desalting was done using ETOH. LPA was used as a precipitation-assistant agent. For desalting and extraction, 200 µl of the eluted oligos were mixed with 20 µl sodium acetate (3M), 5 µl Linear polyacrylamide (LPA as a carrier; 2.5 µg/µl), and 170 µl cooled isopropyl alcohol. After centrifugation at 15000 g for 45 min, the precipitate was washed 2 additional times using 500 µl cooled ethanol and gently dried at ambient temperature, and reserved (or amplified to get ready for the next round). LPA (2.5 µg/µl) was prepared by the method of Gaillard [52].

Negative selection

Counter selection of eluted oligos was only done at the third round and beyond. Oligos eluted from the positive selection were exposed to platelet-free WBC/RBC cells suspended in binding buffer and unbound oligos were collected in the supernatant, amplified by PCR, single-stranded by exonuclease, desalted, purified, and aliquoted at -20 °C until the next round.

Monitoring the efficiency of the enrichment process

A total of 13 rounds of cell-SELEX were applied. That means; 13 times in a duplicate or triplicate experiments oligonucleotides from the initial library, or single-strand preparation from the previous round of SELEX, were exposed to target cells (platelets) then bound DNAs were eluted and exposed to non-target cells (RBCs and WBCs to remove non-specific oligos) and DNA from the final supernatant was extracted and PCR amplified. The stringency of the conditions, e.g., incubation time, elution time, duration of washing steps, and so on, was gradually increased to further enrichment of high-affinity platelet-specific oligonucleotides and efficient depletion of useless DNAs from the process. PCR products (double-strand) from each round of SELEX were incubated with Lambda exonuclease to single-strand FITC-labeled DNA preparation and used in the next round of SELEX. A total of 10 ssDNA samples from 4-13 rounds of SELEX were collected and used individually for flow cytometric detection of the efficiency of the enrichment process. Oligonucleotides originated from the first

round of SELEX were hugely amplified and then single-stranded and used as control library (e.g., for drawing quadrans in flow cytometry diagrams. Briefly, $5x10^5$ freshly prepared and washed platelets were exposed to ssDNA samples in 100 µl binding buffer, incubated at 18 °C for 30 minutes in a dark place, and fluorescence signals for $5x10^4$ cells were acquired by the instrument.

Sequencing of the select aptamers

Selected ssDNA from the 12th round of cell-SELEX were amplified by PCR using non-labeled primers and Taq DNA Polymerase. Oligos from the 13th round of SELEX were omitted to prevent confronting with repeated emergence of identical colonies after cloning. PCR product was further purified using electrophoresis and eluted DNA was cloned to pTG19-T vector using a standard cloning kit (Vivantis; TA010). *E. coli* (TOP10) was used as a competent cell, and transformed cells were layered on LB-agar media enriched with ampicillin and IPTG X-GAL. White colonies were selected individually and reproduced in liquid media. Plasmids originating from a single white colony were extracted using alkaline lysis buffer and sequenced. Clustal Omega online multiple sequence alignment tool was used for alignment of the sequences. Configuration of the sequences was determined using RNA structure 6.2 software.

Affinity and specificity of the selected aptamers

Seven representative colonies were selected, reproduced individually and their plasmids were extracted. Seven aptamer sequences were amplified using FITC-labeled and phosphorylated primers. Phosphorylated strands were digested and FITC-labeled strands were further resolved by electrophoresis and extracted in the pure form. Seven FITC labeled aptamers (each in pure form) were considered for affinity determination experiments.

For affinity determination, in triplicate experiments, fixed numbers of target cells ($5x10^5$ platelets) were exposed, in binding buffer, to nine serial dilutions (0-1600 nM oligos) of FITC-labeled aptamers and incubated in a dark place at 18°C for one hour. After a washing step, fluorescence data related to $5x10^4$ cells were acquired by flow cytometry. Control library was used in parallel as a control. Each experiment was done using one aptamer, therefore, one-site saturation equation $Y=Bmax*X/(K_D + X)$ was used for the calculation of dissociation constant in which, Y is the mean fluorescence intensity, X is the aptamer concentration and Bmax is the maximum fluorescence intensity.

Specificity of the aptamers were tested using pure leukocytes, platelets and RBCs prepared from 5 donors with different characteristics regarding gender and age. Briefly, fixed number of the cells were incubated with fixed concentration of the FITC-labeled aptamers. Density of the cell-bound aptamers were assayed by flow cytometry.

Trypsin digestion of ligands

Exposure of platelets, $5x10^5$ cells, to trypsin (Sigma: T4549; 0.25% final concentration; 10 min incubation time; at 37 °C), before and after aptamer binding was used for evaluation of the ligand nature and accessibility. RPMI-1640 enriched with FBS (10%) was used for trypsin inactivation and centrifugal force was used for cell precipitation. One hundred µl containing 50 nm FITC-labeled aptamer was added to the cells and incubated at 18°C for 30 min. After washing, the cells were analyzed using flow cytometry. Control library and untreated cells were also included in the experiment.

In vitro effects of Aptamers on platelet function

We investigated by two conventional methods, Light Transmission Aggregometry (LTA) and platelet function analyzer (PFA-100), how aptamer binding to platelets can interfere with adhesion and aggregation of the cells. For LTA testing freshly prepared platelets (2×10^{11} cells/L in PPP) were exposed to three concentrations (0.01, 0.1, and 1 µM, final concentration) of the aptamers at 37 °C for 30 min. Platelet-aptamer complex (270 µl) was transferred to LTA cuvette and mixed with 30 µl agonist (type I collagen: 5 µg/ml or epinephrine: 10 µM) and agitated (800-1200 rpm). The aggregometer was calibrated using PPP (100% transmittance) and PRP (0%) and the light transmittance of the test and control tubes were measured steadily for 12 minutes. The slop and maximal aggregation rate (MAR) were calculated and compared. Any recognizable changes of platelet aggregation traces within pairs of test and control experiments were also noticed.

For PFA-100 testing, citrated blood was prepared and spiked with three different concentrations (0.01, 0.1, and 1 µM) of aptamers and incubated at 37 °C for 30 minutes before running the test. Both collagen-epinephrin (CEPI) and collagen-ADP (CADP) cartridges were tested.

Ethical considerations

All patients (volunteers) gave informed consent and signed an informed consent form. All samples obtained in this study were approved by the ethics committee of the "Research Ethics Committees of the Ferdowsi University of Mashhad" with the code of IR.UM.REC.1400.076 and the research was conducted according to the principles of the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects", (amended in October 2013).

Authors' Contributions

FA was fully responsible for study design, for conduction of all experimental procedures, and data collection and evaluation. Also, she was responsible for preparation of the manuscript and revising it if it was necessary. MS participated in the study design and supervision of the work. AH contributed in statistical analysis of the data and evaluating integrity of the data and interpretation of the results. GHT was responsible for overall supervision. All authors read and approved the final manuscript.

Acknowledgements

This study was related to the Ph.D. thesis of F.A. approved by the Ferdowsi University of Mashhad. No specific finance support was allocated from Ferdowsi University of Mashhad. F.A. is very grateful to Dr. Shabani, the head of Biotechnology Department of Semnan University of Medical Sciences (SemUMS) for his full support to conduct this research in the "Universal Research Laboratory" of the SemUMS. F.A. is very grateful to Mrs. Babaei for her help and assistance for doing experiments and operating instruments.

Competing Interests

The authors declare that they have no conflicts of interest

References

1. Ni X, Castanares M, Mukherjee A, Lupold SE. Nucleic acid aptamers: clinical applications and promising new horizons. Curr Med Chem. 2011;18(27):4206-14.

2. Xiang D, Zheng C, Zhou SF, Qiao S, Tran PH, Pu C, et al. Superior Performance of Aptamer in Tumor Penetration over Antibody: Implication of Aptamer-Based Theranostics in Solid Tumors. Theranostics. 2015;5(10):1083-97.

3. Xiang D, Shigdar S, Qiao G, Wang T, Kouzani AZ, Zhou SF, et al. Nucleic acid aptamer-guided cancer therapeutics and diagnostics: the next generation of cancer medicine. Theranostics. 2015;5(1):23-42.

4. Sefah K, Meng L, Lopez-Colon D, Jimenez E, Liu C, Tan W. DNA aptamers as molecular probes for colorectal cancer study. PLoS One. 2010;5(12):e14269.

5. Sefah K, Shangguan D, Xiong X, O'Donoghue MB, Tan W. Development of DNA aptamers using Cell-SELEX. Nat Protoc. 2010;5(6):1169-85.

6. Ellington AD, Szostak JW. Selection in vitro of single-stranded DNA molecules that fold into specific ligand-binding structures. Nature. 1992;355(6363):850-2.

7. Chen M, Yu Y, Jiang F, Zhou J, Li Y, Liang C, et al. Development of Cell-SELEX Technology and Its Application in Cancer Diagnosis and Therapy. Int J Mol Sci. 2016;17(12).

8. Ringquist S, Jones T, Snyder EE, Gibson T, Boni I, Gold L. High-affinity RNA ligands to Escherichia coli ribosomes and ribosomal protein S1: comparison of natural and unnatural binding sites. Biochemistry. 1995;34(11):3640-8.

9. Morris KN, Jensen KB, Julin CM, Weil M, Gold L. High affinity ligands from in vitro selection: complex targets. Proc Natl Acad Sci U S A. 1998;95(6):2902-7.

10. Shangguan D, Li Y, Tang Z, Cao ZC, Chen HW, Mallikaratchy P, et al. Aptamers evolved from live cells as effective molecular probes for cancer study. Proc Natl Acad Sci U S A. 2006;103(32):11838-43.

11. Gold L, Ayers D, Bertino J, Bock C, Bock A, Brody EN, et al. Aptamer-based multiplexed proteomic technology for biomarker discovery. PLoS One. 2010;5(12):e15004.

12. Yang M, Jiang G, Li W, Qiu K, Zhang M, Carter CM, et al. Developing aptamer probes for acute myelogenous leukemia detection and surface protein biomarker discovery. J Hematol Oncol. 2014;7:5.

13. Nimjee SM, Lohrmann JD, Wang H, Snyder DJ, Cummings TJ, Becker RC, et al. Rapidly regulating platelet activity in vivo with an antidote controlled platelet inhibitor. Mol Ther. 2012;20(2):391-7.

14. Chang YM, Donovan MJ, Tan W. Using aptamers for cancer biomarker discovery. J Nucleic Acids. 2013; 817350.

15. Forier C, Boschetti E, Ouhammouch M, Cibiel A, Duconge F, Nogre M, et al. DNA aptamer affinity ligands for highly selective purification of human plasma-related proteins from multiple sources. J Chromatogr A. 2017;1489:39-50.

16. Sypabekova M, Bekmurzayeva A, Wang R, Li Y, Nogues C, Kanayeva D. Selection, characterization, and application of DNA aptamers for detection of Mycobacterium tuberculosis secreted protein MPT64. Tuberculosis (Edinb). 2017;104:70-8.

17. Wu Q, Wu L, Wang Y, Zhu Z, Song Y, Tan Y, et al. Evolution of DNA aptamers for malignant brain tumor gliosarcoma cell recognition and clinical tissue imaging. Biosens Bioelectron. 2016;80:1-8.

18. Mehennaoui S, Poorahong S, Jimenez GC, Siaj M. Selection of high affinity aptamer-ligand for dexamethasone and its electrochemical biosensor. Sci Rep. 2019;9(1):6600.

19. Floege J, Ostendorf T, Janssen U, Burg M, Radeke HH, Vargeese C, et al. Novel approach to specific growth factor inhibition in vivo: antagonism of platelet-derived growth factor in glomerulonephritis by aptamers. Am J Pathol. 1999;154(1):169-79.

20. Zheng X, Hu B, Gao SX, Liu DJ, Sun MJ, Jiao BH, et al. A saxitoxin-binding aptamer with higher affinity and inhibitory activity optimized by rational site-directed mutagenesis and truncation. Toxicon. 2015;101:41-7.

21. Krishnegowda M, Rajashekaraiah V. Platelet disorders: an overview. Blood Coagul Fibrinolysis. 2015;26(5):479-91.

22. Lam FW, Vijayan KV, Rumbaut RE. Platelets and Their Interactions with Other Immune Cells. Compr Physiol. 2015;5(3):1265-80.

23. Liu M, Zaman K, Fortenberry YM. Overview of the Therapeutic Potential of Aptamers Targeting Coagulation Factors. Int J Mol Sci. 2021;22(8).

24. Amisten S. A rapid and efficient platelet purification protocol for platelet gene expression studies. Methods Mol Biol. 2012;788:155-72.

25. Wrzyszcz A, Urbaniak J, Sapa A, Wozniak M. An efficient method for isolation of representative and contamination-free population of blood platelets for proteomic studies. Platelets. 2017;28(1):43-53.

26. Schror K. Aspirin and platelets: the antiplatelet action of aspirin and its role in thrombosis treatment and prophylaxis. Semin Thromb Hemost. 1997;23(4):349-56.

27. Friedman EA, Ogletree ML, Haddad EV, Boutaud O. Understanding the role of prostaglandin E2 in regulating human platelet activity in health and disease. Thromb Res. 2015;136(3):493-503.

28. Zhuo Z, Yu Y, Wang M, Li J, Zhang Z, Liu J, et al. Recent Advances in SELEX Technology and Aptamer Applications in Biomedicine. Int J Mol Sci. 2017;18(10).

29. Ohuchi S. Cell-SELEX Technology. Biores Open Access. 2012;1(6):265-72.

30. Thon JN, Italiano JE. Platelet formation. Semin Hematol. 2010;47(3):220-6.

31. Saboor M, Ayub Q, Ilyas S, Moinuddin. Platelet receptors; an instrumental of platelet physiology. Pak J Med Sci. 2013;29(3):891-6.

32. Bury L, Malara A, Momi S, Petito E, Balduini A, Gresele P. Mechanisms of thrombocytopenia in platelet-type von Willebrand disease. Haematologica. 2019;104(7):1473-81.

33. Ayabe K, Goto S. Is there a 'therapeutic window' for antiplatelet therapy? Eur Heart J Cardiovasc Pharmacother. 2017;3(1):18-20.

34. Marginean A, Banescu C, Scridon A, Dobreanu M. Anti-platelet Therapy Resistance - Concept, Mechanisms and Platelet Function Tests in Intensive Care Facilities. J Crit Care Med (Targu Mures). 2016;2(1):6-15.

35. Oney S, Nimjee SM, Layzer J, Que-Gewirth N, Ginsburg D, Becker RC, et al. Antidote-controlled platelet inhibition targeting von Willebrand factor with aptamers. Oligonucleotides. 2007;17(3):265-74.

36. McKeague M, Derosa MC. Challenges and opportunities for small molecule aptamer development. J Nucleic Acids. 2012;2012:748913.

37. Bibby DF, Gill AC, Kirby L, Farquhar CF, Bruce ME, Garson JA. Application of a novel in vitro selection technique to isolate and characterise high affinity DNA aptamers binding mammalian prion proteins. J Virol Methods. 2008;151(1):107-15.

38. Chen Z, Liu H, Jain A, Zhang L, Liu C, Cheng K. Discovery of Aptamer Ligands for Hepatic Stellate Cells Using SELEX. Theranostics. 2017;7(12):2982-95.

39. Duan Y, Wang L, Gao Z, Wang H, Zhang H, Li H. An aptamer-based effective method for highly sensitive detection of chloramphenicol residues in animal-sourced food using real-time fluorescent quantitative PCR. Talanta. 2017;165:671-6.

40. Tang XL, Wu SM, Xie Y, Song N, Guan Q, Yuan C, et al. Generation and application of ssDNA aptamers against glycolipid antigen ManLAM of Mycobacterium tuberculosis for TB diagnosis. J Infect. 2016;72(5):573-86.

41. Berg K, Lange T, Mittelberger F, Schumacher U, Hahn U. Selection and Characterization of an alpha6beta4 Integrin blocking DNA Aptamer. Mol Ther Nucleic Acids. 2016;5:e294.

42. Zhou L, Li P, Yang M, Yu Y, Huang Y, Wei J, et al. Generation and characterization of novel DNA aptamers against coat protein of grouper nervous necrosis virus (GNNV) with antiviral activities and delivery potential in grouper cells. Antiviral Res. 2016;129:104-14.

43. Spiga FM, Maietta P, Guiducci C. More DNA-Aptamers for Small Drugs: A Capture-SELEX Coupled with Surface Plasmon Resonance and High-Throughput Sequencing. ACS Comb Sci. 2015;17(5):326-33.

44. Baig IA, Moon JY, Lee SC, Ryoo SW, Yoon MY. Development of ssDNA aptamers as potent inhibitors of Mycobacterium tuberculosis acetohydroxyacid synthase. Biochim Biophys Acta. 2015;1854(10 Pt A):1338-50.

45. Moon J, Kim G, Park SB, Lim J, Mo C. Comparison of whole-cell SELEX methods for the identification of Staphylococcus aureus-specific DNA aptamers. Sensors (Basel). 2015;15(4):8884-97.

46. Moosavian SA, Jaafari MR, Taghdisi SM, Mosaffa F, Badiee A, Abnous K. Development of RNA aptamers as molecular probes for HER2(+) breast cancer study using cell-SELEX. Iran J Basic Med Sci. 2015;18(6):576-86.

47. Mozioglu E, Gokmen O, Tamerler C, Kocagoz ZT, Akgoz M. Selection of Nucleic Acid Aptamers Specific for Mycobacterium tuberculosis. Appl Biochem Biotechnol. 2016;178(4):849-64.

48. Mayer G, Hover T. In vitro selection of ssDNA aptamers using biotinylated target proteins. Methods Mol Biol. 2009;535:19-32.

49. Jin YR, Ryu CK, Moon CK, Cho MR, Yun YP. Inhibitory effects of J78, a newly synthesized 1,4-naphthoquinone derivative, on experimental thrombosis and platelet aggregation. Pharmacology. 2004;70(4):195-200.

50. Trowbridge IS, Johnson P, Ostergaard H, Hole N. Structure and function of CD45: a leukocyte-specific protein tyrosine phosphatase. Adv Exp Med Biol. 1992;323:29-37.

51. Ramstrom S, O'Neill S, Dunne E, Kenny D. Annexin V binding to platelets is agonist, time and temperature dependent. Platelets. 2010;21(4):289-96.

52. Gaillard C, Strauss F. Ethanol precipitation of DNA with linear polyacrylamide as carrier. Nucleic Acids Res. 1990;18(2):378.

How to cite this article
Alemi F, Sankian M, Haghparast A, Bassami M R, Hashemi Tabar G. The identification of single strand DNA aptamers which specifically bind to platelets using cell-SELEX technique. Iran J Vet Sci Technol. 2021; 13(2): 68- 83.
DOI: https://doi.org/ 10.22067/ijvst.2021.72518.1079
URL: https://ijvst.um.ac.ir/article_41409.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Aug- 04
Accepted after revision: 2021- Dec- 12
Published online: 2021- Dec- 31

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2021.71710.1065

Effects of oxidized glutathione, cysteine and taurine supplementations on motility charateristics of different goat spermatozoa types

Mazen Alomar

Division of Animal Production, Department of Agriculture, Atomic Energy Commission, Damascus, Syria.

ABSTRACT

This study aimed to determine the influences of antioxidants additives on motility characteristics of different goat spermatozoa types including frozen/thawed, fresh, and chilled samples. Ejaculates from five Shami bucks were collected during breeding and non-breeding seasons. Spermatozoa samples from the three types were incubated in media containing 2 and 4 mM oxidized glutathione (GSSG), 5 and 10 mM L-cysteine,10 and 25 mM taurine, and no additives (control). Motility characteristics were analyzed by a computer-aided sperm analyzer (CASA). Except for taurine, the addition of antioxidants resulted in a significant ($p < 0.05$) increase in the percentage of motile sperm (MOT %) after spermatozoa thawing. When fresh sperm samples were collected during the non-breeding season and treated with both GSSG and L-cysteine, the values of the velocity parameters VAP, VSL, and VCL increased significantly ($p < 0.05$). No significant effects were noted for the velocity parameters when 10 and 25 mM of taurine were added to the chilled spermatozoa, while GSSG and L-cysteine had principally affected MOT % of this spermatozoa type. The rapid spermatozoa subpopulation was the most influenced category by the three antioxidants compared to the slow and medium grades, especially in the case of fresh and frozen/thawed types. In conclusion, the effects of different antioxidants on goat spermatozoa motility largely depend on the used concentration and also on the type of spermatozoa pattern.

Keywords

Reactive oxygen species, Antioxidants, Goat, Spermatozoa, Motility.

Number of Figures: 3
Number of Tables: 3
Number of References:: 35
Number of Pages: 9

Abbreviations

CASA: computer-aided sperm analyzer
CAT: catalase
GSH: glutahione
GSSG: oxidized glutathione
GSH-Px: glutathione peroxidase
GSH: reduced glutathione
GLM: general linear model procedure
MDA: malondialdehyde

Corresponding author: *Mazen Alomar*
Email: *ascientific15@aec.org.sy*
Tel: *+96 (31)1213-2580*

Introduction

It is well known that the sperm plasma membrane is rich in polyunsaturated fatty acids which are susceptible to oxidative stress damage resulting from reactive oxygen species (ROS) during aerobic incubation [1]. To block the effects of oxidative stress, a wide array of antioxidants have been used. Antioxidants could scavenge ROS directly and also they may prevent the propagation of lipid peroxidation in sperm membranes. It must be noted that both spermatozoa and seminal plasma possess antioxidant systems such as taurine, cysteine, reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) [2]. However, in different animal species, the indigenous antioxidant systems were insufficient to protect the spermatozoa from oxidative stress [3, 4]. Therefore, extra antioxidants supplementation was highly recommended to protect spermatozoa during liquid storage. In this respect, the positive effects of adding antioxidants at different temperature degrees to the liquid equine, sheep, and goat semen have been demonstrated in several studies [5, 6, 7].

Glutathione (L-γ-glutamyl-L-cysteinyl glycine; GSH) is a tri-peptide ubiquitously distributed in cells and it plays an important role as an intracellular defense mechanism against oxidative stress. Moreover, GSH is a principally important natural antioxidant in semen, can easily mobilize the system for the removal of peroxides by a reaction that results in the generation of oxidized glutathione [8]. In the sperm membrane, oxidized glutathione (GSSG) can reduce the mobility of sulfhydryl-containing proteins. As Sulfhydryl groups are under redox control, the change in the redox status of the membrane could be linked to ROS production that occurs during cooling and freezing-thawing of spermatozoa [9].

Taurine is one of the major non-enzymatic antioxidants. This sulfonated amino acid was found in both seminal plasma and oviductal fluid [10, 11]. When applied at an appropriate dose, it improves spermatozoa motility and displays antioxidative properties, elevating the CAT level, in association with SOD concentration [12].

Abbreviations-Cont'd

MOT %: percent motility
PMOT %: percent of sperm showing progressive motility
ROS: reactive oxygen species
TAL: tyrode albumin lactate
TEY: tris-egg yolk
SOD: superoxide dismutase
VCL: curvilinear velocity
VAP: average path velocity
VSL: straight line velocity

Cysteine is another amino acid, which has been shown to penetrate the cell membrane easily, enhancing intracellular glutathione biosynthesis both *in vitro* and *in vivo* [13]. This antioxidant has been reported to prevent loss of sperm viability, motility, and membrane integrity during liquid storage and also in the frozen state [14]. Anyhow, cysteine and taurine, as antioxidants, have been used in the cryopreservation process of humans [15], boar [16], bull [17, 18], ram [19], and goat sperm [20] to enhance the post-thaw motility and fertility of spermatozoa.

Despite the existence and the importance of all the previously mentioned studies, no reports have addressed the applications of antioxidants on motility characteristics of different goat spermatozoa types especially in the cases of thawed-frozen, fresh, and chilled sperm samples collected during breeding and non-breeding seasons. These three types are highly important and essential for the different assisted reproductive technologies. For that, the main objective of this study was to evaluate the effects of oxidized glutathione, L-cysteine, and taurine supplementation on the motility of different goat spermatozoa types assessed by computer sperm analyzer system.

Results

Table 1 shows the effects of antioxidants addition to TAL thawing medium on CASA motility parameters of frozen/thawed goat spermatozoa. Except for taurine at a concentration of 10 mM, the addition of all antioxidants in this study and all the used concentrations resulted in a significant increase ($p < 0.05$) of the MOT % parameter.

Table 2 shows the effects of adding antioxidants on fresh sperm motility characteristics. Cysteine and GSSG significantly ($p < 0.05$) increased the values of the MOT %, VAP, VSL, and VCL. When 10 mM of taurine was added, the values of VAP, VSL, and VCL were lower compared to the control, while 25 mM of this agent was able to significantly raise all the values of the analyzed motility parameters compared to control and those treated with 10 mM taurine.

Table 3 shows the effects of antioxidants addition on CASA motility parameters of chilled goat spermatozoa at 5 °C. Except for the positive effect on MOT % parameter when the spermatozoa were treated with GSSG and L-cysteine, as well as the positive effect of GSSG at 2 mM on VAP, VCL, and VSL parameters, the results showed that the antioxidants used in this study did not increase the values of different motility characteristics and no significant differences ($p > 0.05$) was noted between the values of control and the treated spermatozoa with antioxidants.

Figures 1 and 2 show the effects of the three antioxidants addition on the distribution of motility categories of both frozen/thawed and fresh samples. The positive effects were evident by increasing the percentages of rapid spermatozoa after GSSG and L-cysteine supplementations for the two concentrations. The per-

Table 1.
Effects of oxidized glutahione (GSSG), L-cysteine, and taurine on CASA motility parameters of thawed-frozen goat spermatozoa samples.

Parameter/ Treatment	MOT (%)	PMOT (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)
Control	57.9 + 2.5 [a]	8.4 +1.5 [a]	61.2 +2.4 [a]	38.2 + 3.1 [a]	128.1 + 6.4 [a]
GSSG 2 mM	69.9 + 1.5 [b]	10.8+0.91 [b]	69.7 + 4.4 [b]	44.7 + 2.7 [b]	140.9 + 7.9 [b]
GSSG 4 mM	64.5 + 2.9 [b]	9+1.58 [a]	63.1 +4.6 [a]	40.9 + 3.1 [a]	136.2 + 7.6 [a]
L-Cysteine 5 mM	67.8 + 3.6 [b]	12.1 +2.1 [b]	72.2 +8.1 [b]	46.2 + 2.5 [b]	145.8 + 7.6 [a]
L-Cysteine 10 mM	66 + 3.1[b]	10.3 +1.2 [b]	68.1 +5.4 [b]	43.3 + 4.3 [b]	138.1 + 6.9 [a]
Taurine 10 mM	56 +7.1 [a]	7.8 +2.1 [a]	61.6 +6.2 [a]	43.1 +4.6 [a]	134.6 + 9 [a]
Taurine 25 mM	70.3 + 3 [b]	11.7 + 1.5 [b]	72.2 +5.1 [b]	47 + 3.5 [b]	145.7 + 8.1 [b]

Mean + SD (3 replicates; for each replicate and each condition: 3 fields of 150-250 spermatozoa each were analyzed by CASA).
Data with different superscripts in the same column are significantly different ($p < 0.05$).

Table 2.
Effects of oxidized glutahione (GSSG), L-cysteine, and taurine on CASA motility parameters of fresh goat spermatozoa samples.

Parameter/ Treatment	MOT (%)	PMOT (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)
Control	67.9 +3.5 [a]	15.1 + 3.6 [a]	88. 7 + 8.4 [a]	61. 6 + 9.7 [a]	182.4 + 9.3 [a]
GSSG 2 mM	85.1 +5.9 [b]	25.2 + 6.2 [b]	106.9 + 11.5[b]	80.3 +17.7 [b]	205.2 +13.1 [b]
GSSG 4 mM	82.4 + 4.7 [b]	21.4 + 2.6 [b]	108 + 7.5 [b]	73.6 + 7.6 [b]	204.7 + 7.8 [b]
L-Cysteine 5 mM	84.6 +10.8 [b]	23.9 + 6.4 [b]	103.1 +16.1 [b]	73 +12.3 [b]	200 +9 [a]
L-Cysteine 10 mM	83.2 + 3.3 [b]	21.6 + 2.7 [b]	105. 9+11.6 [b]	74.1 + 12.3 [b]	195.8 +9.5 [b]
Taurine 10 mM	69.8 + 1 [a]	14.6 + 1.7 [a]	78.7 +7.1 [a]	53 + 4.7 [a]	169.7 + 8.6 [a]
Taurine 25 mM	75.3 + 4.2 [b]	18.8 + 1.6 [b]	97.2 + 8 [c]	70 + 5.8 [c]	196.4 + 8.5 [c]

Mean + SD (3 replicates; for each replicate and each condition: 3 fields of 150-250 spermatozoa each were analyzed by CASA).
Data with different superscripts in the same column are significantly different ($p < 0.05$).

Table 3.
Effects of oxidized glutahione (GSSG), L-cysteine and taurine on CASA motility parameters of chilled goat spermatozoa samples.

Parameter/ Treatment	MOT (%)	PMOT (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)
Control	70.2 + 1 [a]	12.4 + 1.3 [a]	71 + 3.8 [a]	46.1 + 1.9 [a]	145.2 + 7.1 [a]
GSSG 2 mM	76.3 + 3.4 [b]	13.1 + 1.5 [a]	75.3 + 2.1 [b]	49.4 +1.7 [b]	154.7 + 7.3 [b]
GSSG 4 mM	77. 6 + 4.5 [b]	11.8 + 3.3 [a]	70.8 + 3.3 [a]	45.2 + 3.7 [a]	147.7 + 8.5 [ab]
L-Cysteine 5 mM	76.6 + 4.1 [b]	12.8 + 3.2 [a]	70.5 + 4.5 [a]	44.8 + 4.2 [a]	148.4 + 6.7 [a]
L-Cysteine 10 mM	78 + 3.2 [b]	13 + 3.2 [a]	74.4 + 6 [a]	46.2 + 3.7 [a]	153.3 + 10.6 [a]
Taurine 10 mM	72 + 5.3 [a]	11.4 +1.4 [a]	68. 9 + 6.6 [a]	45.2 + 4.9 [a]	146.4 + 9 [a]
Taurine 25 mM	68.8 + 5.3 [a]	9.5 + 0.9 [a]	68.8 + 4.2 [a]	42.1 + 2.4 [a]	144.4 + 10.2 [a]

Mean + SD (3 replicates; for each replicate and each condition: 3 fields of 150-250 spermatozoa each were analyzed by CASA).
Data with different superscripts in the same column are significantly different ($p < 0.05$).

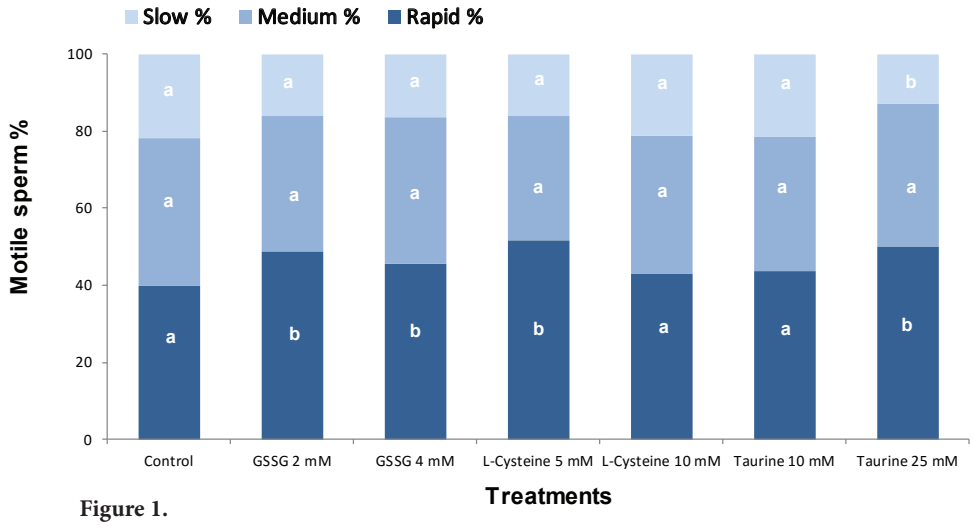


Figure 1.
Effects of antioxidants; oxidized glutahione (GSSG), L-cysteine, and taurine on the distribution of motility subpopulation of frozen/thawed goat spermatoza samples. Different letters within each subpopulation category and between treatments significantly differ ($p < 0.05$).

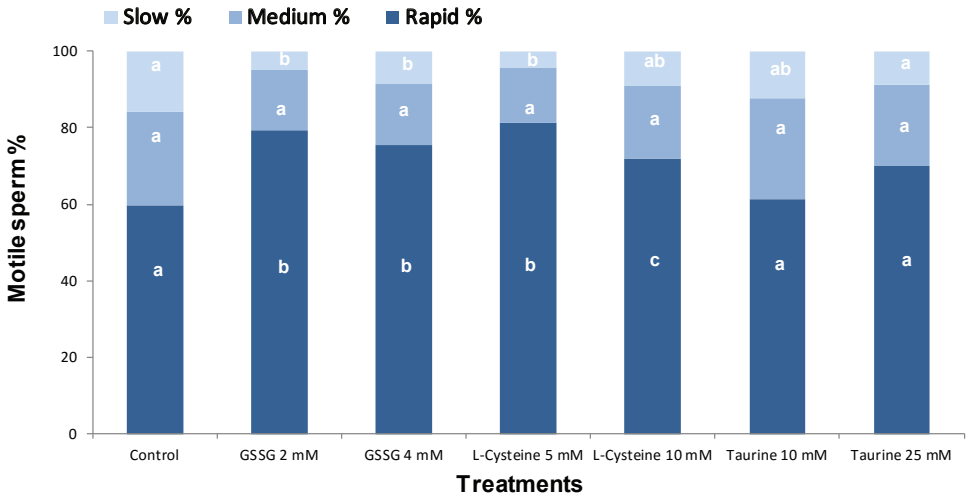


Figure 2.
Effects of antioxidants; oxidized glutahione (GSSG), L-cysteine, and taurine on the motility subpopulations of fresh goat spermatozoa samples. Different letters within each subpopulation category and between treatments significantly differ ($p < 0.05$).

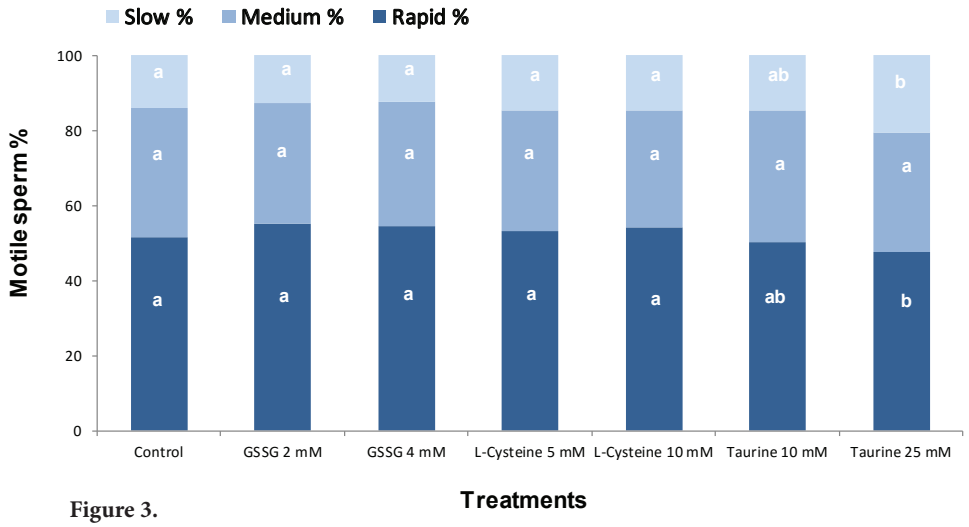


Figure 3.
Effects of antioxidants; oxidized glutahione (GSSG), L-cysteine, and taurine on the distribution of motility subpopulations of chilled goat spermatozoa samples. Different letters within each subpopulation category and between treatments significantly differ ($p < 0.05$).

centage of medium category did not differ ($p > 0.05$) between control samples and antioxidant treatments for all used concentrations. Figure 3 shows the effects of antioxidants addition on the distribution of motility categories of chilled spermatozoa samples. There were no significant differences between the control and the spermatozoa treated with GSSG and cysteine for rapid, medium, and slow categories ($p > 0.05$). When the samples were treated with taurine at a concentration of 25 mM, the percentage of rapid sperm decreased and the percentage of slow motility increased significantly ($p < 0.05$) compared to control.

Discussion

The effects of adding antioxidants on motility characteristics of three different goat spermatozoa types were investigated in the present research. The influences of the same antioxidants on frozen spermatozoa samples from different animal species were previously studied [21, 22, 23]. However, in this study, we focused on other types including frozen/thawed and unfrozen samples. The process of thawing induces ROS production in semen and if optimal supplementation of antioxidants could scavenge ROS, thus it consequently may decrease the deleterious effects of the cryopreservation process. Pieces of information on the use of antioxidants in thawing medium to improve post-thaw motility of frozen goat spermatozoa are lacking. Moreover, treating fresh and chilled semen with antioxidants during the non-breeding season may present an interesting technique in goat semen manipulations.

In this study and for the three spermatozoa types, oxidized glutathione was able to significantly increase the values of MOT % whatever the concentration was. The addition of glutathione (reduced, or oxidized) to the freezing medium of bull semen had reduced the loss of spermatozoa motility by 35 % [24]. In agreement with our results, 5 mM of GSSG had the best protective effect on ram sperm motility after semen thawing [21]. Furthermore, according to the previous authors, a toxic effect on post-thawing spermatological indicators was noted when higher concentrations (10 or 20 mM) of GSSG were used. Anyhow, it must be stressed out that GSSG may not be an efficient agent when added outside the cells, and it cannot prevent peroxidation as vitamin E [25]. The effects of GSSG in semen medium and the mechanism of action of GSSG on spermatozoa motility need more profound researches.

Our results also showed that L-cysteine had positive effects on motility parameters with relatively better results regarding the 5 mM level. Cysteine is rapidly oxidized into cystine outside the cell. Cystine and cysteine are transported into the cell by different transporters. However, cysteine had exhibited significant cryoprotective activity on post-thaw motility parameters of frozen bull spermatozoa [22]. This amino acid can improve intracellular glutathione biosynthesis and such a process may protect the membrane lipids through indirect radical scavenging properties [26]. Moreover, it was suggested that cysteine plays an antioxidative role in preventing malondialdehyde (MDA) production, resulting in higher SOD activity [22]. In contrast to our study and all the previous reports, Yildiz et al. [23] found that cysteine did not have beneficial effects on motility rates after ram semen freezing. Such variability in literature data and our study could be attributed to extender components, antioxidant dose differences, the time of spermatozoa exposure to antioxidants, and also to semen preservation protocols.

Compared to the other two antioxidants, taurine only enhanced the spermatozoa motility characteristics of fresh and frozen/thawed samples with better values for the 25 mM level. When it was applied at the same dose, this antioxidant improved ram sperm motility during cryopreservation [12]. Based on the data of Sariozkan et al. [22], taurine had a beneficial effect by raising the CAT level in the presence of ROS. In the present study, the addition of taurine to chilled spermatozoa did not cause any improvement in velocity parameters and this was similar to results obtained by Sariozkan and co-workers [22] on the bull samples. It was also reported that motility of bovine spermatozoa cooled to 5 °C was maintained close to pre-storage levels in taurine-containing citrate extender up to 48 h.

The chilled spermatozoa were less affected by antioxidants treatments than the other two spermatozoa types. In contrast, progressive sperm motility was significantly higher for chilled buck sperm samples treated by vitamin E and glutathione compared to control [27]. It should be noted that the time of incubation for the chilled samples in this study was relatively short compared to the previous study. Anyhow, a longer period of incubation at 5 - 4 °C may be necessary to demonstrate any eventual positive effects of adding these antioxidants to goat spermatozoa.

The most interesting finding from our study was the ability of antioxidants to improve motility levels of fresh goat spermatozoa collected during the non-breeding season. This type of spermatozoa may be usefully utilized in different reproductive assisted technologies after antioxidants supplementations during this critical period of reproduction. Based upon our preliminary experiments (data not shown) high levels of motility were noted from the control samples of both fresh and chilled types collected during breeding seasons and this probably was responsible for masking any significant effects of the antioxidants supplementations to such spermatozoa types during this period. Indeed, the spermatozoa show their best performances during the breeding season and normally they do not need any additional improvements concerning their motility characteristics. In contrast, the motility levels of cryo-preserved samples during the non-breeding season were very low with no clear effects of antioxidants (data not shown). These

preliminary results may have practical application in choosing when it will be the best time to add the antioxidants systems to each type of goat semen.

Our CASA system was able to distinguish three clear motile subpopulations in the different sperm samples depending on the values of the VAP parameter. Researchers have identified the existence of sperm subpopulations in species as boars, stallions, dogs, bulls, and gazelles, which were defined by specific movement characteristics [28, 29, 30]. Moreover, sperm subpopulations had different sensitivities to respond to external stimuli such as sugars, caffeine, and bicarbonate [31, 32]. Our results showed that antioxidants could not only affect the principal CASA motility characteristics, but also the distributions of spermatozoa subpopulations. The rapid spermatozoa represent the most progressive spermatozoa and it is probably suitable for being a part of the fertilizing population. This category was the most affected by antioxidants treatment especially in the cases of frozen/thawed and fresh types. In contrast, the medium subpopulation category was not influenced by the three different antioxidant treatments. It seems that this class of spermatozoa which presents a transitional stage between the rapid and slow categories may do not have sufficient time to be affected by the different antioxidants treatments. Anyhow, despite the simplicity of motility distribution of the subpopulations provides by this CASA system, the results clearly showed the importance of analyzing such distribution and which subpopulations are the most affected by antioxidants supplementations.

In the present study, we analyzed the effects of each of the three antioxidants separately; future investigations could clarify the effects of simultaneous inclusion of antioxidants' complex components on motility characteristics. In this regard, the supplementation of goat semen extenders with combinations of antioxidants had improved sperm viability and reduced oxidative stress parameters [33]. Despite the great importance of motility assessment, the beneficial effects of antioxidants on spermatozoa may not be only detectable by the evaluation of sperm motility in vitro. Further studies should include the in vitro and in vivo tests including viability, membrane integrity, and most importantly fertilization and pregnancy rates for these three spermatozoa types. Finlay, as ROS plays a very important role in the capacitation and acrosome reaction processes [34, 35]. Therefore, caution must be taken when using antioxidants to not stop these important physiological processes.

In conclusion, our data showed the advantages of adding antioxidants to thawed medium on the motility characteristics of frozen goat spermatozoa. This study also revealed that media supplementation with L-cysteine or GSSG helps in improving the motility of fresh sperm samples collected during the non-breeding season. Chilled spermatozoa collected during the non-breeding season were the less affected spermatozoa type by the different treatments. Thus, the effects of antioxidants largely depend on the temperature of sperm preservation, the used concentration, and also the type of spermatozoa pattern.

Materials & Methods

Area of study, chemicals, and media

This study was carried out at Der-Al-Hajar Animal Production Research Station, 33 km southeast of Damascus. All the chemicals were purchased from Roth (Carl Roth Gmbh-Karlsruhe-Germany). The tris-egg yolk (TEY) medium was prepared as 300 mOsm/kg solution, containing 28.5 mM g citric acid monohydrate, 29.5 mM tris (hydroxymethyl) aminomethane, and 19.8 mM glucose in 80 ml of distilled water. Twenty ml of egg yolk was then added bringing the total volume of this medium to 100 ml. The TAL solution at 300 mOsm/kg had contained 112.94 mM NaCl, 3.22 mM KCl, 0.33 mM NaH_2PO_4, 23 mM $NaHCO_3$, 0.49 mM $MgCl_2.6H_2O$, 2.04 mM $CaCl_2.2H_2O$, 1 mM $C_3H_3O_3Na$, 35 mM $C_3H_5NaO_3$ and 6 g bovine serum albumin in one liter of distilled water. The two media were held constant at pH 7.0.

Animals, semen preparation, and experimental design

Semen was obtained from five sexually-experienced Shami bucks, a native breed of Syrian goat, aged between 3 and 4 years. Semen was collected with the aid of an electro-ejaculator (Minitube - Electro Ejaculator, Germany) administrating a series of 20 cycles pulses of short electrical stimuli with each cycle (two seconds impulse, then two seconds interval) delivering a slightly higher intensity (from 0 Volt to 20 Volt maximum) until semen production. It must be noted that the same five bucks were always used in all the experiments of this study during breeding and non-breeding season. Upon collection, the semen was immediately evaluated for its general appearance and volume. For each animal and after semen collection, sperm concentration was estimated using a haematocytometer. Then an initial analysis of sperm motility was performed using the CASA system (Hamilton Thorne Biosciences, USA) whereas sperm samples at a concentration of $\geq 1 \times 10^9$ spermatozoa/ml were employed. All ejaculations with no or poor motility status were immediately excluded before conducting the analyses.

Three types of semen samples including fresh, chilled, and frozen were used in the present study. Semen was collected during breeding and non-breeding seasons. For both fresh and chilled samples the semen was collected during the non-breeding season in February while for the frozen samples the semen was collected during the breeding season in August. The selection of these exact seasons was based on preliminary experiments which help us on showing the most important effects of antioxidants for each type of spermatozoa in each specific season.

A total of 45 ejaculates were collected in this study. A mixture of semen from five bucks was used in each assay to diminish the effect of individual variation between the animals. It must be stressed out that the number of spermatozoa from each buck was the same in the semen mix. For the frozen spermatozoa the samples were prepared by mixing fresh semen collected during the breeding season (in August) from the five animals and diluted in a tris-based medium without egg yolk at a ratio of 1:9 (semen to tris medium, v:v) and centrifuged at 1000 × g for 15 min. The seminal plasma was discarded and the sperm pellet was suspended to a final concentration of 200×10^6 spermatozoa/mL with tris medium containing both filtered egg yolk (20 %, v:v) and 7 % glycerol. The diluted sperm suspensions were equilibrated at 5 °C for 3 h and loaded into 0.5 mL straws. They were then frozen in nitrogen vapor for 20 min at 10 to 15 cm of height above the nitrogen liquid and transferred to a liquid nitrogen container for storage at −196 °C until use.

Three experiments were conducted in the present study. In the first experiment, the motility characteristics of thawed spermatozoa incubated with the three antioxidants in TAL solution were analyzed. In this experiment, straws thawing were carried out by

immersing the straws in a water bath at 37 °C for 30 sec. Thawed spermatozoa from 0.5 ml straws were directly incubated after thawing in 0.5 ml of TAL medium containing 0 (control), 8 and 4 mM GSSG, 10 and 20 mM L-cysteine, 20 and 50 mM taurine making the final concentration 4 and 2 mM, 5 and 10 mM, 10 and 25 mM respectively. The sperm incubation period lasted for 60 minutes at 37 °C. This experiment was replicated three times in three different weeks in August and the spermatozoa concentrations were 50 x 10^6 for the control and each treatment.

In the second experiment, sperm motility of fresh semen samples collected during the non-breeding season was analyzed. In this experiment, fresh samples of 50 x 10^6 sperm/ml were incubated in tris medium at 37 °C for 60 minutes with 0 (control), 2 and 4 mM oxidized glutathione, 5 and 10 mM L-cysteine, 10, and 25 mM taurine. This experiment was replicated three times in three different weeks in February and the spermatozoa concentrations were 50 x 10^6 for the control and each treatment.

In the third experiment, the motility of chilled spermatozoa incubated with the three antioxidants in the TEY solution was analyzed. The seminal plasma of freshly collected semen was directly removed after collection and the spermatozoa at 50 x 10^6/ml of concentration were incubated in 1 ml of TEY medium without glycerol containing 0 (control), 4 and 2 mM GSSG, 5 and 10 mM L-cysteine, 10 and 25 mM taurine for 60 minutes at 5 °C. This experiment was replicated three times in February and the spermatozoa concentrations were 50 X 10^6 for the control and each treatment.

Motility analyses

The motility characteristics were assessed using a Hamilton-Thorne motility analyzer (HTM version 12.3, Hamilton Thorne Biosciences, USA). Five microliters of diluted semen were loaded in the analyze system lame (2X-CEL of dual-sided sperm analysis chamber of 20 µm depth for the Hamilton Thorne Biosciences System), and for each sample, three fields were counted (each field counted about 150-200 spermatozoa). The motility characteristics included in the analysis were: the percent motility (MOT%), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), and the percent of sperm showing progressive motility (PMOT %: VAP ≥ 50 µm/s and STR ≥ 80 %).

Moreover, spermatozoa subpopulations were defined by the Hamilton Thorne CASA system in three categories, each of the categories is assigned an arbitrary number from 3-1 increasing correspondingly from the lowest to the highest velocity; Rapid (3): fraction of all cells moving with VAP > path velocity (VAP = 25 µm/s), Medium (2): fraction of all cells moving with VAP cutoff (5 µm/s) < VAP < path velocity (VAP = 25 µm/s), Slow (1): fraction of all cells moving with VAP < VAP cutoff (5 µm/s) or VSL < VSL cutoff (11µm/s).

The HTM settings used for goat spermatozoa were negative phase contrast optics at a recording rate of 60 frame/s, light adjustment 50-110, minimum contrast 70, minimum cell size 5 pixels, non motile head size 10 pixels, non motile head intensity 80, low VAP cut off 20mm/s, low VSL cut off 5 mm/s, static size limit 0.60/4.32 (min/max), static intensity limit 0.20/1.92 (min/max), static elongation 7/91 (min/max).

Statistical analysis

Minitab program (Minitab Coventry, United Kingdom) was used for the statistical analysis. The normality of values distribution was first tested with the Shapiro-Wilk test. Data regarding the different antioxidants effects on motility values were subjected to a factorial analysis of variance (ANOVA, general linear model procedure, GLM) followed by multiple pairwise comparisons using a post-hoc (*Tukey's* test). The results are presented as mean + SD and the threshold of signification was set at $p < 0.05$.

Acknowledgements

The author would like to thank the Atomic Energy Commission of Syria for their support while conducting the experiments.

Competing Interests

The author declares that there is no conflict of interest.

References

1. Alvarez JG, Touchstone JC, Blasco L, Storey BT. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. J Androl. 1987; 8(5):338-48.

2. Agarwal A, Prabakaran SA. Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. Indian J Exp Biol. 2005;43(11):963-74

3. Baumber J, Ball BA, Linfor JJ. Assessment of the cryopreservation of equine spermatozoa in the presence of enzyme scavengers and antioxidants. Am J Vet Res. 2005; 66(5):772-9.

4. Nair SJ, Brar AS, Ahuja CS, Sangha SP, Chaudhary KC. A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. Anim Reprod Sci. 2006; 96(1-2):21-9. 5.

5. 5. Maxwell WM, Stojanov T. Liquid storage of ram semen in the absence or presence of some antioxidants. Reprod Fertil Dev. 1996; 8:1013-l 020.

6. Upreti GC, Jensen K, Munday R, Duganzich DM, Vishwanath R, Smith JF. Studies on aromatic amino acid oxidase activity in ram spermatozoa: role of pyruvate as an antioxidant. Anim Reprod Sci. 1998; 51(4):275-87.

7. Ball BA, Medina V, Gravance CG, Baumbe J. Effect of antioxidants on preservation of motility,viability and acrosomal integrity of equine spermatozoa during storage at 5 degrees C. Theriogenology. 2001; 56(4):577-89.

8. Meister A, Anderson ME. Glutathione. Ann Rev Biochem. 1983;52:711-760.

9. Mazur P, Katkov II, Katkova N, Critser JK. The enhancement of the ability of mouse sperm to survive freezing and thawing by the use of high concentrations of glycerol and the presence of an Escherichia coli membrane preparation (Oxyrase) to lower the oxygen concentration. Cryobiology. 2000 ; 40(3):187-209.

10. Holmes RP, Goodman HO, Shihabi ZK, Jarow JP. The taurine and hypotaurine content of human semen. J Androl. 1992;13(3):289-92.

11. Sharma RK, Agarwal A. Role of reactive oxygen species in male infertility. Urology. 1996;48: 835–850.

12. Bucak MN, Ateşşahin A, Varişli O, Yüce A, Tekin N, Akçay A. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen Microscopic and oxidative stress parameters after freeze-thawing process. Theriogenology. 2007; 67(5):1060-7.

13. Sagara J, Miura K, Bannai S. Cysteine uptake and glutathione level in fetal brain cells in primary culture and in suspension. J Neurochem. 1993; 61:1667–1671.

14. Szcześniak-Fabiańczyk B, Bochenek M, Smorag Z, Ryszka F. Effect of antioxidants added to boar semen extender on the semen survival time and sperm chromatin structure. Reprod Biol. 2003; 3(1):81-7. 15.

15. Lopes S, Jurisicova A, Sun JG, et al. Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Hum Reprod 1998;13:896–900.

16. Funahashi H, Sano T. Selected antioxidants improve the function of extended boar semen stored at 10 C. Theriogenology. 2005;63:1605–1616.

17. Chen Y, Foote RH, Brockett CC. Effect of sucrose, trehalose, hypotaurine, taurine, and blood serum on survival of frozen bull sperm. Cryobiology. 1993;30:423–431.

18. Szcześniak-Fabiańczyk B, Bochenek M, Smorag Z, Ryszka F. Effect of antioxidants added to boar semen extender on the semen survival time and sperm chromatin structure. Reprod Biol. 2003;3(1):81-7.

19. Bucak MN, Atessahin A, Yuce A. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze–thawing process. Small Rum Res. 2008;75:128–134.

20. Atessahin A, Bucak MN, Tuncer PB, Kizil M. Effect of Antioxidant Additives on Microscopic and Oxidative Parameters of Angora Goat Semen Following the Freeze-Thawing Process. Small Ruminant Research.2008; 77: 38-44.

21. Uysal O, Bucak MN. Effect of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen thawed ram semen. Acta Veterinaria Brno. 2007;76:383–390.

22. Sariözkan S, Bucak MN, Tuncer PB, Ulutaş PA, Bilgen A. The influence of cysteine and taurine on microscopic-oxidative stress parameters and fertilizing ability of bull semen following cryopreservation. Cryobiology. 2009;58(2):134-8.

23. YILDIZ S, ÖZTÜRKLER Y, ARI UÇ, LEHİMCİOĞLU NC, ATAKİŞİ E, KULAKSIZ R. The Effects of L-Ergothioneine, N-acetylcystein and Cystein on Freezing of Ram Semen. Kafkas Univ Vet Fak Derg. 2015; 21(1): 81-86.

24. Chatterjee S, de Lamirande E, Gagnon C. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. Mol Reprod Dev. 2001; 60:498-506.

25. Keskes-Ammar L, Feki-Chakroun N, Rebai T, Sahnoun Z, Ghozzi H, Hammami S, Zghal K, Fki H, Damak J, Bahloul A. Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. Arch Androl. 2003; 49(2):83-94.

26. Bansal AK, Bilaspuri GS. Impacts of oxidative stress and antioxidants on semen functions. Vet Med Int. 2010;2010:686137.

27. Sarangi A, Singh P, Virmani M, Yadav AS, Sahu S, Ajithakumar HM, Kumari A, Rath AP. Effect of antioxidants supplementation on the quality of Beetal buck semen stored at 4°C. Vet World. 2017;10(10):1184-1188.

28. Abaigar T, Holt WV, Harrison RA, del Barrio G. Sperm subpopulations in boar (Sus scrofa) and gazelle (Gazella dama mhorr) semen as revealed by pattern analysis of computer-assisted motility assessments. Biol Reprod. 1999; 60(1):32-41.

29. Quintero-Moreno A, Miró J, Teresa Rigau A, Rigau T, Rodriguez-Gil JE. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology. 2003;59:1973–90.

30. Rivera MM, Quintero-Moreno A, Barrera X. Effects of constant, 9- and 16-h light cycles on sperm quality, semen storage ability and motile sperm subpopulations structure of boar semen. Reprod Dom Anim. 2006; 41:386–393.

31. Harrison RAP, Ashworth PJC, Miller NGA. Bicarbonate/CO2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membrane. Mol Reprod Dev. 1996;45:378–391.

32. Rigau T, Farré M, Ballester J, Mogas T, Peña A, Rodríguez-Gil JE. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology. 2001;56(5):801-15.

33. Daramola JO, Adekunle EO, Oke OE, Onagbesan OM, Williams TJ., Iyasere OS, James I J, Oyewusi IK, Oyewusi JA. Effects of pyridoxine in combination with different antioxidants on viability and oxidative stress parameters of cryopreserved goat buck semen. Arch. Zootec. 2017;66:15-21.

34. Aitken RJ, Paterson M, Fisher H, Buckingham DW, van Duin M. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. J Cell Sci. 1995;108 (Pt 5):2017-25.

35. O, Flaherty C, de Lamirande E, Gagnon C. Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. Free Radic Biol Med. 2006;41:528-540.

How to cite this article

Alomar M. Effects of oxidized glutathione, cysteine and taurine supplementations on motilitycharateristics of different goat spermatozoa types. Iran J Vet Sci Technol. 2021;13(2): 84-92.
DOI: https://doi.org/10.22067/ijvst.2021.70946.1055
URL: https://ijvst.um.ac.ir/article_41468.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Sep- 17
Accepted after revision: 2021- Dec- 18
Published online: 2021- Dec- 31

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2021.72388.1078

In vitro acaricidal activity of *Melia azedarach* ripe fruit extract against *Hyalomma excavatum* (Acari: Ixodidae)

Samira Gilvari[a], Ali Moshaverinia[a], Amir Moghaddam Jafari[b], Saeed Yaghfoori[c], Maryam Akaberi[d]

[a] Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[b] Department of Basic Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[c] Laboratory of Hormozgan Veterinary Head Office, Bandar Abbas, Iran.
[d] Department of Pharmacognosy, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

ABSTRACT

The current study aimed to evaluate the effect of dichloromethane extract of *Melia azedarach* ripe fruit on larvae and adult females of *Hyalomma excavatum* at concentrations of 0.25, 0.5, 1, 2, and 4%, using the larval immersion test (LIT) and adult immersion test (AIT). The results showed that in LIT, the percentage mortality of larvae was significantly higher at concentrations 1, 2, and 4% than that in the control group after 24 h. While the mortality rates varied from 8.66% to 72.66% after 24 h post-treatment, complete mortality of the examined larvae was achieved at a concentration of 4% after 48 h post-exposure whereas, it was13.33% in the negative control group. In AIT, the percentage inhibition of oviposition in the treatment groups was significantly greater than that in the control group ($p < 0.01$). The maximum inhibition of oviposition was 17.72%, which was achieved at a concentration of 4% and it was 0% in the control group. The difference between reproductive index in treatment and control groups was not statistically significant ($p > 0.01$). This study showed that the ripe fruit extract of *M. azedarach* was toxic to *H. excavatum* under laboratory conditions.

Keywords

Melia azedarach, Hyalomma excavatum, Azadirachtin, plant-based acaricide

Number of Figures: 1
Number of Tables: 3
Number of References: 36
Number of Pages: 7

Abbreviations

DCM: Dichloromethane
LIT: Larval immersion test
AIT: Adult immersion test
AZA: Azadirachtin
IO: Inhibition of oviposition
RI: Reproductive index

Corresponding author: *Ali Moshaverinia*
Email: *moshaverinia@um.ac.ir*
Tel: *+98 (51)3880-5639*

Introduction

Hard ticks (*Arachnida: Acarina: Ixodidae*) are obligate blood-feeding ectoparasites that affect both human and animal health via sucking blood and transmission of some pathogenic agents such as *Babesia spp., Theileria spp., Anaplasma spp.,* and *Nairovirus* [1, 2]. Anxiety, irritation, stress, skin damage, weight loss, tick paralysis, decrease in milk production, loss of production, and anemia are direct adverse consequences of infestation with the hard ticks [2].

Nowadays, tick control relies on using synthetic pesticides. Although these compounds are more available and possess fast-killing effects, intensive and repeated use of them to control tick infestations has resulted in developing resistance to an array of acaricides. *Rhipicephalus (Boophilus) microplus* resistance to permethrin was recorded in the USA and Mexico [3]. Resistance to cypermethrin and deltamethrin in *Hyalomma anatolicum* has been reported from India [1, 4]. Tick resistance to conventional pesticides and increased demand for organic products has accelerated the research on plant-based acaricides [5].

Melia azedarach, belonging to the *Meliaceae* family, is a well-known source of various bioactive components with insecticidal properties. This deciduous tree species native to Indomalaya and Australasia is now cultivated in most subtropical and tropical regions of the world [6, 7]. Acaricidal, insecticidal and larvicidal efficacy of *M. azedarach* extract against agriculture pests, mosquitoes, important veterinary ticks, and mites have already been reported [8-11]. *M. azedarach* has been reported to have a complex mixture of compounds including saponins, terpenoids, flavonoids, tannins, alkaloids, and limonoids [6]. Limonoids particularly azadirachtin (AZA) constitute the biologically active components of *M. azedarach* fruits. However, other limonoids such as nimbin, nimbolinin, and salannin have also been reported from the *M. azedarach* fruits [6, 12, 13] AZA is the most important limonoid and biopesticide of this plant which its toxicity and adverse effects on feeding, growth, fecundity, and oviposition of arthropods, especially for phytophagous insects have been proven [14-16]. This compound is found in different parts of the *M. azedarach* tree and the highest level of AZA is generally found in seeds [17]. Although several methods have been reported for the identification and quantitative determination of azadirachtin, most studies have used high-performance liquid chromatography (HPLC) for this purpose worldwide [17]. The AZA content in various parts of trees is influenced by several factors such as genetic, climatic conditions, harvesting time, geographical area, and time of collection/storage of plant materials [17].

Adult *Hyalomma excavatum* ticks (known as large Anatolian *Hyalomma*) infesting cattle, sheep, horses, goats, camels, and donkeys are found almost all over Iran except the Caspian Sea area [18-21]. This *Hyalomma* species serves as a vector for some pathogens particularly *Theilera annulata* and Razmi et al. (2003) reported a high rate of *T. annulata* infection in examined *H. excavatum* collected from cattle in Mashhad area, Iran [21, 22].

Due to the high prevalence of *H. excavatum* in most parts of Iran and the necessity of searching for less hazardous and eco-friendly alternatives for synthetic acaricides, the present study aimed to evaluate acaricidal effects of *M. azedarach* fruit extract on *H. excavatum* under laboratory conditions.

Results

Larval immersion test

In larval immersion test (LIT), the percentage mortality of larvae was significantly higher at concentrations 1, 2, and 4% than the control group after 24 h. The mortality rates varied from 8.66% to 72.66%, 24 h post-treatment, and 100% mortality of examined larvae was achieved at concentrations 4%; 48 h post-exposure. The extract killed 100% of the tick larvae at all concentrations after 72 h, while the mortality rate was 17.30% in the control group (Figure 1, Table 1).

The LC50 and 99 (lethal concentrations of 50 and 99%) values of this extract against examined tick larvae were calculated at 24 and 48 h post-exposure and is presented in Table 2.

Adult immersion test

In adult immersion test (AIT), the percentage inhibition of oviposition in treatment groups was sig-

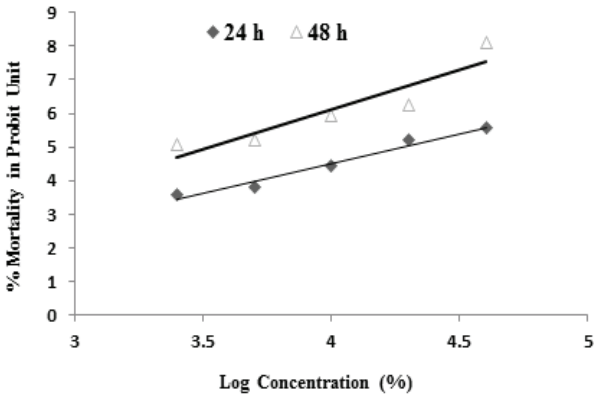


Figure 1.
Linear regression curve of percentage mortality of *H. excavatum* larvae in Probit unit versus logarithm concentration of *M. azedarach* ripe fruit extract.

nificantly greater than the control group ($p < 0.01$). The maximum inhibition of oviposition was 17.72%, which was achieved at a concentration of 4% and this parameter was concentration-dependent. The difference between reproductive indexes in the treatment and control groups was not statistically significant ($p > 0.01$). The detailed data about the inhibition of oviposition and reproductive index is presented in Table 3.

Discussion

The LIT results demonstrated a different level of larval mortality. Besides, the percentage of mortality of exposed larvae was concentration and time-dependent. These findings confirm several similar studies that investigated the effectiveness of *M. azedarach* crude extract against ticks, mites, and mosquitoes [8, 10, 11]. Borges et al. (2003) showed that the hexane extract of *M. azedarach* ripe fruit was effective against *Rhipicephalus* (*Boophilus*) *microplus* larvae in a concentration and time-dependent manner [8]. The acaricidal effects of this extract against *Demanyssus gallinae* and *Tetranychus urticae* have been reported [10, 23]. Furthermore, Selvaraj and Mosses (2011) observed that leaf and seed extract of this tree produced significant larval mortality in all larval stages of *Anopheles stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti* [11]. Azadirachtin is recognized as the main active ingredient and the most important component of *Azadirachta indica* and *M. azedarach*. Feed deterrency, growth reduction, increase in mortality, abnormal/delayed molts and down-regulation of insects' reproductive organs have been observed after applying AZA [14]. The other known bioactive components of *M. azedarach* include meliartenin, meliacaprin, meliacin, meliantrol, melianol, salannin, nimbin, and pinoresinol bis-epi-pinoresinol with anti-feeding/growth activities, repellency, inhibition of oviposition, and embryogenesis properties [24, 25]. In the present study, a high mortality rate in examined tick larvae was observed after a short time post-exposure. This fast-killing effect may be related to inhibition of cell division and protein synthesis in cells of ectoparasites [14].

In this study, egg production in exposed ticks was significantly lower than that in the control group, and this extract at the concentration of 4% produced 17.72% inhibition of egg production. In a similar study, the ripe fruit extract of *M. azedarach* at the concentration of 0.25% caused complete egg production inhibition in *Boophilus microplus* [8]. Variation in efficacy with different concentrations may be associated with genetic characteristics of the plant, edaphoclimatic conditions, and even duration of storage affecting the chemical composition of a plant extract [26, 27]. Reproductive disruption in examined ticks can be attributed to azadirachtin that affects reproductive tissues at molecular/cellular levels and disrupts endocrine processes by altering ecdysteroids and juvenile hormone titers [14]. Sousa et al. (2013) observed a

Table 1.
Means ± SD of mortality rates of *Hyalomma excavatum* larvae in treatment and control groups exposed to different concentrations of *M. azedarach* ripe fruit extract 24, 48, and 72 h post-exposure.

Time (h)	Control group (water + tween)	Case groups (% *M. azedarach* extract)				
		0.25	0.5	1	2	4
24	3.33 ± 5.77 ^{Aa}	8.66 ± 3.21 ^{Aa}	15.33 ± 4.50 ^{ABa}	29.00 ± 9.64 ^{Ba}	58.33 ± 17.55 ^{Ca}	72.66 ± 16.16 ^{Ca}
48	13.33 ± 7.63 ^{Aa}	53.66 ± 9.81 ^{Bb}	59.33 ± 7.37 ^{Bb}	82.00 ± 23.06 ^{Db}	89.66 ± 17.89 ^{Db}	100.00 ± 0.00 ^{Db}
72	17.30 ± 8.32 ^{Aa}	100.00 ± 0.00 ^{Bc}	100.00 ± 0.00 ^{Bc}	100.00 ± 0.00 ^{Bb}	100.00 ± 0.00 ^{Bb}	100.00 ± 0.00 ^{Bb}

Different capital letters within rows and small letters within columns indicate significant differences ($p < 0.05$).

Table 2.
The LC50 and LC99 values of *M. azedarach* ripe fruit extracts against *H. excavatum* Larvae.

Time (h)	Slope (95% CL)	R^2	LC50 (%) (95% CL)	LC99 (%) (95% CL)
24	1.77 ± 0.17	0.97	1.86 (1.77-1.96)	38.40 (36.48-40.32)
48	2.33 ± 0.56	0.85	0.33 (0.31-0.35)	3.32 (3.15-3.48)

CL: Confidence Limit

Table 3.
Means ± SD of mortality rates of *Hyalomma excavatum* larvae in treatment and control groups exposed to different concentrations of *M. azedarach* ripe fruit extract 24, 48, and 72 h post-exposure.

Tick reproduction	Control group (water + tween)	Case group (% *M. azedarach* extract)				
		0.25	0.5	1	2	4
Inhibition of oviposition (%)	0.00 ± 0.00	4.14 ± 2.19	6.66 ± 2.89	8.74 ± 3.97[a]	14.26 ± 6.07[a]	17.72 ± 6.15[a]
Reproductive index	0.63 ± 0.06	0.60 ± 0.05	0.59 ± 0.03	0.57 ± 0.03	0.54 ± 0.06	0.52 ± 0.08

[a] Significant differences compared with the control group (p <0.05).

reduction in ovary weight, morphological changes in oocysts, vacuolization, chorion deformity, and disorganization of the yolk granules of engorged females *Rhipicephalus (Boophilus) microplus* treated with *M. azedarach* hexanoic extract [28]. To fully understand the various acaricidal effects of *M. azedarach*, further studies are required to identify all its bioactive acaricide components with their special effects at cellular and molecular levels.

Presently, there is scant published data on LC50 values for *Hyalomma spp.* exposed to *M. azedarach* extract. The current study recorded an LC50 value of 1.86% for *H. excavatum* treated with dichloromethane extract of *M. azedarach* 24h post-exposure. LC50 values of 0.26 and 4.17% were reported for *Hyalomma dromedarii* nymphal stage exposed to petroleum ether and ethyl alcohol extract of *M. azedarach*. Also, these extracts showed a significant effect on *H. dromedarii* eggs with LC50 values of 3.14 and 1.77%, respectively [29]. LC50 value of 1.78% was recorded for *Dermanyssus gallinae* tereated with Hexan extract of *M. azedarach* [10]. These variations in LC50 values can be attributed to the type of solvent used for plant extraction, susceptibility of exposed ectoparasite species, and its developmental stage.

Besides the laboratory studies, Borges et al. (2005) and Sousa et al. (2011) evaluated the efficacy of hexane extract of ripe fruits of *M. azedarach* against all developmental stages of *R. microplus* [30, 31]. Standardized laboratory and farm tests need to be developed to add along with this incremental process for finding natural pesticides.

In conclusion, the findings of this study showed that *M. azedarach* ripe fruit extract was effective against larvae and engorged females of *H. excavatum* ticks, and more studies are required to investigate its efficacy in field trials.

Materials & Methods

Collection of ticks

Adult males and females of *H. excavatum* were taken from an active tick colony rearing in the Faculty of Veterinary Medicine's parasitology laboratory, Ferdowsi University of Mashhad, Iran. The identification of ticks was made using a stereomicroscope based on morphological criteria [21].

The developmental stages of ticks

Unfed mixed sex adult ticks were experimentally fed on healthy pathogen-free rabbits (male) at room temperature. 180 adult engorged female ticks were used for adult immersion test (AIT), but a group was placed into tubes individually and kept at 28 ºC, 80% RH to oviposit. Eggs were harvested and divided into groups of 300 eggs and transferred into perforated tubes. Newly hatched larvae were maintained at 28 ºC, 80% RH in an incubator and used for larval immersion test [32].

Preparation of the crude extract

The collected *M. azedarach* ripe fruits from the campus of the Ferdowsi University of Mashhad, Mashhad, Iran, were dried in the shade at room temperature and powdered using a grinding machine. The powder was extracted with dichloromethane (DCM) in the Soxhlet extraction apparatus and the solvent was removed by a rotatory evaporator. The residue was serially diluted with distilled water to obtain desired concentrations of 0.25, 0.5, 1, 2, and 4% and Tween 20 was used as emulsifier to ensure complete solubility of the materials in water [8, 10].

Larval immersion test

Larval immersion test (LIT) was performed based on the methodology described by Singh et al. (2017) [33]. Approximately 300 14-21 day old larvae were immersed in 0.5 ml of each desired concentration for 10 min. Then, the larvae were transferred on a filter paper to dry and 100 larvae were taken and placed in a folded filter paper packet (7.0 cm by 7.0 cm) using an aspirator. The pockets were sealed with adhesive tape and incubated at 28 °C and 80% RH. The packets were opened after 24, 48, and 72 h for counting live and dead larvae [33]. The mortality rate was corrected using the Abbott formula if mortality in the control group was between 0 and 5%:
Corrected mortality % = % test mortality - % control mortality/100 - % control mortality × 100 [34].
Distilled water + Tween 20 was used as a negative control and the larval immersion test for each concentration was repeated three times.

Adult immersion test

The AIT was performed as described by Godara et al. (2015) and the FAO (2004) [35, 36]. The engorged female ticks were weighed and allocated to groups with 10 ticks and each group was immersed in 30 ml of the prepared concentrations, namely 0.25, 0.5, 1, 2, and 4% for 5 min. The control group was treated with distilled water + Tween20. After sieving, the retained ticks were placed onto a clean tissue paper towel for drying and kept separately in a Petri dish. The ticks were stored in an incubator at 28 °C and relative humidity of 80% to oviposit. Eggs laid by every tick were weighted and incubated to hatch

[35, 36]. There were three replications for each concentration and control.
The percentage Inhibition of Oviposition (IO) and Reproductive Index (RI) was calculated using the following formula:

Reproductive Index (RI) = Average weight of eggs laid / Average weight of live tick

Percentage inhibition of oviposition (IO) = RI of control ticks – RI of treated ticks / RI of control ticks × 100

Statistical analysis

The data were subjected to SPSS software ver. 25 (IBM corporation, USA). Statistical analysis of the data was performed using the variance analysis (ANOVA and General Linear Model) followed by Duncan's multiple range test with a probability level < 0.01.
The lethal concentrations for 50% (LC50) and 99% (LC99) with their respective 95% confidence limits (CL) were determined using regression analysis equation to the probit transformed data of mortality.

Authors' Contributions

AM and AMJ created the original idea. SG carried out the experiments, and AM, AMJ, SY, and MA directed the project. All authors analyzed and interpreted the data. AM and SG contributed to the writing of the manuscript.

Acknowledgements

The authors would like to thank the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran for providing material and for financial support and providing the facilities that make this project possible.

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Nandi A, Singh H, Singh NK. Esterase and glutathione S-transferase levels associated with synthetic pyrethroid resistance in Hyalomma anatolicum and Rhipicephalus microplus ticks from Punjab, India. Exp Appl Acarol. 2015;66(1):141-57.

2. Radostits OM, Gay CC, Hinchcliff KW, Constable PD. Veterinary Medicine E-Book: A textbook of the diseases of cattle, horses, sheep, pigs and goats: Elsevier Health Sciences; 2006.

3. Thomas DB, Klafke G, Busch JD, Olafson PU, Miller RA, Mosqueda J, et al. Tracking the increase of acaricide resistance in an invasive population of Cattle Fever Ticks (Acari: Ixodidae) and implementation of real-time PCR assays to rapidly genotype resistance mutations. Ann Entomol Soc Am. 2020;113(4):298-309.

4. Gupta S, Gupta S, Kumar S. Cypermethrin resistance in Hyalomma anatolicum and Rhipicephalus microplus ticks of arid and semi-arid zone of Haryana, a northern state of India. Int J Trop Insect Sci. 2021;41(1):703-9.

5. Webster A, Souza UA, Martins JR, Klafke G, Reck J, Schrank A. Comparative study between Larval Packet Test and Larval Immersion Test to assess the effect of Metarhizium anisopliae on Rhipicephalus microplus tick larvae. Exp Appl Acarol. 2018;74(4):455-61.

6. Sharma D, Paul Y. Preliminary and pharmacological profile of Melia azedarach L.: An overview. J App Pharm Sci. 2013;3(12):133-8.

7. Venson I, Guzmán JS, Talavera FF, Richter H. Biological, physical and mechanical wood properties of Paraiso (Melia azedarach) from a roadside planting at Huaxtla, Jalisco, Mexico. J Trop For Sci. 2008;20(1):38-47.

8. Borges L, Ferri P, Silva W, Silva W, Silva J. In vitro efficacy of extracts of Melia azedarach against the tick Boophilus microplus. Med Vet Entomol. 2003;17(2):228-31.

9. Carpinella MC, Miranda M, Almirón WR, Ferrayoli CG, Almeida FL, Palacios SM. In vitro pediculicidal and ovicidal activity of an extract and oil from fruits of Melia azedarach L. J Am Acad Dermatol. 2007;56(2):250-6.

10. Sariosseiri A, Moshaverinia A, Khodaparast MHH, Kalidari GA. In vitro acaricidal effect of Melia azedarach ripe fruit extract against Dermanyssus gallinae (Acari: Dermanyssidae). Persian J Acarol. 2018;7(2):203-208.

11. Selvaraj M, Mosses M. Efficacy of Melia azedarach on the larvae of three mosquito species Anopheles stephensi, Culex quinquefasciatus and Aedes aegypti (Diptera: Culicidae). Eur Mosq Bull. 2011;29(1):116-21.

12. Ambrosino P, Fresa R, Fogliano V, Monti SM, Ritieni A. Extraction of azadirachtin A from neem seed kernels by supercritical fluid and its evaluation by HPLC and LC/MS. J Agric Food Chem. 1999;47(12):5252-6.

13. Carpinella MC, Defago MT, Valladares G, Palacios SM. Antifeedant and insecticide properties of a limonoid from Melia azedarach (Meliaceae) with potential use for pest management. J Agric Food Chem. 2003;51(2):369-74.

14. Nisbet AJ. Azadirachtin from the neem tree Azadirachta indica: its action against insects. An Soc Entomol Brasil. 2000;29(4):615-32.

15. Prakash G, Emmannuel C, Srivastava AK. Variability of azadirachtin in Azarirachta indica (neem) and batch kinetics studies of cell suspension culture. Biotechnol Bioprocess Eng. 2005;10(3):198-204.

16. Prakash G, Bhojwani SS, Srivastava AK. Production of azadirachtin from plant tissue culture: state of the art and future prospects. Biotechnol Bioprocess Eng. 2002;7(4):185-93.

17. Fernandes SR, Barreiros L, Oliveira RF, Cruz A, Prudêncio C, Oliveira AI, et al. Chemistry, bioactivities, extraction and analysis of azadirachtin: State-of-the-art. Fitoterapia. 2019;134(1):141-50.

18. Hosseini-Chegeni A, Hosseini R, Tavakoli M, Telmadarraiy Z, Abdigoudarzi M. The Iranian Hyalomma (Acari: Ixodidae) with a key to the identification of male species. Persian J Acarol. 2013;2(3):503-529.

19. Nabian S, Rahbari S, Changizi A, Shayan P. The distribution of Hyalomma spp. ticks from domestic ruminants in Iran. Med Vet Entomol. 2009;23(3):281-3.

20. Rahbari S, Nabian S, Shayan P. Primary report on distribution of tick fauna in Iran. Parasitol Res. 2007;101(2):175-7.

21. Walker AR. Ticks of domestic animals in Africa: a guide to identification of species: Bioscience Reports Edinburgh; 2003.

22. Razmi GR, Ebrahimzadeh E, Aslani M. A study about tick vectors of bovine theileriosis in an endemic region of Iran. J Vet Med. 2003;50(6):309-10.

23. Kadıoğlu I. Acaricidal effects of different plant parts extracts on two-spotted spider mite (Tetranychus urticae Koch). Afr J Biotechnol. 2011;10(55):11745-50.

24. Castillo LE, Jiménez J, Delgado M. Secondary metabolites of the Annonaceae, Solanaceae and Meliaceae families used as biological control of insects. Trop Subtrop Agroecosystems. 2010;12(3):445-62.

25. Isman MB. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annu Rev Entomol. 2006;51(1):45-66.

26. Maccioni A, Falconieri D, Porcedda S, Piras A, Gonçalves MJ, Alves-Silva JM, et al. Antifungal activity and chemical composition of the essential oil from the aerial parts of two new Teucrium capitatum L. chemotypes from Sardinia Island, Italy. Nat Prod Res. 2021;35(24): 6007-6013.

27. Yakkundi SR, Thejavathi R, Ravindranath B. Variation of azadirachtin content during growth and storage of neem (Azadirachta indica) seeds. J Agric Food Chem. 1995;43(9):2517-19.

28. Sousa LADD, Rocha TL, Sabóia-Morais SMT, Borges LMF. Ovary histology and quantification of hemolymph proteins of Rhipicephalus (Boophilus) microplus treated with Melia azedarach. Rev Bras Parasitol Vet. 2013;22(3):339-45.

29. Abdel-Ghany HS, Fahmy MM, Abuowarda MM, Abdel-Shafy S, El-Khateeb RM, Hoballah EM. In vitro acaricidal effect of Melia azedarach and Artemisia herba-alba extracts on Hyalomma dromedarii (Acari: Ixodidae): embryonated eggs and engorged nymphs. J Parasit Dis. 2019;43(4):696-710.

30. Borges LMF, Ferri PH, Silva WC, Silva WJ, Melo LS, Souza LAD, et al. Ação do extrato hexânico de frutos maduros de Melia azedarach (Meliaceae) sobre Boophilus microplus (Acari: Ixodidae) em bezerros infestados artificialmente. Rev Biol Trop. 2005;34(1):53-59.

31. Sousa LADD, Júnior HBP, Soares SF, Ferri PH, Ribas P, Lima EM, et al. Potential synergistic effect of Melia azedarach fruit extract and Beauveria bassiana in the control of Rhipicephalus (Boophilus) microplus (Acari: Ixodidae) in cattle infestations. Vet Parasitol. 2011;175(3-4):320-4.

32. Abdel-Shafy S, Zayed A. In vitro acaricidal effect of plant extract of neem seed oil (Azadirachta indica) on egg, immature, and adult stages of Hyalomma anatolicum excavatum (Ixodoidea: Ixodidae). Vet parasitol. 2002;106(1):89-96.

33. Singh N, Saini S, Singh H, Sharma S, Rath S. In vitro assessment of the acaricidal activity of Piper longum, Piper nigrum, and Zingiber officinale extracts against Hyalomma anatolicum ticks. Exp Appl Acarol. 2017;71(3):303-17.

34. Sabatini G, Kemp D, Hughes S, Nari A, Hansen J. Tests to determine LC50 and discriminating doses for macrocyclic lactones against the cattle tick, Boophilus microplus. Vet Parasitol. 2001;95(1):53-62.

35. Godara R, Parveen S, Katoch R, Yadav A, Katoch M, Khajuria J, et al. Acaricidal activity of ethanolic extract of Artemisia absinthium against Hyalomma anatolicum ticks. Exp Appl Acarol. 2015;65(1):141-8.

36. FAO. Module 1. Ticks: Acaricide resistance; diagnosis management and prevention. Guidelines resistance management and integrated parasite control in ruminants. 2004.

How to cite this article
Gilvari S, Moshaverinia A, Moghaddam Jafari A, Yaghfoori S, Akaberi M. In vitro acaricidal activity of Melia azedarach ripe fruit extract against Hyalomma excavatum (Acari: Ixodidae). Iran J Vet Sci Technol.2021; 13(2): 93-99.
DOI: https://doi.org/10.22067/ijvst.2021.72388.1078
URL: https://ijvst.um.ac.ir/article_41503.html

Iranian Journal of Veterinary Science and Technology

Received: 2020- Feb- 20
Accepted after revision: 2021- Jun- 08
Published online: 2021- Dec- 31

Short Communication

DOI: 10.22067/ijvst.2021.68564.1013

Recovery effects of pomegranate seed powder on the testes following cadmium poisoning in Japanese quail (*Coturnix japonica*); a stereological and lipid peroxidation study

Rahmat Allah Fatahian Dehkordi[a], Shahab Bahadoran[b], Mohammadreza Alijani[c], Abdollnaser Mohebi[b], Hanie Mohammadi[c]

[a] Department of Basic Sciences, Faculty of Veterinary Medicine, University of Shahrekord, Shahrekord, Iran.
[b] Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Shahrekord, Shahrekord, Iran.
[c] DVM Student, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran.

ABSTRACT

This study aimed to investigate the effects of pomegranate seed powder on cadmium-poisoned testicular tissue in Japanese quail. A total of 270 day-old Japanese quail chicks were assigned to six treatment groups, control group, cadmium 50 ppm (group II), pomegranate seed powder (1 and 0.5 %; groups III and IV, respectively), pomegranate seed powder 1% + Cd 50 ppm (group V), pomegranate seed powder 0.5% + Cd 50 ppm (group VI). Stereological parameters in testes and TBARS, cholesterol, and triglyceride levels were determined. Testicular components showed a significant reduction in area surface and volume density in the cadmium-exposed groups compared with controls ($p < 0.05$). It was found that in the cadmium induction group, TBARS, cholesterol, and triglyceride levels were significantly higher compared to the normal level ($p < 0.05$). The results showed that pomegranate seed powder could increase the area surface and volume density of testicular germinal ingredients and decrease the content of TBARS, cholesterol, and triglyceride ($p > 0.05$) in cadmium poisoning testis. These results show that cadmium has destructive effects and pomegranate seed powder has prevented the development of these effects on stereological and lipid parameters.

Keywords

Japanese quail, Pomegranate seed powder, Stereology, TBARS, Histology

Number of Figures: 2
Number of Tables: 1
Number of References: 29
Number of Pages: 6

Abbreviations

TBARS: Thiobarbituric acid reactive substances
Cd: Cadmium
H&E: Hematoxylin and eosin
ANOVA: Analysis of variance
Spem: Spermatogina
Spem-1: Primary spermatocyte
Spet: Spermatid

Corresponding author:
Rahmat Allah Fatahian Dehkordi
Email: fatahian-r@sku,ac.ir
Tel: +98(93)5624-9361

Cadmium is a natural heavy metal that in its industrial form, depending on its dosage, causes poisoning in humans and animals [1]. It has been reported that cadmium inflicts damage on the reproductive system of birds that can occur either experimentally [2] or by being environmentally exposed to this heavy metal [3]. Given that 1 to 2% of the acute dose of cadmium is taken up by the testes [4], however, the damage to the process of spermatogenesis is very severe [5]. Low doses of cadmium alter the immunological microenvironment in the testicles, which results in increased testicular autoimmunity [6]. Testicular poisoning with cadmium is caused by complex intra-network reactions, including the destruction of the blood testicular barrier or inhibition of oxidative stress leading to germ cell apoptosis, edema, and intra-testicular bleeding [7-9].

Pomegranate (Punica granatum) fruit, juice, seed, and peel due to a high content of polyphenols, especially ellagitannins, tannins, and anthocyanins have high antioxidant capacities [10]. Consumption of pomegranate extract significantly increases the sperm quality, density of the spermatogenic cell, antioxidant activity, and testosterone levels in male rats [11]. It has also been shown that pomegranate contains hydrolyzed and concentrated tannins that exhibit anti-cancer properties in vitro and in vivo [12, 13].

Qualitative patterns, including atrophy, hypoplasia, hyperplasia, and or hypertrophy can be stated by stereological methods through measurable and comparative data [14]. However, the impact of various situations on the process of spermatogenesis and or survival of the germ cell line requires simple methods and quantification. This study aimed to determine the protective effects of pomegranate seed powder against the toxic effects of Cd in quail using biochemical and histo-stereological methods. All the chemicals and reagents used in this study were of molecular biology grade and were purchased from Sigma-Aldrich Co. Ripe pomegranates are purchased from the local market and after separating the skin, seeds (protein, 13.6; fat, 29.6, fiber, 39.3) were washed with water and after drying, seeds were powdered using a hammer mill and sieved through a 40-mesh sieve [15]. All protocols used in this experiment were approved by the Institutional Animal Care and Use Committee of Shahrekord University. Two hundred and seventy-one-day-old unsexed quail (Japanese) chicks were used in this study. The birds were fed by the standard basic diet until the 7th day of age (one week, habituation) before starting the experiment. All diets were formulated to meet the nutrient requirements of the Japanese quails. On day 7, the birds were randomly assigned to six treatments, with three replicates (cages) of fifteen quails each.

Group I: received basic diet and ordinary water. Group II: received a basic diet and 50 ppm cadmium orally. Group III: birds were fed with a basic diet + 1% pomegranate seed powder. Group IV: birds were fed with a basic diet + 0.5% pomegranate seed powder. Group V: received a basic diet along with 1% pomegranate seed powder and 50 ppm cadmium. Group VI: received basic diet along with 0.5% pomegranate seed powder and 50 ppm cadmium.

Blood samples were centrifuged at 1500 rpm for 15 minutes and TBARS, cholesterol, and triglyceride in serum samples were measured. Testes specimens fixed in Merck formalin and processed through paraffin embedding, cut into 5 µm sections, stained with hematoxylin and eosin (H&E) techniques for stereological evaluation.

Stereology

By the count of all point, the total area determined (Figure 1), and finally the volume wass estimated by:

$$V{:=}k \times t \times a(p) \times \sum^{P}(\text{testis})$$

In which $\sum^{P}$ is total area, k is k^{th} section is mounted on a glass slide, $a(p)$ is the area of each point.

The volume of the seminiferous tubules, the inter-tubular tissues, and the germinal epithelium was estimated [16] by:

$$V_{V(\text{structur/ref})} = P_{(\text{structure})} / P_{(\text{total})}$$

In which $P_{(structure)}$ and $P_{(total)}$ represent respectively, the total numbers of points hitting the structures of interest, and the reference space $V_{(ref)}$ and summed over all sections and fields from one testis.

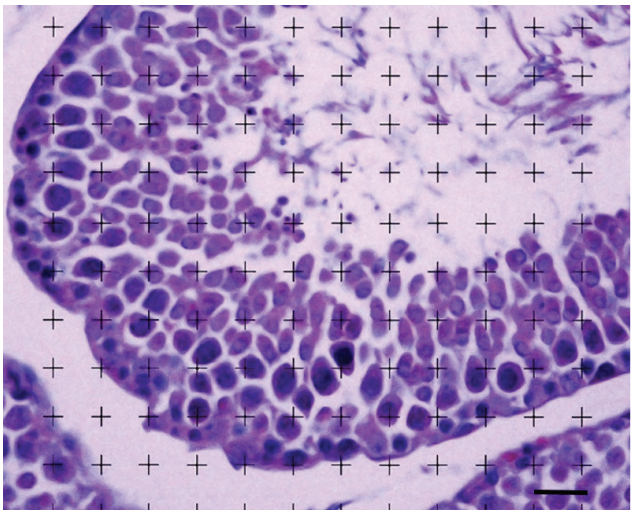


Figure 1.
Photomicrograph of testis in quail. Point-counting method was used to evaluate stereological parameters. The area surface and volume density of the spermatogonia, primary spermatocyte, and spermatid were determined by calculation of the points hitting with the micrograph; (scale bar=20 µm).

$$V_{(structure)} = V_{V(structure/testis)} \times V_{final}(mm^3)$$

The total volume was obtained by multiplying the density by the final testis volume [16].

Biochemical analysis

TBARS was determined calorimetrically using the method of Buege and Aust (1978), briefly, 0.1 mL of serum was treated with 2 mL of TBA-TCA-HCl reagent, and absorbance of the supernatant was measured against reference blank at 535 nm. Concentrations were calculated using an extinction coefficient of 1.56 9 105 mol^{-1} L cm^{-1} and expressed as nmol mL^{-1} [17]. Blood biochemical parameters including triglyceride and cholesterol were measured by commercial kits of Pars Azmoun Company and according to the protocols of this company. Data were analyzed by one-way analysis of variance (ANOVA) using the SPSS V. 23/0 software (SPSS Inc., Chicago, IL). The means were considered significantly different at $p < 0.05$.

The Vv (spem/tes) (Table 1) was significantly decreased in the cadmium-exposed group compared to the control group ($p < 0.05$). In addition, the Vv (spem-1/tes) showed a significant decrease in groups that received cadmium compare to the control group ($p < 0.05$). The Vv (spet/tes) also were significantly decreased in the group exposed to the cadmium compared to the control groups ($p < 0.05$). Nevertheless, the administration of pomegranate seed powder (1 and 0.5%) along with cadmium (50ppm, V and VI groups) inverted the mean of all stereological volumetric parameters to near normal. But these differences between V and VI groups with the control group were not statistically significant ($p > 0.05$).

The area surface (Table 1) in all stereological parameters of spermatogonia, primary spermatocytes, and spermatids was decreased in the cadmium groups compared to the control birds ($p < 0.05$). Following a similar pattern of volume density, pomegranate seed powder (1 and 0.5%) reversed the area surface of the measured parameters mentioned non-significantly toward normal in cadmium-exposed quails (V and VI groups; $p > 0.05$).

The mean TBARS level (Figure 2 and Table 2) of the control group (group I) compared with those of the other groups had no significant difference in this study except for the cadmium group (group II). Nevertheless, a significant increase had happened in the cadmium group (32.27 ± 4.32) in comparison with the TBARS level of the control group (20.3±3.21; $p < 0.05$). On the other hand, pomegranate seed powder (1 and 0.5%) was able to bring the level of TBARS in cadmium poisoned quail closer to the control group, although this reduction was not significant ($p > 0.05$).

It should be noted that there was a significant increase in the cholesterol and triglyceride values (Table 2) of the cadmium group (181.41 ± 7.28 and 277.08 ± 86.16 respectively) compared with those of the control group (148.99 ± 25.07 and 194.78 ± 7.49 respectively; $p < 0.05$). Pomegranate seed powder (1 and 0.5%) groups (groups V and VI) showed a decrease in cholesterol and triglyceride values in cadmium-poisoned birds' groups, although this decrease was non-significant ($p > 0.05$). Therefore, the administration of pomegranate seed powder in two different doses ($p > 0.05$) reversed the levels of cholesterol and triglyceride to near normal in cadmium-exposed birds.

This study showed that the volume density of spermatogonia, primary spermatocyte, and spermatid affected by cadmium administration in a dose of 50 ppm. Meanwhile, similar changes were observed regarding the area surface of structural components mentioned in the face of cadmium. As the results showed, a significant decrease was seen in area sur-

Table 1.
Effect of pomegranate on stereological parameters in the testes of cadmium-exposed birds

		Area surface			Volume density		
		spem	spem-1	spet	spem	spem-1	spet
I	Control	0.16 ± .07 [a]	0.21 ± 0.10 [a]	0.17 ±. 04 [a]	0.25±0.13 a	0.43 ±.17 [a]	0.25 ± 0.13 [a]
II	Cd	0.07 ±. 04 [b]	0.12 ± .05 [b]	0.10 ±.06 [b]	0.10±.04 b	0.23 ±.19 [b]	0.10 ±.04 [b]
III	Pome. 1%	0.13 ± .03	0.23 ±. 06	0.17 ±.06	0.28±.13	0.50 ±.26	0.28 ±.13
IV	Pome. 0.5%	0.13 ± .06	0.23 ±. 07	0.20 ±.07	0.24±.19	0.50 ±.25	0.24 ±.19
V	Pome. 1% + Cd	0.12 ± .03	0.21 ±. 08	0.14 ±.04	0.22±.13	0.41 ±.17	0.22 ±.13
VI	Pome. 0.5% + Cd	0.12 ±.02	0.20 ± .07	0.14 ±.07	0.21±.12	0.41 ±.18	0.21 ±.12

[a, b] Significant difference compared with controls in each column at $p < 0.05$; Spem: spermatogina, Spem-1: primary spermatocytes, Spet: spermatid.

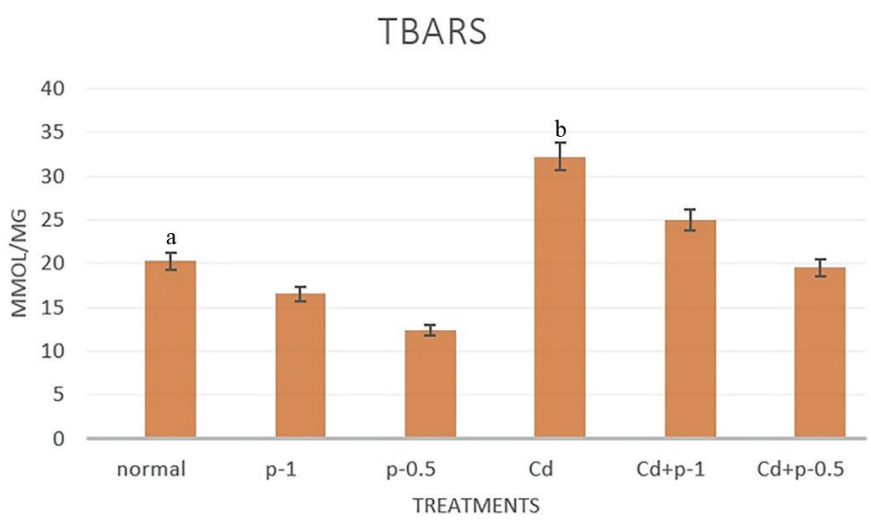


Figure 2.
Effect of pomegranate on TBARS levels of testes of cadmium-exposed birds. Groups: normal (control), p-1 (pomegranate 1%), p-0.5 (pomegranate 0.5%), Cd (cadmium), Cd+p-1 (cadmium+ pomegranate 1%),Cd+p-0.5 (cadmium+ pomegranate 0.5%); Dissimilar letters indicate significant differences.

face and volume density in the cadmium-treated birds in comparison with those of the control group. In agreement with our findings, Blanco et al. displayed a significant decreasing in the volume density of the testicular structure and decreasing tubular densities and epithelial percentages in the animals that were exposed to cadmium [18]. Adamkovicova et al. showed that the decrease in the epithelial layer volume of the seminiferous tubules in the face of cadmium can be due to the penetration of cells into the lumen leading to lumen shrinkage and a significant decrease in the percentage of epithelial volume fraction [19]. Therefore, we infer that a decrease in the volume density of measured parameters in our study could be another reason for the reduction of the epithelium layer volume in Adamkovicova's research. Numerous studies have shown that alteration in quantitative parameters, similar to measured parameters of this study, is related to toxic effects of cadmium which can result in the miss of germination epithelium [20, 21].

The results of this study show that no significant differences were observed between the control group and the pomegranate seed powder group in all the evaluated parameters. The results of this study also revealed that the area surface and the volume density of spermatogonia, primary spermatocyte, and spermatid were increased in groups V and VI compared to the cadmium group.

Cadmium exposure for 35 days caused higher TBARS, cholesterol, and triglyceride values in the serum of exposed birds to cadmium in the comparison control group. These are according to other findings that cadmium produces exceeding amounts of reactive oxygen species (ROS), which leads to oxidative stress damage and causes of injury and disease expansion [9, 22-26]. An increment in TBARS capacity was considered as a sign of oxidative damage [27, 28]. The present study showed that TBARS value in group II increases than control, but in groups V and VI, the TBARS value was at a low level compared with group II. Therefore, oral administration of pomegranate seed powder (both 1 and 0.5%) to cadmium treated quails non-significantly (p >0.05) decreased the altitude of TBARS when compared to the group treated with cadmium alone. Administration of pomegranate seed powder reversed the concentration of TBARS in cadmium exposed birds; such that, this shift caused the amount of TBARS value to deviate to the normal level, although these changes were not significant. Like TBARS, similar results were obtained in the cholesterol and triglyceride levels. It was observed that the levels of cholesterol and

Table 2.
Effect of pomegranate on biochemical parameters in the serum of cadmium-exposed birds

Groups		parameters		
		TBARS mmol/mg	Cholesterol mg/d	Triglyceride mg/d
I	Control	20.30 ± 3.21 [a]	148.99 ± 25.07 [a]	194.78 ± 7.49 [a]
II	Cd	32.27 ± 4.32 [b]	181.41 ± 7.28 [b]	277.08 ± 86.16 [b]
III	Pome. 1%	16.57 ± 2.17	139.36 ± 17.45	152.37 ± 21.16
IV	Pome. 0.5%	12.35 ± 2.01	123.32 ± 15.37	142.10 ± 15.42
V	Pome. 1% + Cd	25.00 ± 2.64	153.78 ± 24.26	224.34 ± 24.61
VI	Pome. 0.5% + Cd	19.58 ± 2.85	168.95 ± 16.56	208.27 ± 28.14

[a, b] Significant difference compared with controls in each column; Each value represents the mean ± SEM

triglyceride in the serum exposed to cadmium had an incremental distribution pattern than to controls; vice versa, the amount of these parameters in groups V and VI showed a declining process when compared with the cadmium-induced group (II).

Saleh et al. (2018) have reported that the phenolic compounds present in the pomegranate peel powder and extract may have the same antioxidant activity as α-tocopherol acetate in the meat of broiler chickens and decreased the oxidation rate and TBARS index, although their mechanism of action may be different [29]. The results of the present study indicated that the TBARS content in samples treated with the pomegranate powder was non significantly lower than that of the Cd group. In conclusion, these results indicate that Cd induces destructive effects on the stereological indices and lipid peroxidation markers and pomegranate seed powder has prevented the development of these effects in the testis of Japanese quails.n the meat of broiler chickens and decreased the oxidation rate and TBARS index, although their mechanism of action may be different [29]. The results of the present study indicated that the TBARS content in samples treated with the pomegranate powder was non significantly lower than that of the Cd group. In conclusion, these results indicate that Cd induces destructive effects on the stereological indices and lipid peroxidation markers and pomegranate seed powder has prevented the development of these effects in the testis of Japanese quails.

Authors' Contributions

RG, RA and HM performed the experiments. RF, IK, and BK designed the research project and drafted the manuscript.

Acknowledgements

The authors would like to thank the Shahrekord University for financial support.

Conflict of interest

The authors declare that they have no conflicts of interest.

References

1. Järup L, Akesson A. Current status of cadmium as an environmental health problem. Toxicol Appl Pharmacol. 2009;238(3):201-8.

2. Toman R, Massanyi P, Lukáč N, Ducsay L, Golian J. Fertility and content of cadmium in pheasant (Phasianus colchicus) following cadmium intake in drinking water. Ecotoxicol Environ Saf. 2005;62(1):112-7.

3. Dauwe T, Janssens E, Kempenaers B, Eens M. The effect of heavy metal exposure on egg size, eggshell thickness and the number of spermatozoa in blue tit Parus caeruleus eggs. Environ Pollut. 2004;129(1):125-9.

4. Sarkar A, Ravindran G, Krishnamurthy V. A brief review on the effect of cadmium toxicity: from cellular to organ level. Int J Adv Biotechnol Res. 2013;3(1):17-36.

5. Marettová E, Maretta M, Legáth J. Toxic effects of cadmium on testis of birds and mammals: a review. Anim Reprod Sci. 2015;155:1-10.

6. Ogawa Y, Itoh M, Hirai S, Suna S, Naito M, Qu N, et al. Cadmium exposure increases susceptibility to testicular autoimmunity in mice. J Appl Toxicol. 2013;33(7):652-60.

7. Tremellen K. Oxidative stress and male infertility—a clinical perspective. Hum Reprod Update. 2008;14(3):243-58.

8. Turner TT, Lysiak JJ. Oxidative stress: a common factor in testicular dysfunction. J Androl. 2008;29(5):488-98.

9. Chen YW, Yang CY, Huang CF, Hung DZ, Leung YM, Liu SH. Heavy metals, islet function and diabetes development. Islets. 2009;1(3):169-76.

10. Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG, et al. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J Nutr Biochem. 2005;16(6):360-7.

11. Türk G, Sönmez M, Aydin M, Yüce A, Gür S, Yüksel M, et al. Effects of pomegranate juice consumption on sperm quality, spermatogenic cell density, antioxidant activity and testosterone level in male rats. Clin Nutr. 2008;27(2):289-96.

12. Gali-Muhtasib HU, Younes IH, Karchesy JJ, el-Sabban ME. Plant tannins inhibit the induction of aberrant crypt foci and colonic tumors by 1,2-dimethylhydrazine in mice. Nutr Cancer. 2001;39(1):108-16.

13. Castonguay A, Gali H, Perchellet E, Gao X, Boukharta M, Jalbert G, et al. Antitumorigenic and antipromoting activities of ellagic acid, ellagitannins and oligomeric anthocyanin and procyanidin. Int J Oncol. 1997;10(2):367-73.

14. Noorafshan A. Stereology as a valuable tool in the toolbox of testicular research. Ann Anat. 2014;196(1):57-66.

15. Elgindy AAE, Elsarha ES. Biochemical and technological studies on the effect of pomegranate peel in addition to pan bread and its effect on the pollution of cadmium chloride in mice. Adv Environ Biol. 2015;9(27):33-43.

16. Noorafshan A. Stereology as a valuable tool in the toolbox of testicular research. Ann Anat. 2014;196(1):57-66.

17. Buege JA, Aust SD. [30] Microsomal lipid peroxidation. Methods Enzymol. 52: Elsevier; 1978. p. 302-10.

18. Blanco A, Moyano R, Vivo J, Flores-Acuna R, Molina A, Blanco C, et al. Quantitative changes in the testicular structure in mice exposed to low doses of cadmium. Environ Toxicol Pharmacol. 2007;23(1):96-101.

19. Adamkovicova M, Toman R, Cabaj M, Massanyi P, Martiniakova M, Omelka R, et al. Effects of subchronic exposure to cadmium and diazinon on testis and epididymis in rats. Sci World J. 2014;2014:632581.

20. do Carmo Cupertino M, Novaes RD, Santos EC, Bastos DSS, dos Santos DCM, Fialho MdCQ, et al. Cadmium-induced testicular damage is associated with mineral imbalance, increased antioxidant enzymes activity and protein oxidation in rats. Life Sci. 2017;175:23-30.

21. Cupertino MC, Novaes RD, Santos EC, Neves AC, Silva E, Oliveira JA, et al. Differential Susceptibility of Germ and Leydig Cells to Cadmium-Mediated Toxicity: Impact on Testis Structure, Adiponectin Levels, and Steroidogenesis. Oxid Med Cell Longev. 2017;2017:3405089.

22. Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal. 2012;24(5):981-90.

23. Pillai A, Gupta S. Antioxidant enzyme activity and lipid peroxidation in liver of female rats co-exposed to lead and cadmium: effects of vitamin E and Mn2+. Free Radic Res. 2005;39(7):707-12.

24. Kara H, Cevik A, Konar V, Dayangac A, Yilmaz M. Protective effects of antioxidants against cadmium-induced oxidative damage in rat testes. Biol Trace Elem Res. 2007;120(1-3):205-11.

25. Samarghandian S, Azimi-Nezhad M, Shabestari MM, Azad FJ, Farkhondeh T, Bafandeh F. Effect of chronic exposure to cadmium on serum lipid, lipoprotein and oxidative stress indices in male rats. Interdiscip Toxicol. 2015;8(3):151-4.

26. Manca D, Ricard A, Trottier B, Chevalier G. In vitro susceptibilities of rat tissues to cadmium-induced lipid peroxidation: comparison of evolved hydrocarbons and thiobarbituric acid reactive substances. In Vitro Toxicol. 1990;3(3):255-67.

27. Chakraborty S, Singh OP, Dasgupta A, Mandal N, Das HN. Correlation between lipid peroxidation-induced TBARS level and disease severity in obsessive–compulsive disorder. Prog Neuropsychopharmacol Biol Psychiatry. 2009;33(2):363-6.

28. Jakovljevic B, Novakov-Mikic A, Brkic S, Bogavac MA, Tomic S, Miler V. Lipid peroxidation in the first trimester of pregnancy. J Matern Fetal Neonatal Med. 2012;25(8):1316-8.

29. Saleh H, Golian A, Kermanshahi H, Mirakzehi M. Antioxidant status and thigh meat quality of broiler chickens fed diet supplemented with α-tocopherolacetate, pomegranate pomace and pomegranate pomace extract. Ital J Anim Sci. 2018;17(2):386-95.

How to cite this article

Fatahian Dehkordi RA, Bahadoran S, Alijani M, Mohebi A, Mohammadi H. Recovery effects of pomegranate seed powder on the testes following cadmium poisoning in Japanese quail (*Coturnix japonica*); A stereological and lipid peroxidation study. Iran J Vet Sci Technol. 2021; 13(2):100- 105.
DOI: https://doi.org/10.22067/ijvst.2021.68564.1013
URL: https://ijvst.um.ac.ir/article_41503.html

Iranian Journal of Veterinary Science and Technology

Received: 2020-
Accepted after revision: 2021-
Published online: 2021-

Case Report

DOI: 10.22067/ijvst.2021.72504.1083

Rare case of hemangiopericytoma in a domestic short-haired cat

Farzad Hayati[a], Ensiyeh Sajadian Jaghargh[a], Mohsen Maleki[b], Mahdi Hassankhani[c], Hossein Khoribabalhakam[a], Fatemeh Namazi[d]

[a] Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[b] Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[c] Department of Small Animal Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
[d] Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran.

ABSTRACT

A two-year-old queen was referred to the surgery section of veterinary hospital with lateral recumbency position and the primary diagnosis of dystocia. The queen was highly lethargic and dehydrated, with a pale mucous membrane, a rectal temperature of 37.3 °C, and no vaginal discharge during first inspection. The primary examination revealed a big mass in the abdominal cavity, which was followed by other diagnostic investigations. Due to a huge and encroaching tumor and the patient's owner's refusal to accept any further therapies, the queen was euthanized during an exploratory celiotomy. This report describes a large Hemangiopericytoma (HPCy) in a domestic short-haired queen diagnosed based on physical examination, radiography, ultrasonography, histomorphology, and immunohistochemical reactivity. The results of pathology revealed a whorls around blood vessels and the "fingerprint" pattern of tumor with negative S-100 immunohistochemistry staining. In dogs, HPCy is a common neoplasm, but it is a seldom finding in cats. There is no previous report of HPCy in the abdominal cavity as observed in the presented case to the authors' knowledge.

Keywords

hemangiopericytoma, cat, immunohistochemistry, abdominal cavity, histomorphology

Abbreviations

HPCy: Hemangiopericytoma
DSH: Domestic short-haired
CBC: Cell blood count

Number of Figures: 2
Number of Tables: 2
Number of References: 16
Number of Pages: 6

Corresponding author: *Farzad Hayati*
Email: *hayati@um.ac.ir*
Tel: *+98 915 317 2177*

Introduction

Hemangiopericytoma (HPCy) is a common neoplasm in middle-aged or older dogs [1,2]. These tumors represent 7% of canine cutaneous tumors and are most often found in the lower extremities [3]. According to the literature, it is uncommon in cats [4]. It accounts for around 2.5 percent of all soft tissue sarcomas in humans and is classified as a malignant neoplasm [3]. HPCys [1,5] are a kind of soft tissue sarcoma that arises from vascular pericytes. HPCy is a locally infiltrative tumor that recurs following surgical excision on rare occasions, although it seldom metastasizes [4]. These tumors usually appear as solitary, well-circumscribed, lobulated, and pseudocapsulated masses of gray-white to red-brown color, occasionally reaching considerable dimensions [3,4]. The hallmark of this tumor microscopically is whorls around blood vessels and the "fingerprint" pattern formed by the neoplastic cells [2,4,6]. It should be differentiated from endothelioma, angiosarcoma, fibrosarcomas, leiomyosarcomas, schwannoma, peripheral nerve sheath tumor, and seldom dermatofibrosarcoma (a variant of malignant fibrous histiocytoma) and, maybe, glomus tumor [5,6]. The definitive diagnosis of HPCy is immunohistochemical findings [4]. This study describes a case report of hemangiopericytoma in a domestic short-haired (DSH) cat referred to a veterinary clinic, Iran.

Case Presentation

A two-year-old female DSH cat weighing 3.5 kg was presented to a veterinary hospital, Tehran, Iran, with clinical signs of lateral recumbency position, anorexia, and abdominal enlargement. The owner assumed she was pregnant because of her swollen abdomen. The queen was highly lethargic and dehydrated, with a pale mucous membrane, a rectal temperature of 37.3 °C, and no vaginal discharge during the first inspection. Cell blood counts (CBC) and biochemical profiles were determined using a blood sample (Table 1). An ultrasonographic examination showed a heterogeneous mass with a nearly distinct border. The mass was evaluated using Doppler, and power Doppler revealed significant vascularity in the mass (Figure 1). Medetomidine (0.02 mg/kg) was used to pre-anesthetize the patient. After injection of diazepam (0.2 mg/kg, IV) and ketamine hydrochloride (6 mg/kg, IV), the patient was intubated and positioned in a dorsal recumbency position. Anesthesia was maintained by isoflurane (1.5%) inhalation. After the aseptic preparation of the surgical region, the midline incised from the xiphoid to a midline point between the umbilicus and pubis. A large soft tissue mass was in the abdominal cavity and around the lumbar vertebral column, and the uterus and other abdominal organs' appearance were grossly normal. Due to a huge and encroaching tumor and the patient's owner's refusal to accept any further therapies, the queen was euthanized during an exploratory celiotomy. The mass was removed and shown to be white, multilobulated, and enormous, weighing around 900 grams (Figure 2A, 2B). The specimens of mass were fixed in 10 percent formalin for histological analysis. The tissue specimens were placed in ethanol (70% for 24 h, 90% for 1 h, and 100% for 1 h) for graded dehydration, then cleaned in xylene and embedded in paraffin. Sections were made at 5 µm thickness, stained with hematoxylin and eosin for the histopathological evaluation. Immunohistochemical analysis was performed to differentiate the tumor from the peripheral nerve sheath tumor and confirm the histopathological diagnosis. On formalin-fixed, paraffin-embedded tissues, immunohistochemistry expression of S-100 protein was performed, and the sections were processed using the avidin-biotin-peroxidase complex method. Histopathologically, the tissue sections showed that the tumor contained multiple layers of spindle-shaped cells arranged around a central thin-wall vessel, a fingerprint pattern, with the collagenous stroma. The neoplastic cells had homogenous eosinophilic cytoplasm with prominent nuclei. The mitotic figures were rare (Figure 2C, 2D). By the immunohistochemical examination, the tumor cells stained negative for S-100 protein (Figure 2E). Based on histopathological and immunohistochemical findings, an HPCy was diagnosed.

Results & Discussion

HPCy has been found in both dogs and men [5]. It is, however, infrequently documented in cats [3]. HPCy is most often observed in the legs and hindlimbs of both humans and dogs [6]. In cats, it is seen on the thoracic side and the left thigh, according to prior case reports [3,5]. In dogs, metastatic HPCy is unusual [6], with just 5 individuals having metastasized to the lungs, sublumbar lymph nodes, or chest [7-10], whereas in men, the metastatic rate ranges from 11.7 percent to 56.5 percent [6]. Dogs have a broad variety of recurrence rates (ranging from 26 to 60%) [6]. In a DSH cat, it was found in the belly cavity and surrounding the lumbar spinal column.

In our study, the preoperative findings were in terms of the physical examination, ultrasonography, CBC, and serum biochemical profile (Table 1). Mild normocytic normochromic- non-regenerative anemia (because of mild anemia: HCT 31, MCV normal, normochromic: MCHC normal) besides low serum iron

Table 1.
Measurement of CBC and serum biochemicals in the patient.

Hematology	Value	Unit	Reference Range
HCT	31	%	37-55
Hb	102	g/l	120-180
RBC	4.77	$\times 10^{12}$cell/l	5.5-8.5
MCV	65	fl	60-72
MCH	25	Pg	21-26
MCHC	330	g/l	310-360
NRBC	0	cell/100 wbc	0-1
RDW	13.8	%	10.6-14.3
Reticulocyte	0.7	%	1: Non regenerative 1-4: weak regenerative 5-20: moderate regenerative 21-50: strong regenerative
PLT	750	$\times 10^9$ cell/l	150-500
WBC	18.6	$\times 10^9$ cell/l	4.5-17
Neutrophil	12.7	$\times 10^9$ cell/l	3-11.5
Lymphocyte	3.35	$\times 10^9$ cell/l	1-4.8
Monocyte	1.8	$\times 10^9$ cell/l	0-1.3
Eosinophil	0.3	$\times 10^9$ cell/l	0.1-1.2
Basophil	0	$\times 10^9$ cell/l	0-0.1
Band Neutrophil	0.45	$\times 10^9$ cell/l	<0.3
Total Protein	94	g/l	54-74
Albumin	37	g/l	27-45
Globulin	57	g/l	19-37
BUN	310	mg/l	70-280
Creatinine	12	mg/l	3-13
ALP	267	IU/l	10-140
ALT	55	IU/l	17-95
AST	39	IU/l	5-65
GGT	2	IU/l	0-8
Total Bilirubin	1	mg/l	0-4
Glucose	1410	mg/l	750-1300
Triglyceride	830	mg/l	290-1200
Cholesterol	1970	mg/l	1300-3700
Na	151	mEq/l	145-150
K	4.1	mEq/l	3.9-5.5
Cl	109	mEq/l	106-127
Corrected Chloride	112	mEq/l	107-113
Total Ca	119	mg/l	90-115
Ionized Ca	62	mg/l	50-58
P	37	mg/l	28-61
Serum Iron	690	µg/l	970-2630
TIBC	2970	µg/l	2800-4890
Transferrin Saturation	23.23	%	27-66
ACTH Stimulation Test			
Basal cortisol	50	µg/l	20-60
1 Hour after Injection	170	µg/l	60-180

concentration and low TIBC indicates anemia due to chronic disease or acute inflammation. Neutrophilic leukocytosis with monocytosis and left shift indicates chronic active inflammatory response which is anticipated from the case presentation with inflammatory/neoplastic reaction. Hyperproteinemia regarding the hyperglobulinemia and normal serum albumin concentration is compatible with active antigenic stimulation by neoplastic tissue. Differential diagnoses of hypercalcemia and normophosphatemia (increased total and ionized serum Ca2+ concentration alongside normal serum phosphorus concentration), regarding the case history, were hypoadrenocorticism and focal osteolytic lesions [11]. Hypoadrenocorticism was ruled out by an ACTH stimulation test and a normal Na/K concentration. As a result, hypercalcemia and normophosphatemia are thought to be associated with localized spinal bone osteolysis caused by tumor invasion. The liver and renal functions were found to be normal. The tumor's intra-abdominal or bone metastatic pathology may cause a moderate (less than 3 times) rise in serum ALP activity. Thrombocytosis may occur because of inflammation.

Based on these findings, the abdominal tumor's presumptive diagnosis was made, but we performed the histopathological and immunohistochemical studies for the definitive diagnosis. Microscopically, whorls around blood vessels and the "fingerprint" pattern have been identified as a feature of this tumor [2, 4, 6]. The tissue slices in this research had a hypercellular pattern that looked like a fingerprint, which is a common occurrence. In canines and men, HPCy histopathological categorization is a valuable diagnostic technique for predicting prognosis, recurrence, and metastatic rates. Based on tissue differentiation, mitotic count, and necrosis quantification, this categorization might range from grade I to III (poor diagnostic) [1, 12]. The tumor showed features consistent with grade II in this case. As it is mentioned previously, immunohistochemistry is useful in ruling out other spindle cell tumors, which present the same whorl pattern [4, 6]. The neoplastic cells stained positively for vimentin, anti-muscle actin, and laminin and negative for S-100 protein, factor VIII-related antigen, lysozyme, and UEA I lectin [2, 4, 5, 6, 13]. S-100 is detected in 100% of schwannomas, 100% of neurofibromas (weaker than schwannomas), 50% of malignant peripheral nerve sheath tumors (may be weak and/or localized), paraganglioma stromal cells, histiocytoma, and clear-cell sarcomas [14]. The absence of immunoreactivity against S-100 helped rule out a diagnosis of other soft tissue sarcomas.

Treatment options for HPCy include surgery and radiation. Chemotherapy and other systemic therapies have had mixed results [15]. Without radiation, the canine HPCy recurrence rate was found to be 26 percent [9] and 38 percent [16]. Meantime to recurrence with or without radiotherapy after surgery 27 and 16 months, respectively. Maruo et al. suggested that conservative resection and intraoperative radiotherapy may control local tumors (e.g HPCy) without an adequate margin [1]. Baldi et al. used electrochemotherapy for the treatment of thoracic HPCy in a cat. Electrochemotherapy is a new treatment for tumors [5].

In conclusion, a hemangiopericytoma was diagnosed based on the physical examination, radiography, ultrasonography, histomorphology, and immunohistochemical reactivity in the cat. To the authors' knowledge, this is the third reported hemangiopericytoma and the largest among them in cats. This tumor is rare in cats; therefore, the diagnosis of HPCy in cats needs more information.

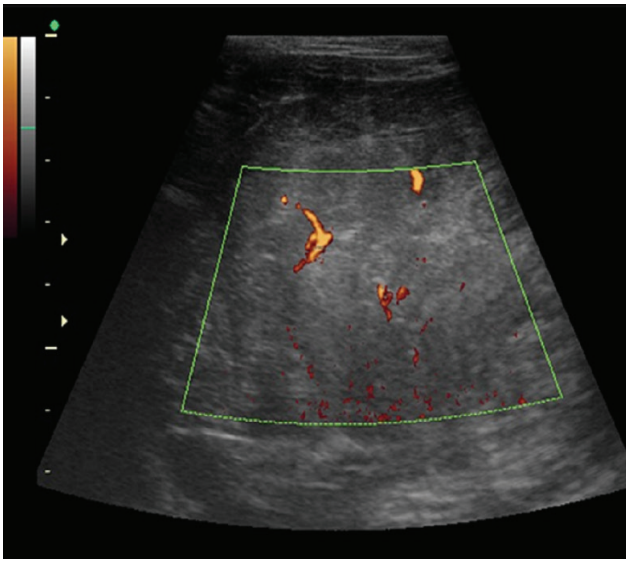


Figure 1.
Ultrasonography of the mass.

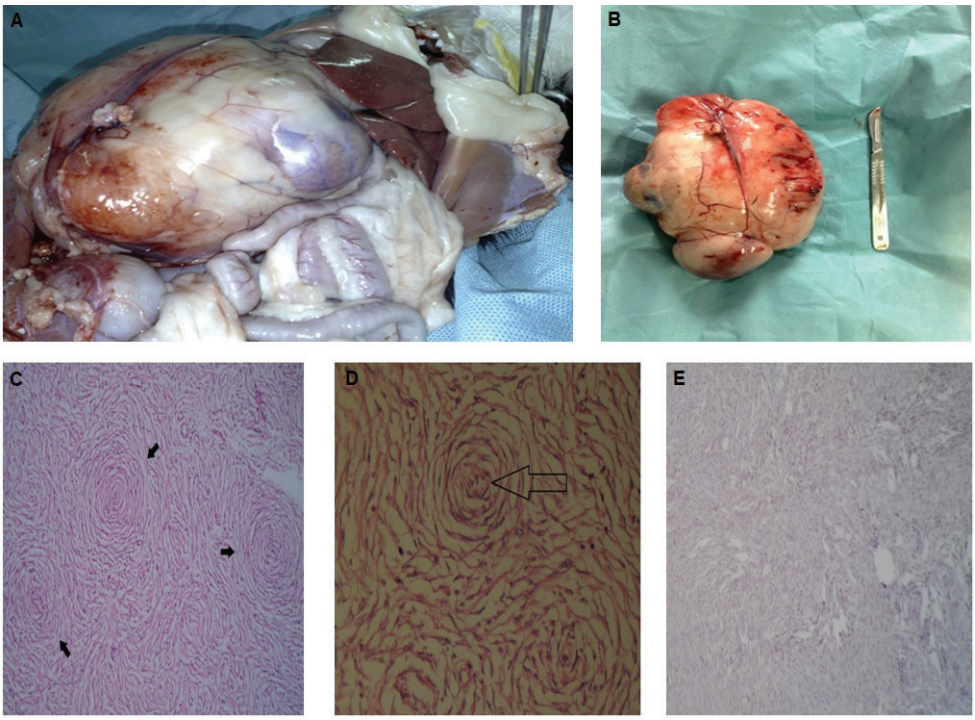


Figure 2.
A) The large mass in the abdominal cavity close to kidney. B) The excised mass. C&D) Spindle shaped cells are arranged around a central capillary, "fingerprint pattern", in the collagenous stroma, H&E staining. E) Negative immunolabelling with S-100.

Authors' Contributions

ESJ and HK performed review literature and manuscript writing. MM and FN performed the pathological analysis MH examined the animal and performed review literature. FH performed surgery, review literature, and manuscript writing.

Acknowledgements

We thank Dr. Saeed Ahrari for his diagnostic comments.

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Takuya M, Shida T, Fukuyama Y, Hino Y, Yamada T, Madarame H. Evaluation of intraoperative radiation therapy for incompletely resected or recurrent canine hemangiopericytoma: seventeen cases. Int J Appl Res Vet M 10. 2012; 132-136.

2. Handharyani E, Ochiai K, Kadosawa T, Kimura T, Umemura T. Canine hemangiopericytoma: an evaluation of metastatic potential. J Vet Diagn Invest. 1999;11(5):474-8.

3. Blutke A, Knebel J, Brühschwein A, Breuer W, Hermanns W. Hemangiopericytoma in a cat: a case report. Vet Med-Czech. 2012;57(5).

4. Silva EOd, Romero F, Green KT, Martins MIM, Bracarense APFRL. Hemangiopericytoma in a female dog with direct invasion of abdominal cavity and pulmonary metastasis. Cienc Rural. 2014;44(2):358-61.

5. Baldi A, Spugnini EP. Thoracic haemangiopericytoma in a cat. Vet Rec. 2006;159(18):598-600.

6. Perez J, Bautista MJ, Rollon E, Lara FCn-Md, Carrasco L, Mulas JMndl. Immunohistochemical characterization of hemangiopericytomas and other spindle cell tumors in the dog. Vet Pathol. 1996;33(4):391-7.

7. Richardson RC, Render JA, Rudd RG, Shupe RE, Carlton WW. Metastatic canine hemangiopericytoma. Javma-J Am Vet Med A. 1983;182(7):705-6.

8. Pantekoek J, Schiefer B. Metastasising canine fibrosarcoma originally diagnosed as haemangiopericytoma. J Small Anim Pract. 1975;16(12):259-65.

9. Postorino NC. Prognostic variables for canine hemangiopericytoma: 50 cases (1974-1984). J Am Anim Hosp Assoc. 1988;24:501-9.

10. Bostock DE, Dye MT. Prognosis after surgical excision of canine fibrous connective tissue sarcomas. Vet Pathol. 1980;17(5):581-8.

11. Schenck PA, Chew DJ, Nagode LA, Rosol TJ. Disorders of calcium: hypercalcemia and hypocalcemia. Fluid, electrolyte, and acid-base disorders in small animal practice. 2006;4:120-94.

12. Trojani M, Contesso G, Coindre JM, Rouesse J, Bui NB, De Mascarel A, et al. Soft tissue sarcomas of adults; study of pathological prognostic variables and definition of a histopathological grading system. Int J Cancer. 1984;33(1):37-42.

13. Gross TL, Ihrke PJ, Walder EJ, Affolter VK. Mesenchymal neoplasms and other tumors. Skin diseases of the dog and cat Clinical and Histopathologic Diagnosis 2nd ed, Blackwell Science, Oxford. 2005:722-7.

14. Nonaka D, Chiriboga L, Rubin BP. Differential expression of S100 protein subtypes in malignant melanoma, and benign and malignant peripheral nerve sheath tumors. J Cutan Pathol. 2008 Nov;35(11):1014-9.

15. Allen H. Hemangiopericytoma in the dog. 2003.

16. Graves GM, Bjorling DE, Mahaffey E. Canine hemangiopericytoma: 23 cases (1967-1984). Javma-J Am Vet Med A. 1988;192(1):99-102.

How to cite this article

Hayati F, Sajadian Jaghargh E, Maleki M, Hassankhani M, Khoribabalhakam H, Namazi F. Rare case of hemangiopericytoma in a domestic short-haired cat. Iran J Vet Sci Technol. 2021;13(2):106- 111.
URL: https://ijvst.um.ac.ir/article_41469.html

Iranian Journal of Veterinary Science and Technology

Received: 2021-
Accepted after revision: 2021-
Published online: 2022-

Abstracts (in Persian)

ارزیابی فعالیت ضد باکتریایی cLF–کایمرا و پتانسیل هم افزایی آن با ونکومایسین در برابر استافیلوکوکوس اورئوس مقاوم به متی سیلین

سحر روشنک[1]، فخری شهیدی[1*]، عاطفه سرادقی توپکانلو[1]، زانا پیرخضرانیان[2]، محمد هادی سخاوتی[2]

۱گروه علوم و صنایع غذایی، دانشکده کشاورزی، دانشگاه فردوسی، مشهد، ایران.
۲گروه علوم دامی، دانشکده کشاورزی، دانشگاه فردوسی، مشهد، ایران.

چکیده

مصرف بی رویه آنتی بیوتیک ها باعث شیوع میکروارگانیسم های مقاوم به آنتی بیوتیک شده است. بنابراین نیاز مبرم به کشف عوامل ضدباکتریایی جدید یا ترکیبی از عوامل موجود، به عنوان یک راهکار درمانی ایمن برای مبارزه با عفونت های مختلف، وجود دارد. در مطالعه حاضر، اثر هم افزایی cLF-کایمرا که یک پپتید ضد میکروبی (AMPs) است و آنتی بیوتیک ونکومایسین با استفاده از روش چک برد علیه استافیلوکوکوس اورئوس مقاوم به متی سیلین (MRSA) مورد بررسی قرار گرفت. cLF-کایمرا دارای اثر ضد میکروبی بر MRSA و استافیلوکوکوس اورئوس حساس به متی سیلین (MSSA) بود (MIC به دست آمده به ترتیب ۵۱۲ و ۲۵۶ میکروگرم بر میلی لیتر بود). ترکیب cLF-کایمرا با ونکومایسین دارای اثر هم افزایی (FIC =۳۷۵/۰) بود. نتایج نشان داد که در غلظت FIC ، خروج مواد از سیتوپلاسمی از سلول های باکتریایی و تعداد سلول های زنده مانده، به ترتیب به طور قابل توجهی بیشتر و کمتر از زمانی است که از پپتیدها یا آنتی بیوتیک ها به تنهایی استفاده شد. تجزیه و تحلیل تصاویرمیکروسکوپی الکترونی در غلظت FIC ، آسیب شدید غشای سلول های باکتریایی را نشان داد. در نتیجه ، استفاده از cLF-کایمرا و ونکومایسین در این غلظت مصرف هر دو ماده را کاهش می دهد.

واژگان کلیدی

اثر هم افزایی، پپتیدهای ضد میکروبی، آنتی بیوتیک ها، باکتری های مقاوم

* نویسنده مسئول: فخری شهیدی
mohri@um.ac.ir

Iranian Journal of Veterinary Science and Technology

Received: 2021- Apr- 28
Accepted after revision: 2021- Jul- 02
Published online: 2021-Dec- 01

Abstracts (in Persian)

تاثیر کاهش دهندۀ تروگزروتین بر سطوح سیتوکین‌های التهابی در موش‌های صحرایی مبتلا به سپسیس ایجاد شده با سوراخ کردن تجربی کورروده

علی شاکر[1]، سیامک کاظمی درآبادی[1]*، یاسر جعفری خطایلو [2]

۱گروه علوم درمانگاهی، دانشکده دامپزشکی دانشگاه تبریز، تبریز، ایران.
۲گروه پاتوبیولوژی، دانشکده دامپزشکی دانشگاه تبریز، تبریز، ایران.

چکیده

سپسیس عامل اصلی مرگ و میر در بیمارانی است که تحت عمل جراحی قرار می‌گیرند و درمان آن در حال حاضر شامل احیای قلبی و کاهش دادن خطرات زودهنگام عفونت است. تروگزروتین ترکیبی معمول در سبزیجات، میوه‌ها و دانه‌ها است که برخی فعالیت‌های زیستی مانند اثرات ضد پلاکتی، ضد سروتونینی، آنتی اکسیدانی و ضد التهابی دارد. به همین دلیل فرض را بر این قرار دادیم که این ماده می‌تواند سطوح اینترلوکین ۱ و فاکتور نکروز کننده توموری آلفا را در سرم موش‌های صحرایی مبتلا به سپسیس کاهش دهد. در این پژوهش از بیست و چهار موش صحرایی نر و بالغ نژاد اسپاراگ-داولی استفاده شد. موش‌های صحرایی به طور مساوی و تصادفی به سه گروه تقسیم شدند: گروه بدل، گروه کنترل و گروه تیمار. گروه‌های کنترل و تیمار تحت جراحی لیگاتور و سوراخ کردن کورروده قرار گرفتند. به حیوانات گروه تیمار تروگزروتین (۱۳۰ میلی‌گرم/کیلوگرم) به صورت زیرجلدی و دو بار در روز به مدت ۳ روز یا تا زمان مرگ حیوان تزریق شد. موش‌های صحرایی نجات یافته سه روز پس از لیگاتور و سوراخ کردن کورروده و پس از گرفتن ۱/۵ میلی‌لیتر خون آسان کشی شدند. سطوح سرمی اینترلوکین ۱ و فاکتور نکروز کننده توموری آلفا با روش الایزا اندازه گیری شد. تفاوت میزان مرگ و میر میان گروه کنترل و دو گروه دیگر معنادار بود. نتایج افزایش معناداری در اینترلوکین ۱ و فاکتور نکروز کننده توموری آلفا در گروه کنترل در مقایسه با گروه بدل نشان داد. افزون بر آن، سطوح سرمی آن‌ها در گروه تیمار در مقایسه با گروه کنترل به طور معناداری کاهش یافته بود. به عنوان نتیجه گیری می‌توان گفت که یافته‌های ما نشان دادند که تروگزروتین در موش‌های صحرایی مبتلا شده به شوک سپتیک می‌تواند با کاهش دادن سیتوکین‌های التهابی مانند اینترلوکین ۱ و فاکتور نکروز کننده توموری آلفا زنده مانی بیمار را افزایش دهد.

واژگان کلیدی

پاسخ ایمنی؛ حیوانات آزمایشگاهی؛ پریتونیت؛ شوک سپتیک؛ تروگزروتین

* نویسنده مسئول: سیامک کاظمی درآبادی
s.kazemi@tabrizu.ac.ir

Iranian Journal of Veterinary Science and Technology

Received: 2021- Jul- 06
Accepted after revision: 2021- Aug- 10
Published online: 2021-Dec- 01

Abstracts (in Persian)

یافته های انگل شناسی و آسیب شناسی کوکسیدیوز ناشی از آلودگی تجربی با آیمریا آهساتا در بره ها

نادر احمدی صالح بابری[1]، ایرج کریمی[2]، حسین نورانی[3]، حمید رضا عزیزی[2]، غلامرضا رزمی[3]*

۱دانش آموخته دکتری انگل شناسی دامپزشکی، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران.
۲گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه شهرکرد، شهرکرد، ایران.
۳گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران.

چکیده

این مطالعه به منظور روند بیماری زایی آیمریا آهساتا طی دوره ۴۲ روزه در بره ها با تاکید بر بررسی مورفولوژی پاتولوژی و انتشارجراحات در بافت های آلوده انجام شد. دوازده بره به تعداد مساوی در دو گروه مورد و کنترل پس از تایید سلامتی انها تقسیم شدند. بره های گروه مورد با تعداد اووسیست هاگدار شده ۱×۱۰^{۵} آیمریا آهساتا آلوده شدند. هفت روز پس از آلودگی میزان اووسیست در هر گرم مدفوع (OPG) در هر بره آلوده تعیین گردید. در روز های ۷، ۱۴،۲۱، ۲۸ ، ۳۵و ۴۲ پس از آلودگی یک بره از هر گروه کالبدگشایی شدند . ضایعات ماکروسکوپی در شیردان روده کوچک و بزرگ، عقد های مزانتریک، طحال و کبد مورد بررسی قرار گرفتند . از روز ۱۴ تا ۲۱ضایعات ماکروسکپی ملایم تا شدید شامل پرخونی و ادم در مخاط روده کوچک بهمراه کانون های سفید و کوچک به قطر یک تا دو میلی متر بویژه در ایلیوم وژوژنوم مشاهده گردید. از روز هفتم تا انتهای مطالعه مراحل مختلف زندگی انگل با انتشار سلول های اماسی، هیپرپلازی سلول های اپیتلیال و همچنین تخریب این سلول دیده شد. نتایج بدست آمده نشان دهنده بیماری زایی آیمریا اهساتا در بره ها بودند و همچنین بیشتر ضایعات ماکروسکوپی و میکروسکوپی این تک یاخته در ژوژنوم دیده شدند.

واژگان کلیدی

آیمریا آهساتا، گوسفند، پاتولوژی، تعداد اووسیست

* نویسنده مسئول: غلامرضا رزمی
razmi@um.ac.ir

Iranian Journal of Veterinary Science and Technology

Received: 2021- May-30
Accepted after revision: 2021- Aug- 28
Published online: 2021- Dec- 11

Abstracts (in Persian)

ارزیابی برخی سایتوکاین های التهابی و فاکتورهای ایمنولوژیک در گاوهای شیری مبتلا به کتوز تحت بالینی

نگار کریمی[1]، حسام الدین سیفی[1،2]، محمد حیدرپور[1،2]*

۱ گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران.
۲ قطب علمی سقط جنین و مرگ و میر نوزاد دامهای نشخوارکننده، دانشکده دامپزشکی،دانشگاه فردوسی مشهد، مشهد، ایران.

چکیده

تغییر در پروفایل سیتوکینی و تضعیف سیستم ایمنی و همچنین کتوز تحت بالینی، از چالش های قابل ملاحظه در حوالی زایش هستند. بنابراین، هدف مطالعه حاضر مقایسه برخی سیتوکین های التهابی و فاکتورهای ایمونولوژیک بین دو گروه گاوهای سالم و مبتلا به کتوز تحت بالینی است. نمونه های سرم از ۳۰ گاو شیری که از نظر بالینی سالم بودند در ابتدای دوره خشکی، یک هفته قبل از زمان زایش مورد انتظار و یک هفته پس از زایش گرفته شدند. دام ها به دو گروه سالم (N=۲۰) و کتوز تحت بالینی (N=۱۰) بر اساس نقطه تمیز بتا-هیدروکسی بوتیرات سرم ۱/۲ mmol/L در یک هفته بعد از زایش تقسیم شدند. غلظت های ایمونوگلوبولین، اینترلوکین۴-، اینترلوکین۱۰-، اینترفرون-گاما، فاکتور نکروز تومور-آلفا، و هاپتوگلوبین با الایزا اندازه گیری شدند. آنالیز آماری با نرم افزار و با استفاده از آزمون های آنالیز واریانس مختلط و مستقل انجام شد. نتایج این مطالعه نشان داد که سطوح کلی اینترلوکین۴- (p=۰/۰۳۳)، اینترلوکین۱۰- (p=۰/۰۴۹)، فاکتور نکروز تومور-آلفا (p=۰/۰۲۸)، و هاپتوگلوبین (p=۰/۰۱۸) به طور معنی داری در گروه کتوز تحت بالینی از گروه کنترل بالاتر بود. علاوه بر این، اثر زمان × کتوز تحت بالینی بر میزان اینترلوکین۴- (p =۰/۰۲۸) و هاپتوگلوبین (p =۰/۰۲۲) معنی دار بود. همچنین مشخص شد که اینترلوکین۴- (p =۰/۰۰۸)، اینترلوکین۱۰- (p =۰/۰۰۹)، فاکتور نکروز تومور-آلفا (p =۰/۰۱)، و هاپتوگلوبین (p =۰/۰۰۲) همگی در زمان یک هفته بعد از زایش در گروه کتوز تحت بالینی به میزان معنی داری بالاتر از گروه کنترل بودند. می توان نتیجه گرفت که رخداد کتوز تحت بالینی در گاوهای شیری می تواند با تغییرات ایمونولوژیک و التهابی در حوالی زایش ارتباط داشته باشد.

واژگان کلیدی

فاکتورهای ایمونولوژیک؛ التهاب؛ کتوز؛ کتوز تحت بالینی

* نویسنده مسئول: محمد حیدرپور
heidarpour@um.ac.ir

Iranian Journal of Veterinary Science and Technology

Received: 2021- May- 28
Accepted after revision: 2021- Sep- 11
Published online: 2021-Dec- 18

Abstracts (in Persian)

شواهد تقابل سیستم های کانابینوئیدرژیک و دوپامینرژیک با گیرنده های MC3/ MC4 ملانوکورتینی در تنظیم اخذ غذای در جوجه های نوزاد

محمد بامری[۱]، مرتضی زنده دل*[۲]، بیتا وزیر[۱]، احمد اصغری[۳]، نگار پناهی[۱]

۱گروه علوم پایه، دانشکده دامپزشکی، دانشگاه آزاد اسلامی واحد علوم و تحقیقات تهران، تهران، ایران.
۲گروه علوم پایه، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران.
۳گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه آزاد اسلامی واحد علوم و تحقیقات تهران، تهران، ایران.

چکیده

مطالعه حاضر با هدف بررسی نحوه تأثیر مکانیسم های مرکزی دوپامینرژیک و کانابینوئیدرژیک بر مصرف غذا ناشی از ملانوکورتین در جوجه های نوزاد انجام شد. به همین منظور۹ آزمایش طراحی شد. در آزمایش ۱، جوجه ها با سرم فیزیولوژی و (۲/۵ ، ۵ و ۱۰ نانومول) بصورت داخل بطن- مغزی تزریق شدند. در آزمایش ۲، جوجه ها با سرم فیزیولوژی، (۱۰ نانومول)، ال- دوپا (۱۲۵ نانومول) و تزریق همزمانMTII و ال- دوپا تزریق شدند. آزمایشات مراحل ۳-۹ مشابه آزمایش شماره ۲ بود، اما جوجه ها با ۶ -هیدروکسی دوپامین (۱۵۰ نانومول)، SCH23390 (۵ نانومول)، AMI-193 (۵ نانومول)، NGB2904 (۶/۴ نانومول)،) L-741,742 (۶ نانومول)، SR141716A (۶/۲۵ میکروگرم) وAM630 (۵ میکروگرم) بجای ال- دوپا تزریق شدند. سپس مصرف تجمعی غذا تا ۱۲۰ دقیقه بعد از تزریق اندازه گیری شد. با توجه به نتایج بدست آمده، تزریق MTII (۲/۵، ۵ و ۱۰ نانومول) بطور وابسته به دوز، موجب کاهش مصرف غذا در جوجه های نژاد تخمگذار تا ۱۲۰ دقیقه پس از تزریق در مقایسه با گروه کنترل شد($p < ۰.۰۵$). تزریق ال- دوپا و SR141716A موجب تقویت اثرات هیپوفاژیک ناشی از MTII شد ($p <۰.۰۵$)، در حالیکه تزریق ۶ -هیدروکسی دوپامین موجب مهار اثرات هیپوفاژیک ناشی از MTI شد($p <۰.۰۵$). علاوه بر این، تزریق SCH23390 و AMI-193 موجب تضعیف اثرات هیپوفاژیک ناشی از MTII در جوجه ها شد ($p <۰.۰۵$)، اما L-741,742، NGB2904 و AM630 اثری بر هیپوفاژی ناشی از MTII نداشت ($p >۰.۰۵$). این نتایج نشان داد هیپوفاژی ناشی از MTII احتمالا از طریق گیرنده های D_2، D_1 و CB_1 در جوجه های نوزاد تخمگذار میانجی گری می شود.

واژگان کلیدی

دوپامین؛کانابینوئید؛ ملانوکورتین؛ اخذ غذا؛ جوجه تخمگذار

* نویسنده مسئول: مرتضی زنده دل

zendedel@ut.ac.ir

Iranian Journal of Veterinary Science and Technology

Abstracts (in Persian)

Received: 2021- Jun- 20
Accepted after revision: 2021- Oct- 24
Published online: 2021-Dec- 20

تشخیص مولکولی و آنالیز فیلوژنتیکی کک پولکس ایریتانس در مناطق مختلف ایران

شهین صیدی، موسی توسلی*، فرناز ملکی فرد

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

چکیده

کک انسان به عنوان یک انگل خارجی باعث ایجاد مشکلات بهداشتی جدی در سطح جهانی می شود. این مطالعه به منظور بررسی تنوع مولکولی پولکس ایریتانس (کک انسان) بر اساس ژنوم میتوکندریایی و هسته ایی در چهار منطقه مختلف ایران انجام شد. در این بررسی در مجموع ۱۰۱۹ کک پولکس ایریتانس جمع آوری گردید و تحت آنالیز مرفومتریک و مولکولی قرار گرفت. پس از استخراج DNAبا استفاده از پرایمرهای اختصاصی در توالی های ITS2، ITS1 و COX1 واکنشPCR انجام شد و ۳۴ نمونه محصولPCR تعیین توالی گردید. تنوع درون گونه ایی در کک پولکس ایریتانس بر اساس نشارکر میتوکندریایی (COX1) ۰/۱۵ درصد ارزیابی گردید. همردیف سازی توالی نوکلئوتیدی COX1 در مناطق مختلف تنها یک جابه جایی در اسید نوکلئوتید شماره ۱۶۲ را نشان می دهد که در ایزوله همدان اسید نوکلوتید آدنین به جای اسید نوکلئوتید گوانین قرار می گیرد. براساس نشانگر هسته ای (ITS1) تنوع درون گونه در ده جمعیت ۰/۴۱ درصد دیده شد. توالی ITS1 دارای سه واحد تکراری و متوالی به طول 99bp بوده که از اسید نوکلئوتید های شماره ۱۴۶، ۲۴۶ و ۳۳۲ شروع شدند. براساس تعیین توالی نوکلئوتیدی ITS2 هیچ تنوع مولکولی در ده جمعیت مورد مطالعه وجود نداشت. نتایج تجزیه و تحلیل ژنوم هسته ایی و میتوکندریایی نشان داد که این قطعات برای نشان دادن تنوع درون گونه ایی، تمایز در سطح گونه و جنس پولکس ایریتانس مفید هستند.

واژگان کلیدی

کک، ایران؛ نشانگر رونویسی شده داخلی I؛ نشانگر رونویسی شده داخلی II؛ سیتوکروم اکسیداز I؛ پولکس ایریتانس

* نویسنده مسئول: موسی توسلی

mtavassoli@urmia.ac.ir

Iranian Journal of Veterinary Science and Technology

Abstracts (in Persian)

Received: 2021- Apr- 09
Accepted after revision: 2021- Oct- 19
Published online: 2021-Dec- 30

ناحیه قاعده ای جانبی آمیگدال نقش مهمی در حافظه مرتبط با نشانه های بصری و اثرات پس از مصرف ترجیح غذایی در موش های صحرایی دارد

مهنار زامیاد[1]، مهدی عباس نژاد[1]*، سعید اسماعیلی ماهانی[1]، وحید شیبانی[2]، مریم رئوف[3]

۱ گروه زیست شناسی، دانشکده علوم، دانشگاه شهید باهنر کرمان، کرمان، ایران.
۲ گروه فیزیولوژی، مرکز علوم تحقیقات کرمان، دانشکده علوم پزشکی کرمان، کرمان، ایران.
۳ گروه درد دهانی صورتی، آکادمی مرکزی دندانپزشکی آمستردام، دانشگاه آمستردام، آمستردام، هلند.

چکیده

هسته قاعده ای جانبی آمیگدال (BLA) نقش مهمی در رفتار یادگیری مرتبط با غذا دارد. با رویکردی جدید، به ارزیابی نقش BLA در ترجیح غذایی و حافظه مرتبط با نشانه های بصری مربوط به غذا پرداختیم. ۳۲ سر موش صحرایی نر بالغ نژاد ویستار با وزن ۲۰۰-۲۵۰ گرم مورد استفاده قرار گرفت. تخریب الکتریکی BLA با عبور جریان ۱/۵ میلی آمپر به مدت ۷ ثانیه انجام شد. رفتارهای مرتبط با غذا و ترجیح غذایی با استفاده از دستگاه خودکار ارزیابی گشت. نشانه های بصری هندسی نیز ساخته شد. موش های صحرایی محروم از غذا در ۶ مرحله متوالی با وعده های غذایی متفاوت (گندم، گندم + شکر، آرد سفید و بیسکوییت) مواجه شدند. تعداد دفعات مراجعه، مدت زمان سپری شده در هر ناحیه و درگاه، مسافت پیموده شده در هر مراجعه و میزان کل غذای مصرفی مورد ارزیابی قرار گرفت. تغییرات بیان c-Fos در هیپوکامپ نیز با استفاده از روش وسترن بلات تعیین گشت. گروه کنترل و شم، بیشترین و کمترین ترجیح را به ترتیب به بیسکوییت و آرد سفید نشان دادند. موش های دارای ضایعه BLA منحنی ترجیحی تغییر یافته ای را به نمایش گذاشتند. در گروه شم، افزایش میزان مصرف غذا با افزایش تعداد دفعات مراجعه به هر ناحیه و درگاه و افزایش مدت زمان سپری شده در ارتباط بود. علاوه بر این، کاهش بیان c-Fos در هیپوکامپ در گروه ضایعه BLA مشاهده شد. روی هم رفته، هسته قاعده ای جانبی آمیگدال نقش مهمی در حافظه مرتبط با نشانه های بصری غذا و ترجیح غذاهای پرکالری و شیرین دارد.

واژگان کلیدی

ترجیح غذایی؛ حافظه و یادگیری؛ نشانه های بصری؛ هسته قاعده ای جانبی آمیگدال؛ تخریب؛ موش های صحرایی؛ c-Fos

* نویسنده مسئول: مهدی عباس نژاد
mabbas@uk.ac.ir

Iranian Journal of Veterinary Science and Technology

Received: 2021- Oct- 06
Accepted after revision: 2021- Nov- 16
Published online: 2021- Dec- 30

Abstracts (in Persian)

شناسایی آپتامرهای تک رشته از جنس DNA که به طور اختصاصی به پلاکت ها متصل میشوند با استفاده از تکنیک Cell-SELEX

فاطمه عالمی[1]، مجتبی سنگیان[2]، علیرضا حق پرست[1]، محمدرضا باسامی[1،3]، غلامرضا هاشمی تبار*[1،3]

۱ گروه بیوتکنولوژی، دانشگاه فردوسی مشهد، مشهد، ایران.
۲ مرکز تحقیقات ایمونولوژی، دانشکده پزشکی، دانشگاه علوم پزشکی مشهد، مشهد، ایران.
۳ گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران.

چکیده

آپتامرها اولیگونوکلئوتیدهایی هستند که به آسانی تولید میشوند و با گرایش و اختصاصیت فوق العاده به اهداف خود متصل میشوند. چندین آپتامر ویژه عوامل محلول آبشار انعقادی تولید شده اند اما آپتامرهای ویژه مولکول های سطحی پلاکت، تا به حال تولید نشده اند. هدف ما کشف آپتامرهایی از جنس DNA می‌باشد که می‌توانند به طور اختصاصی به پلاکت های انسانی متصل شوند. از روش cell-SELEX برای کشف آپتامرها استفاده گردید. اولیگونوکلئوتیدهای تک رشته سنتز شده با طول ۷۹ نوکلئوتید به عنوان مخزن اولیه آپتامر استفاده گردید. پلاکت های خالص از طریق حذف ناخالصی ها با استفاده از سانتریفیوژ افتراقی و دانه های مغناطیسی تهیه گردید. پرایمر فوروارد نشاندار شده با FITC برای تکثیر اولیگونوکلئوتیدهای انتخاب شده به روش PCR استفاده گردید و از آنزیم اگزونوکلئاز برای حذف رشته پیرو استفاده شد. پس از انجام ۱۲ مرحله cell-SELEX اولیگونوکلئوتیدهای انتخاب شده تکثیر شده و به وکتور مناسب کلون گردید و پس از تکثیر در باکتری مناسب توالی آنها تعیین گردید. توالی آپتامرهای حاصل از ۲۰۰ کلنی مثبت، هم تراز شده و وجود هفت گروه آپتامری شناسایی گردید. آپتامرهای خالص انتخاب شده از هر گروه، تکثیر شده و شدت و ویژگی گرایش آنها به هدف و نیز هضم پذیری مولکول های هدف آنها ارزیابی شد. تداخل این آپتامرها با دو تست عملکردی پلاکت ها نیز تحقیق شد. میل ترکیبی آپتامرهای شناسایی شده از ۱۰۹ تا ۳۴۰ نانو مولار بود. قرار گرفتن پلاکت ها در معرض تریپسین اتصال بعدی همه هفت آپتامر شاخص را به مولکول های هدف خود ملغا نمود. چهار تا از آپتامرها پس از اتصال به هدف، از مولکول هدف خود در مقابل آنزیم تریپسین محافظت نمودند. هیچ یک از هفت آپتامر تاثیر معنی داری بر نتایج تست های عملکردی پلاکت نداشتند. در این تحقیق هفت آپتامر ویژه پلاکت ها شناسایی و خصوصیات آنها تعیین شد. این آپتامرها ممکن است کاربردهای متنوعی در تشخیص و یا درمان اختلالات پلاکتی داشته باشند.

واژگان کلیدی

آپتامردی ان ای؛ آپتامر ویژه پلاکت؛ پلاکت؛ cell-SELEX

* نویسنده مسئول: غلامرضا هاشمی تبار
Hashemit@um.ac.ir

Iranian Journal of Veterinary Science and Technology

Received: 2021- Sep- 17
Accepted after revision: 2021- Dec- 18
Published online: 2021-Dec- 31

Abstracts (in Persian)

بررسی اثرات کشندگی زیتون تلخ (ملیا آزداراچ) روی کنه هیالوما اکسکاواتوم و تعیین میزان آزادیراکتین آن

سمیرا گیلوری [۱]، علی مشاوری نیا* [۱]، امیر مقدم جعفری [۲]، سعید یغفوری [۳]، مریم اکابری [۴]

۱گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، ایران.
۲گروه علوم پایه، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، ایران.
۳آزمایشگاه مرکزی اداره کل دامپزشکی استان هرمزگان، بندرعباس، ایران.
۴ دانشکده داروسازی، دانشگاه علوم پزشکی مشهد، ایران.

چکیده

هدف از مطالعه حاضر، ارزیابی اثر عصاره میوه رسیده زیتون تلخ (ملیا آزداراچ) روی نوزادان و ماده بالغ کنه هیالوما اکسکاواتوم در غلظت های ۰.۲۵، ۰.۰۵، ۱، ۲، و ۴ ٪ بود که با استفاده از روش غوطه وری نوزادهای کنه و ماده های بالغ انجام شد. همچنین میزان آزادیراکتین در این عصاره مورد بررسی قرار گرفت. میزان مرگ و میر نوزادان کنه ۲۴ ساعت پس از مواجهه با غلظت های ۱، ۲ و ۴ درصد نسبت به گروه کنترل تفاوت معنی داری نشان داد. در حالیکه میزان مرگ و میر نوزادان ۲۴ ساعت پس از مواجهه، در دامنه بین ۶/۸ تا ۶۶/۷۲ ٪ متغیر بود بعد از ۴۸ ساعت مرگ و میر کامل در غلظت ۴ ٪ حاصل شد. در تست غوطه وری ماده های بالغ، درصد ممانعت از تخمگذاری در گروه درمان نسبت به گروه کنترل به طور معنی داری بالاتر بود و بالاترین میزان آن ۱۷.۷۲ ٪ ثبت شد که در غلظت ۴ ٪ بدست آمد. تفاوت معنی داری بین گروه کنترل و درمان در سایر پارامترهای تولیدمثلی دیده نشد. آنالیز کروماتوگرافی مایع با عملکرد بالا میزان آزادیراکتین را ۱۹۴.۱۷ میکروگرم در میلی لیتر نشان داد. یافته های این مطالعه نشان می دهد عصاره میوه زیتون تلخ اثرات سمی و کشنده روی کنه هیالوما اکسکاواتوم در شرایط آزمایشگاهی دارد.

واژگان کلیدی

ملیا آزداراچ، هیالوما اکسکاواتوم، آزادیراکتین، کنه کش های گیاهی

* نویسنده مسئول:علی مشاورنیا
moshaverinia@um.ac.ir

Iranian Journal of Veterinary Science and Technology

Received: 2021- Feb- 21
Accepted after revision: 2021- Jun- 08
Published online: 2021- Dec- 31

Abstracts (in Persian)

اثرات بهبودی پودر دانه انار بر ساختار بیضه به‌دنبال مسمومیت با کادمیوم در بلدرچین ژاپنی (*Coturnix japonica*)، یک مطالعه استریولوژیکی و پراکسیداسیون لیپیدی

رحمت الله فتاحیان دهکردی*[1]، شهاب بهادران[2]، محمدرضا علیجانی[3]، عبدالناصر محبی[2]، حانیه محمدی[2]

۱گروه علوم پایه، دانشکده دامپزشکی، دانشگاه شهرکرد، شهرکرد ، ایران.
۲گروه علوم بالینی، دانشکده دامپزشکی، دانشگاه شهرکرد، شهرکرد، ایران.
۳ دانشجوی DVM، دانشکده دامپزشکی، دانشگاه شهرکرد، شهرکرد، ایران.

چکیده

هدف از این مطالعه بررسی اثرات پودر دانه انار بر بافت بیضه مسموم شده با کادمیوم در بلدرچین های ژاپنی بود. در مجموع ۲۷۰ جوجه بلدرچین ژاپنی در شش گروه قرار گرفتند؛ گروه کنترل، کادمیوم ۵۰ ppm (گروه II)، پودر دانه انار (۱ و ۰/۵درصد؛ گروه های III و IV)، پودر دانه انار۱درصد+ کادمیوم ۵۰ ppm (گروه V)، پودر دانه انار ۰/۵ درصد+ ۵۰ ppm (گروه VI). پارامترهای استریولوژیک، TBARS، سطح کلسترول و تری‌گلیسیرید در بیضه تعیین شد. اجزای بیضه کاهش قابل توجهی در مساحت سطح و چگالی حجم در گروه های در معرض کادمیوم در مقایسه با گروه کنترل نشان داد(p <۰/۵). مشخص گردید که در گروه القای کادمیوم، TBARS، سطح کلسترول و تری‌گلیسیرید در روز ۴۲ در مقایسه با سطح نرمال، به‌طور قابل توجهی بالاتر بود (p <۰/۵). نتایج نشان داد که پودر دانه انار می‌تواند مساحت سطح و تراکم حجم اجزای یاخته‌زای بیضه را افزایش داده و محتوای TBARS، کلسترول و تری‌گلیسیرید را در بیضه‌های مسموم شده با کادمیوم کاهش دهد (p <۰/۵). این نتایج نشان می دهد که کادمیوم اثرات مخربی بر پارامترهای استریولوژیک و پراکسیداسیون لیپید بر بیضه بلدرچین ژاپنی دارد.

واژگان کلیدی

بافت‌شناسی ،TBARS ،بلدرچین ژاپنی، پودر دانه انار، استریولوژی

* نویسنده مسئول: رحمت ا.. فتاحیان دهکردی
fatahian-r@sku.ac.ir

Iranian Journal of Veterinary Science and Technology

Received: 2021- Oct- 02
Accepted after revision: 2021- Dec- 12
Published online: 2021- Dec- 31

Abstracts (in Persian)

گزارش تومور نادر همانژیوپریسایتوما در محوطه شکمی گربه نژاد موکوتاه اهلی

فرزاد حیاتی[1*]، انسیه سجادیان جاغرق[1]، محسن ملکی[2]، مهدی حسنخانی[3]، حسین خوری باب الحکم[1]، فاطمه نمازی[4]

۱گروه علوم درمانگاهی، دانشکده دامپزشکی دانشگاه فردوسی، شهر مشهد، کشور ایران.
۲گروه پاتوبیولوژی، دانشکده دامپزشکی دانشگاه فردوسی، شهر مشهد، کشور ایران.
۳ گروه علوم درمانگاهی، دانشکده دامپزشکی دانشگاه تهران، شهر تهران، کشور ایران.
۴ گروه پاتوبیولوژی، دانشکده دامپزشکی دانشگاه شیراز، شهر شیراز، کشور ایران.

چکیده

گربه ی ماده دوساله با وضعیت به پهلو افتاده و مشکوک به سخت زایی به بخش جراحی بیمارستان دامپزشکی ارجاع شد. در معاینات اولیه حیوان به شدت ضعیف و کم آب، همراه با مخاطات کم رنگ و دمای مقعدی ۳۷.۳ درجه سانتیگراد و فاقد ترشحات واژنی بود. یک توده بزرگ در محوطه شکمی ملامسه گردید و جهت تشخیص تحت ارزیابی دقیق قرار گرفت. به دلیل وسعت تومور و درگیری زیاد در اطراف آن و امتناع صاحب آن از ادامه درمان، بیمار طی سیلیوتومی اکتشافی تحت مرگ با ترحم قرار گرفت. این گزارش به توصیف همانژیوپریسایتوما بزرگ در محوطه شکمی گربه ماده نژاد مو کوتاه اهلی بر اساس معاینه فیزیکی، رادیوگرافی و سونوگرافی، پروفایل خون و بیوشیمی، هیستومورفولوژی و ایمونوهیستوشیمی می پردازد. اگرچه این تومور در سگ شایع است ولی در گربه به ندرت گزارش شده است. بر اساس دانش نویسندگان، تا کنون گزارشی از همانژیوپریسایتوما در محوطه شکمی گربه ارائه نشده است.

واژگان کلیدی

گربه، همانژیوپریسایتوما، محوطه شکمی، ایمونوهیستوشیمی، هیستومورفولوژی

* نویسنده مسئول: فرزاد حیاتی
hayati@um.ac.ir

GUIDE FOR AUTHORS

IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

Guide for authors

SCOPE

Iranian journal of Veterinary Science and Technology (IJVST) publishes important research advances in veterinary medicine and subject areas relevant to veterinary medicine including anatomy, physiology, pharmacology, bacteriology, biochemistry, biotechnology, food hygiene, public health, immunology, molecular biology, parasitology, pathology, virology, large and small animal medicine, poultry diseases, diseases of equine species, and aquaculture. Articles can comprise research findings in basic sciences, as well as applied veterinary findings and experimental studies and their impact on diagnosis, treatment, and prevention of diseases. IJVST publishes four kinds of manuscripts: Research Article, Review Article, Short Communication, and Case Report.

Guide for Authors

GENERAL GUIDELINES

1. Submitted manuscripts should not be previously published elsewhere and should not be under consideration by any other journal.
2. The corresponding author should provide all co-authors with information regarding the manuscript, and obtain their approval before submitting any revisions.
3. The submitted manuscript should be accompanied by a written statement signed by the corresponding author on behalf of all the authors that its publication has been approved by all co-authors, stating that the whole manuscript or a part of it has not been published.
4. Ethics: Authors must state that the protocol for the research project has been approved by the Ethics Committee of the institution within which the work was undertaken. Authors are responsible for animal welfare and all statements made in their work.

OPEN ACCESS POLICY

Iranian Journal of Veterinary Science and Technology is a fully Open Access journal in which all the articles are available Open Access. There is no cost to the reader or author. All costs are covered by the Ferdowsi University of Mashhad Press.

COPYRIGHT

Copyright on any open access article in the Iranian Journal of Veterinary Science and Technology, published by Ferdowsi University of Mashhad Press is retained by the author(s).

- Authors grant Ferdowsi University of Mashhad Press a license to publish the article and identify itself as the original publisher.
- Authors also grant any third party the right to use the article freely as long as its integrity is maintained and its original authors, citation details, and publisher are identified.

The Creative Commons Attribution License 4.0 formalizes these and other terms and conditions of publishing articles. The Copyright assignment form can be downloaded from the IJVST website.

SUBMISSION

Authors should submit their manuscript in electronic format directly through the IJVST website (ijvst.um.ac.ir) along with a letter to the editor signed by the author to whom correspondence should be addressed. Please ensure that Email addresses are university/governmental addresses and full postal addresses are included on the title page of the manuscript. The following files and forms can be downloaded from the IJVST website:

Manuscript (template file can be downloaded from the IJVST website)
Title page (template file can be downloaded from the IJVST website)
Tables (template file can be downloaded from the IJVST website)
Endnote manuscript library file (Vancouver style can be downloaded from the IJVST website)

Copyright assignment form (can be downloaded from IJVST website)
Conflict of interest and author agreement form (can be downloaded from the IJVST website)

For further information, please contact the Editorial Office:
Iranian Journal of Veterinary Science and Technology
Email: ijvst@um.ac.ir;
Tel: +98 51 3880-3742

PREPARATION OF MANUSCRIPT

Manuscripts should be written in English, with Abstract in both English and Persian (where applicable), typewritten in MS Word program, double-spaced, in 12-point "Times New Roman" font on A4 paper size. Authors are requested to reserve margins of 2.5 cm all around the pages. Manuscript should also have line numbers. All pages of the manuscripts should also be enumerated.

Research Articles should contain Title page, Abstract, Keywords, List of Abbreviations, Introduction, Results, Discussion, Materials and methods, References, and Figure legends. Tables and figures should be appended as individual files.

Review Articles should contain Title page, Abstract, Keywords, List of Abbreviations, Introduction, appropriate sections dependeing to the subject, Conclusions and future directions. Tables and figures should be appended as individual files. The review article should provide an update on recent advances in a particular field. Authors wishing to submit review articles should contact the Editor with an outline of the proposed paper prior to submission.

Case Reports should include Title page, Abstract, Keywords, List of Abbreviations, Introduction, Case Presentation, Results and Discussion, and References. Case reports should not exceed 2000 words (excluding the references) and should include no more than two tables or figures. Tables and figures should be appended as individual files.

Short Communications should not exceed 2000 words (excluding the references) and include no more than two tables or figures. They should include Title page, Abstract, Keywords, List of Abbreviations, the text summarizing results with no other divisions, and References. Tables and figures should be appended as individual files.

Title Page

Full Title Page should include title (concise and informative), author(s) (including the complete name, department affiliation, and institution), running head (condensed title) (≤ 50 characters, including spaces), name and address of the authors to whom correspondence and reprint requests

should be addressed, Acknowledgements, Author contributions, and Conflict of interest.

Acknowledgements: Personal acknowledgement, sources of financial support, contributions and helps of other researchers and everything that does not justify authorship should be mentioned in this section, if required.

Author contributions: Authors are required to include a statement to specify the contributions of each author. The statement describes the tasks of individual authors referred to by their initials. Listed below is an example of author contributions statement:
Conceived and designed the experiments: HD, SS. Performed the experiments: SS. Analyzed the data: HD, SS, MMM, ARB. Research space and equipment: HD, MMM, ARB. Contributed reagents/materials/analysis tools: HD. wrote the paper: SS, HD.

Conflict of interest: All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. If there are no conflicts of interest then please state 'The authors declare that there is no conflict of interest'. This form can be downloaded from the IJVST website.

Abstract

Abstract (in English and Persian) no more than 250 words should contain the purpose of the study, findings and the conclusion made on the basis of the findings. Authors who are not native Persian speakers may submit their manuscript with an abstract in English only. Abbreviations and reference citations may not be used in the abstracts.

Keywords

For indexing purposes, each submitted manuscript should include three to seven keywords, following the abstract and preferably chosen from the Medical Subject Headings (MESH). Keywords should express the precise content of the manuscript.

Introduction

Introduction should be as concise as possible, and clearly explain the main objective and hypothesis of the investigation.

Results

Results indicate the results of an original research in a clear and logical sequence. Do not repeat data that are already covered in tables and illustrations. In manuscripts describing more than one animal, all animals should be assigned a case number.

Discussion

Discussion should include the answer to the question proposed in the introduction and empha-

size the new and important aspects of the study and the conclusions that follow from them. It could include the implication, application, or speculation of the findings and their limitations, relate the observations to other relevant studies, and links the conclusions with the goals of the study. Recommendations, when appropriate, may be included.

Materials and methods

Materials and methods should be described in sufficient details to allow other researchers to reproduce the results. Specify any statistical computer programs used .The methods of data collection and use of statistical analysis will be checked by the referees and if necessary, a statistician. Drugs and therapeutic agents, reagents, softwares and equipments should be given in the format: name (trade name, manufacturer name, city, country), e.g. Statview 5 (SAS Institute, Inc., Cary, NC, USA).

Animals: All animal experiments should comply with the ARRIVE (https://arriveguidelines.org/) guidelines and the authors should clearly indicate in the manuscript the ethical code of the study.

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:

Mouse beta actin gene: *Actb*
Bovine beta actin gene: *ACTB*
Chicken beta actin gene: *actb*
Beta actin protein: ACTB

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;

Reverse transcription (if used): amount of RNA, concentration of all reagents: primers concentration (either random primers or oligonucleotides), reverse transcriptase and master mix components;

qPCR: sequence of forward and reverse primers, probes, amplicon size, accession number of Genebank; thermocycler parameters (i.e. denaturation, annealing and extension steps, number of cycles, melting curves); validation of PCR products; non-template controls for reverse transcription and qPCR should be included in all reactions; and

Data analysis: details for the quantitative or relative analysis.

Use of antibodies: Authors must show that the antibodies are validated and their specificity sis con-

firmed.

References

Must be up-to-dated and limited to those that are necessary. Lists of references should be given in numerical order in the text, and in the reference list. Please use Vancouver style. To download the Vancouver Style follow the link in the IJVST website which could be used in the Endnote software.

Example piece of text and reference list :

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;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.

Tables

Please submit tables as individual files and editable text and not as images. Place all table notes below the table body. Each table should have a title which is followed by explanation of results shown in the table. Use of vertical rules must be avoided. Tables should be self-explanatory, and clearly arranged. Tables should provide easier understanding and not duplicate information already included in the text or figures. Each table should be typewritten with double spacing on a separate file and numbered in order of citation in the text with Arabic numerals. Each table should have a concise heading that makes it comprehensible without reference to the text of the article. Explain any non-standard abbreviations in a footnote to the table.

Figures

Figures must be submitted in individual files (format: TIFF, Dimensions: Width: 789 – 2250 pixels

at 300 dpi Height maximum: 2625 pixels at 300 dpi, Resolution: 300 – 600 dpi, file size: less than 10 MB, Text within figures: Arial or Symbol font only in 8-12 point). The text and other labels should be placed in the figure as un-compressed layers. Each figure should have a title which is followed by explanation of results shown in the figure. Figures should be numbered in order of citation in the text with Arabic numerals.

For the use of bar diagrams the following publication should be consulted:

Weissgerber TL, Milic NM, Winham SJ, Garovic VD. Beyond bar and line graphs: time for a new data presentation paradigm. PLoS Biol. 2015 Apr 22;13(4):e1002128.

The bar diagrams should be provided in color and in a well-designed and professional format. Please do not use different shades of gray. The axes of diagrams should have titles and units. Also, the source file of the image (Excel etc.) should be provided for typesetting.

Illustrations should be numbered as cited in the sequential order in the text, with a legend at the end of the manuscript. Color photographs are accepted at no extra charge. The editors and publisher reserve the right to reject illustrations or figures based upon poor quality of submitted materials.

If a published figure is used, the publisher's permission needs to be presented to the office, and the figure should be referenced in its legend.

Use of Italics

Gene symbols, Latin terms (i.e. *in vivo, in vitro, ex vivo, in utero, in situ*, and etc.) and species scientific names (using the binomial nomenclature), should be typed in italics, while the first letter of the genus name must be capitalized (i.e. *Homo sapiens*).

Publication Ethics

PUBLICATION ETHICS

Iranian Journal of Veterinary Science and Technology is aligned with COPE's (Committee on Publication Ethics) best practice guidelines for dealing with ethical issues in journal publishing and adopts the COPE guidelines. The journal members (editor, editorial board and the journal manager) have agreed to meet the purposes and objectives of the Journal.

Ethical guidelines for authors:

Authorship Criteria

IJVST requires authors to confirm that they and their co-authors meet all four criteria for authorship based on the guidelines of The International Committee of Medical Journal Editors (ICMJE) (verbatim as follows):

1. Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
2. Drafting the work or revising it critically for important intellectual content; AND
3. Final approval of the version to be published; AND
4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

The section "Author Contributions" in the manuscript should illustrate and clarify who contributed to the work and how. If a contributor does not meet all four above criteria should be acknowledged in the "Acknowledgements" section of the article.

Author agreements and conflict of interest

Written authorization from all authors for publication of the article is mandatory for IJVST to start the review process. This form entitled "Conflict of interest declaration and author agreement form" must be signed and completed by all authors. This statement and signatures certifies that all authors have seen and approved the manuscript being submitted. Also, the authors by signing this form warrant that the article is the Authors' original work, that the article has not received prior publication and is not under consideration for publication elsewhere, and that the corresponding author shall bear full responsibility for the submission.

Editors and members of editorial board as authors

Editor and members of editorial board are excluded from publication decisions when they are authors or have contributed to a manuscript.

Publication Ethics

Ethical guidelines for Peer reviewers

Iranian Journal of Veterinary Science and Technology (IJVST) follows and adheres to COPE Ethical Guidelines for Peer Reviewers. IJVST peer reviews all submitted manuscripts with contents in the scope of the journal. The process has been explained in the section "Peer Review Process".

Ethical guidelines for Editor

Iranian Journal of Veterinary Science and Technology regarding the responsibilities of the editors follows and adheres to COPE Ethical Guidelines for editors. The main guidelines are summarized in the guide to ethical editing from COPE.

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:

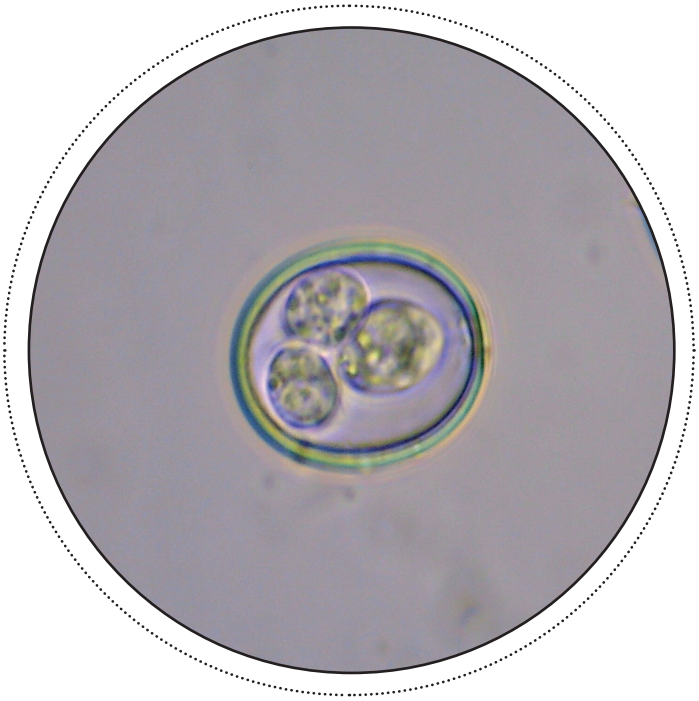
1. TITLE is clear and adequate
2. ABSTRACT clearly presents objects, methods, and results.
3. INTRODUCTION well-structured and provides a rationale for the experiments described.
4. MATERIALS AND METHODS are sufficiently explained and is detailed enough to be reproduced.
5. RESULTS are clearly presented 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.

Final Decision

Based on the reviewers' recommendations a final decision is made by the editor and if needed the help of a member of the editorial board (depending on the field of study). Decisions will include accept, minor revision, major revision with and without re-review, and reject. We aim to reach a final decision on each manuscript as soon as their review results are available.

Author Index

Author index



Iranian Journal of Veterinary Science and Technology

Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Azadi Square, Mashhad, IRAN
P.O. Box: 1793; Postal Code: 9177948974

Tel: 0098 51 3880 3742
Fax: 0098 51 3876 3852

Web: ijvst.um.ac.ir
Email: ijvst@um.ac.ir