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ON THE COVER

Hemangiopericytoma. Histopathological features reveal multiple layers of spindle shaped cells arranged around a central thin-wall vessel in a fingerprint pattern(See page 1).

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RESEARCH ARTICLE

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Prevalence of canine cutaneous neoplasms from Shiraz, Iran

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ABSTRACT

The present study was conducted on 42 dogs with a histopathological diagnosis of skin neoplasia presented in the Shiraz University Veterinary Clinic from April 2012 to December 2017. All cases were reviewed, excluding the mammary gland neoplasms. The histopathological type, prevalence, sex, age, breed and site distribution of the neoplasms were described. In addition, previous studies on canine skin tumors from other geographic regions were evaluated and compared with the results of the present study. Fifteen different histopathological types of tumor were diagnosed. The prevalence of epithelial, mesenchymal, melanocytic and lymphohistiocytic tumors was 61.9%, 35.7%, 2.4% and 0 %, respectively. The three most common tumors were sebaceous gland adenoma (21.42%), squamous cell carcinoma (11.9%), and lipoma (11.9%). The incidences of these tumors were more than other researches. Although there is no obvious explanation for these geographical differences, the possible reasons may be the geographical locations, environmental influences, and the study population and breed. Terriers were the most common type of the dogs in this study (34.4%). The present findings about the dogs age and various skin tumors and the anatomical locations indicates that there is no significant variation in these important parameters among the Iranian dogs and dogs from other parts of the world.

Keywords

Cutaneous neoplasms, Dog, Histopathology, Iran

Abbreviations

WHO: World Health Organization
H&E: Hematoxylin and eosin
UV: Ultraviolet

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Introduction

Neoplastic conditions in dogs can involve the skin, which is the largest organ system of the body [1, 2]. Several studies have been published about the relative incidence, predilection sites, and the effects of age, sex and breed on different skin tumors of dogs from different geographic regions like Denmark [3], United Kingdom [4], USA [5], Greece [6], Grenada [7] and Zimbabwe [8]. It is important to demonstrate the regional variations in the occurrence and type of tumors that appear in dogs. For example, cutaneous histiocytoma has been the most frequently reported neoplasm in dogs in India and Zambia [9, 10]; lipoma was the most common skin tumor in dogs in Korea [11], and mast cell tumor ranked first in studies on dogs from Thailand, Denmark, Greece, Brazil, and Zimbabwe [1, 3, 6, 8, 12].

The exact reasons for these differences are not well understood. The reports show remarkable differences in various geographic regions that could be related mainly to climate, ultraviolet radiation (environmental carcinogenic exposure) and the breeds of dogs (differences in genetic susceptibility) as risk factors for some types of cutaneous tumors [13, 14].

According to our knowledge, there is no available and clear information on the canine cutaneous tumors in Iranian dogs. Thus, the aims of the present study were to identify the most common histologic types of canine cutaneous tumors, tumor frequency, age, sex and site distribution of a defined geographical area. These objectives can be used as a source of histologically confirmed cases for epidemiologic and laboratory studies. There are also comparisons between our findings with the results of surveys in other countries.

Results

Among the 42 neoplastic skin masses examined, fifteen different cutaneous neoplasms were recognized. Of these, 26 (61.9%) were of epithelial origin (8 types), 15 (35.7%) of mesenchymal origin (6 types), 1 (2.4%) of melanocytic origin (1 type) and no lymphohistiocytic origin were seen (Tables 1 and 2). Among the tumors, 8 (19.05%) were malignant whereas 34 (80.95%) were benign (Table 2).

The frequency, mean age in years, and male-to-female ratio of each neoplastic tumor type was shown (Table 1). The three most common tumors were sebaceous gland adenoma (9, 21.42%), squamous cell carcinoma (5, 11.9%), and lipoma (5, 11.9%). These three tumors comprising 45.22% of all cutaneous neoplasms diagnosed during the study (Table 1). The sebaceous gland adenoma was the most common tumor of all cutaneous neoplasms diagnosed during this period.

The mean age of affected dogs for all neoplasms except papilloma varied between 5.2 years (for haemangiopericytoma) and 11.5 years (for hepatoid gland adenoma and mast cell tumor) (Table 1). Tumors of epithelial and mesenchymal origin had a close mean age of occurrence (8.15 and 8.53 years, respectively).

Terriers were the most common type of dogs in this study (34.4%). Four of five cases of lipoma and half of the cases of sebaceous gland adenoma were observed in Terriers. Other breeds represented in this study in descending order of frequency were mixed breed dogs (sheep dogs) (9), German Shepherd Dog (7), Poodles (2), Pekingese (2), Labrador retriever (1), Great Dane (1), Maltese (1), Boxer (1), Shih Tzu (1), Miniature Pinscher (1) and Spaniel (1).

Skin tumors were most frequently located on the head and neck (16), followed in descending order by trunk (12), limbs (11), and multiple sites (3) (Table 3). Among the malignant tumors, 6 of 8 were found in the head and neck.

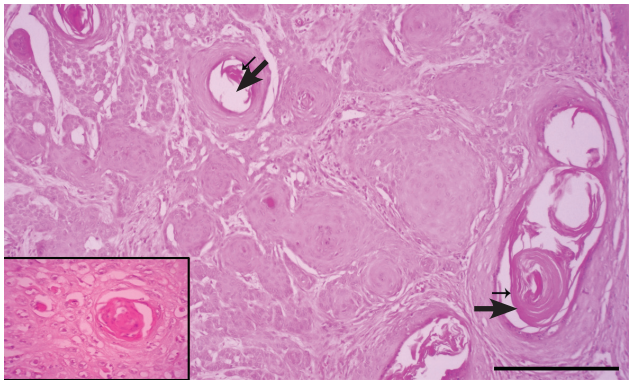

Figure 1
Squamous cell carcinoma. Cords and islands containing of large cells with abundant eosinophillic cytoplasm, ovoid nuclei with a prominent nucleolus, and keratin pearls (arrows). H&E. Scale bar=100 µm. Inset shows the high magnification of tumor cells, 400x.

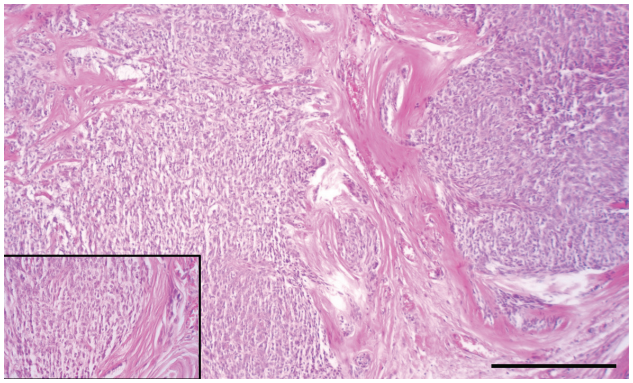

Figure 2
Trichoepithelioma. The round to oval neoplastic cells with lightly eosinophilic cytoplasm and vesicular nuclei and accumulation of keratin. H&E. Scale bar=100 µm. Inset shows the high magnification of tumor cells, 400x.

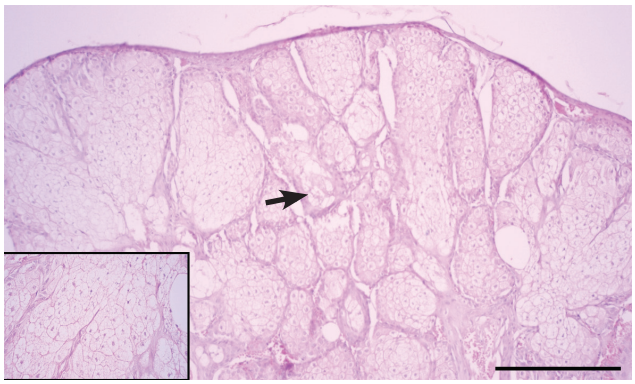

Figure 3
Sebaceous adenoma. Multiple lobules composed of the neoplastic cells with abundant pale, vacuolated cytoplasm, centrally hyperchromatic nuclei, surrounded by a rim of small, basophilic reserve cells (arrow). H&E. Scale bar=100 µm. Inset shows the high magnification of tumor cells, ×400.

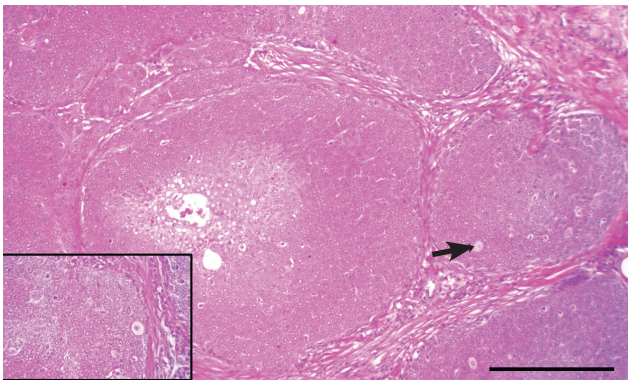

Figure 4
Sebaceous carcinoma. The neoplastic cells with moderate amount of cytoplasm and hyperchromatic, pleomorphic nuclei with prominent nucleoli. Some of these cells contained intracytoplasmic lipid vacuoles (arrow). H&E. Scale bar=100 µm. Inset shows the high magnification of tumor cells, ×400.

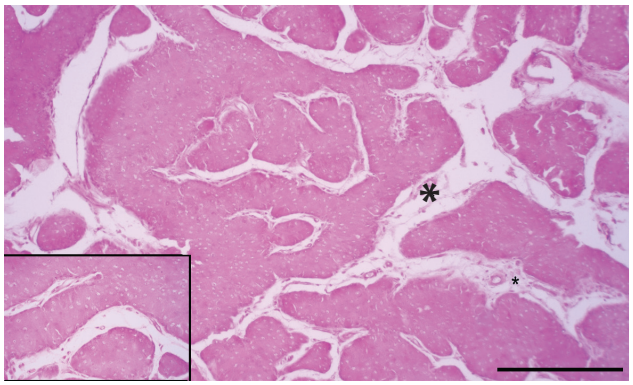

Figure 5
Hepatoid gland adenoma. The polyhedral neoplastic cells with centrally, large, ovoid nuclei with a central nucleolus, abundant eosinophilic cytoplasm and distinct cell borders (resembling hepatocytes), with a fibrovascular stroma (*). H&E. Scale bar=100 µm. Inset shows the high magnification of tumor cells, 400x.

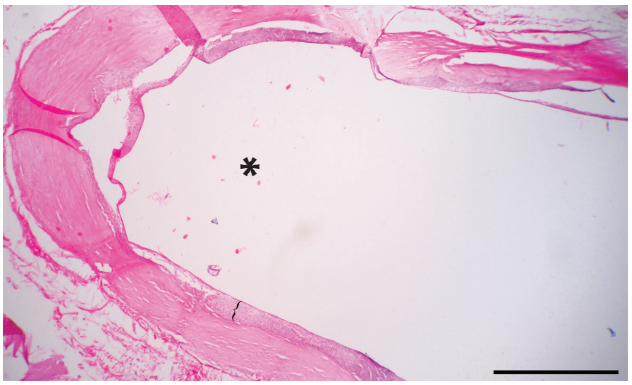

Figure 6
Infundibular Keratinizing Acanthoma. The tumor consisted of a cyst (*) lined by a keratinized stratified squamous epithelium ({). H&E. Scale bar=100 µm. Inset shows the high magnification of tumor cells, 400x.

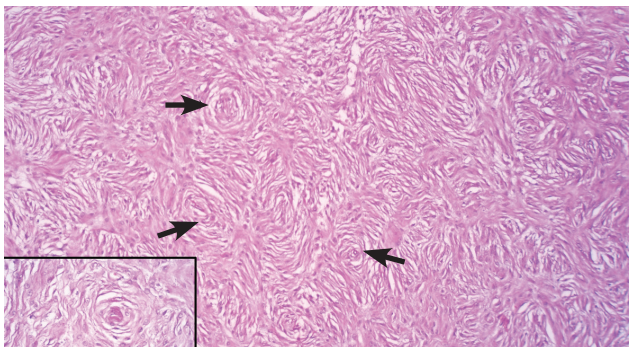

Figure 7
Hemangiopericytoma. Spindle shaped cells arranged around a central capillary in a fingerprint pattern (arrows). H&E. Scale bar=100 µm. Inset shows the high magnification of tumor cells, 400x.

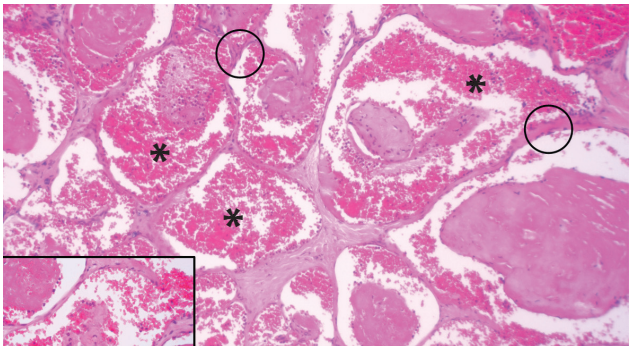

Figure 8
Hemangioma. Variably sized vascular spaces filled with erythrocytes (*) and lined by a layer of uniform endothelial cells (circles). H&E. Scale bar=100 µm. Inset shows the high magnification of tumor cells, 400x.

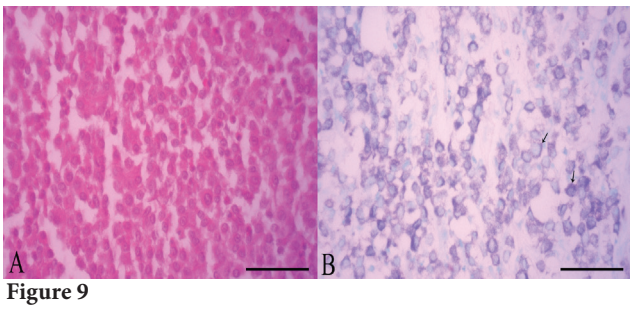

Figure 9
Mast cell tumor. A) Round to polygonal neoplastic cells with moderate amount of cytoplasm and round central to slightly eccentric nuclei. H&E. Scale bar=100 µm. B) Fine metachromatic granules (arrows) dispersed in the cytoplasm. Toluidine blue. Scale bar=100 µm.

Indicative photomicrographs of some cutaneous tumors recorded in the present study are also presented (Figures 1-9).

Discussion

The results of this survey in Shiraz are compared with those from Zimbabwe, Greece, Denmark, Korea, Thailand and the USA (Table 4). In the present study, the most common tumors were sebaceous gland adenoma, squamous cell carcinoma, and lipoma. These three tumors comprised near half of the all diagnosed cutaneous neoplasms. The incidences of these tumors were far more than the other researches [1, 4-6, 8, 11, 12]. There is no obvious explanation for these geographical differences. The squamous cell carcinoma prevalence (11.9%) was close to a survey in Zimbabwe (15.4%) [8]. Squamous cell carcinoma was the second most common tumor in both studies. A possible explanation may be in the environmental influences. Zimbabwe and Shiraz lie in the subtropics and most dogs are kept outdoors and therefore have greater exposure to sunlight. Other tumors, such as haemangiosarcomas and hemangioma, that have been associated with increased exposure to UV light in humans and domestic animals [6] were also seen in both studies. However, a study in tropical Queensland found no increase in UV light-associated tumors number compared with other regions [15]. The discrepancies between these data and the results obtained from previous studies in relative incidence of these diagnosed tumors may be related mainly to differences in the classification system, the diagnostic criteria, the geographical locations and environmental influences, and the breed and study population.

The majority of the diagnosed tumors in the current study were benign (80.95%) in nature, and this finding was in accordance with earlier reports [16, 17]. The percent of malignant tumors (19.05%) was less than the percent detected in Zimbabwe (37%) and Denmark (42.48) [3, 8].

Among Skin tumors, 61.9% and 35.7% were of epithelial origin and mesenchymal origin respectively. Prevalence of skin epithelial versus non-epithelial tumors in the dog varied in different studies. In many studies [8, 18], the most common tumors (around 45-50%) were mesenchymal origin following by epithelial origin (near 40%) and melanocytic origin (5-9%). In comparison to these researches, epithelial tumors were higher in the present study. In Greece, epithelial tumors were the most common (like our finding), and constituted 48% of all skin tumors and mesenchymal tumors constituted 40% [6]. The observed differences in different countries may be a reflection of canine breed population and environmental influences.

For all skin neoplasms, except papilloma that occurs in young dogs [18], the mean age of affected dogs (Table 1) falls within the common range for the occurrence of most neoplasms in dogs, that is, 6 to 14 years [19]. According to our data, the mean age of dogs affected by skin tumors is 8.22 years. It is close to the results of similar researches in Zimbabwe [8], Korea [11], and Germany [20].

Head and neck were the most frequent location for skin tumors, followed in descending order by trunk, limbs, and multiple sites (Table 4). In many researches, trunk detected as the most common site of skin tumors; or greater prevalence of skin tumors found in female dogs. These kinds of data were determined by including mammary tumors and the high prevalence of mammary neoplasms in bitches and also different classifications used by researchers [19]. In the present study, the mammary gland neoplasms were excluded. Due to a limited number of cases for each tumor, drawing valid conclusions on the sex, age, breed and anatomical locations predilection for the most frequently diagnosed tumors were not possible in our overall population. However, the present finding about the dogs age and various cutaneous tumors and anatomical locations shows that there is no significant difference in these important parameters among the Iranian dogs and dogs from other parts of the world.

Twelve breeds, including mixed breed dogs, were represented in this study. Terriers were the most common type of dogs who presented with skin tumors, followed by mixed breed dogs and German Shepherds. Terriers are the common and popular breed in Iran. The differences on the incidence of skin tumors might have appeared due to different popularity of certain breeds in certain geographical regions. Since canine population figures for Iran are not available, relative prevalence among different breeds could not be determined; while there may be a predisposition for cutaneous tumors among some breeds.

It is necessary to document the prevalence of different tumors in various geographic areas so that more definitive information may be gathered for future use.

Documented knowledge on the incidence and type of tumors helps veterinary practitioners to identify an appropriate therapy and anticipate an appropriate prognosis. After an accurate clinical examination, consideration of documented information on sex, age, breed and the histopathological report, tumors can be diagnosed in a reasonable amount of time and clinicians will be able to determine and decide on a proper treatment, and anticipate an appropriate prognosis for many patients.

This study was entirely based on clinical cases submitted from the Veterinary Medical Teaching Hospital of Shiraz University. Therefore, it is anticipated that the results of this study will reflect the prevalence and distribution of various skin tumors in the Iranian dog population. Furthermore, this result would serve as an important reference in future researches. To our knowledge, this kind of information has not been published previously for the Iranian dog population.

Conclusion

Based on the present finding, most of the cutaneous neoplasms in dogs from Shiraz (Iran) were epithelial-origin and the most common tumors were sebaceous gland adenoma, squamous cell carcinoma, and lipoma. Although there is no obvious explanation for differences between these results and similar research findings in other countries, the possible reasons may be the geographical locations, environmental influences, and the study population and breed. The present finding about the dogs ages and various cutaneous neoplasms and anatomical locations reveals that no significant variation is in these parameters among Iranian dogs and dogs from other parts of the world.

Material and methods

The present study was conducted on 42 dogs of different breeds, sex and age group with skin tumors presented to the Shiraz University Veterinary Clinic from April 2012 to December 2017. Only dogs living in the Shiraz region at the time of collection of the specimens were included in the survey. All cases with a histopathological diagnosis of skin neoplasia were reviewed, excluding the mammary gland neoplasms.

Biopsies have been taken from suspected sites under general anesthesia. Specimens were fixed in 10% neutral buffered formalin, embedded in paraffin and sections stained with haematoxylin and eosin. Tumors were diagnosed and classified according to the current World Health Organization (WHO) classification of animal tumors [21, 22]. History relating to sex, breed, age, and site of the tumor were obtained. Initially, the histopathological type, prevalence, sex, age and site distribution of the neoplasms were described. The neoplasms were grouped into 4 broad classes, namely epithelial, mesenchymal, melanocytic and lymphohistiocytic. Location of the tumor on the body was categorized into 4 groups: head and neck, trunk, limbs, and multiple sites.

In addition, published studies on skin tumors of dogs from other geographic regions were evaluated and compared with the results of the present study.

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Author Contributions

Design of study: M.A.H. and F.N. Clinical sampling: M.A.H. Performed pathological studies: F.N. and F.Z.K

Conflict of Interest

The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

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Experimental infection of pheasants with a velogenic chicken isolate of Newcastle disease virus

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ABSTRACT

Newcastle disease (ND) is a highly contagious infection of many avian species, causing enormous losses in poultry production worldwide. The objective of this study was to reveal the clinical feature, virus shedding, and immune response following infection with a velogenic chicken isolate of Newcastle disease virus (NDV) in susceptible and vaccinated pheasants. Eighty day-old pheasant chicks were allotted to four groups. At 30 days of age, the birds in groups 1 and 3 were vaccinated with B1 strain via eye drop. Two weeks later, each bird in groups 1 and 2 was inoculated with 100 µL (50 µL/eye) of NDV-infected allantoic fluid containing 10^5 EID_{50} of viral inoculum. All groups were inspected daily for three weeks. Swab samples were taken at different time points, and verified for NDV infection by using reverse-transcription polymerase chain reaction (RT-PCR). Serological examination was also made by haemagglutination-inhibition assay. Clinically, watery mucoid feces was observed only in one case among the vaccinated challenged birds, whereas the unvaccinated challenged birds showed anorexia, mild depression and head deviation. Out of 20 birds in group 2, one case (5%) died. Based on RT-PCR, virus shedding was only observed among the unvaccinated birds from 5 to 14 days after challenge. The NDV was detected more in tracheal swabs (40%) than in cloacal swabs (30%). The infected birds showed a high seroconversion. In conclusion, the velogenic NDV circulating in Iranian chicken flocks has a low pathogenicity for pheasants, and ocular vaccination with B1 strain could provide a good protection.

Keywords

Immune response, Newcastle disease, Pheasant, Virus shedding

Abbreviations

ND: Newcastle disease
NDV: Newcastle disease virus
EID_{50}: 50% embryo infective dose
HI: hemagglutination-inhibition
RT-PCR: reverse-transcription polymerase chain reaction
cDNA: complementary DNA
bp: base pairs

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Introduction

Newcastle disease (ND) is a highly contagious and fatal disease affecting at least 241 species of domestic and wild birds of both sexes and all age groups [1]. The causative agent is a virulent virus of the avian paramyxovirus serotype I of *Avulavirus* genus belonging to the family *Paramyxoviridae* [2]. Velogenic Newcastle disease virus (NDV) is endemic in many countries of the Middle East, Africa and Asia [3]. The inclusion of ND in the list of notifiable diseases by World Organization for Animal Health (2008) is indicative of its paramount economic impact on the worldwide poultry industry [2]. Among poultry, chickens are the most susceptible, showing the most clinical signs, and ducks are the least susceptible, showing the least clinical signs. Nonvaccinated pheasants are highly susceptible with clinical signs similar to those observed in chickens [3].

The first outbreaks of ND were observed in chickens in 1926 in Java, Indonesia, and Newcastle-upon-Tyne, England [3]. Then, NDV was spread throughout the world, and affected other avian species such as turkeys and quails [4,5]. Several outbreaks of ND have been also reported in pheasants in East Anglia [6], Iraq [7], Great Britain [8], Denmark [9], and South East England [10].

In Iran, NDV is endemic in different parts of the country, causing enormous losses due to high mortality, sub-optimal production, slaughterhouse condemnation of carcasses, and high prevention and treatment expenses. In recent years, outbreaks of ND have been occasionally observed in different avian species in Iran, including Japanese quail [11], ostrich [12], exotic caged birds [13], and broiler chickens [14]. During 2012-2013, some outbreaks with heavy losses occurred among commercial broiler chicken flocks located in southwest Iran. The isolated viruses were classified as genotype VII, and subsequently to subgenotype VIId [15].

Today, pheasant is extensively reared in several countries of the world as a game bird or for the purpose of human consumption. In recent years, commercial production of pheasant has increased in some regions of Iran, and a part of protein demands of Iranian people is provided with the meat of this bird. The NDV may be easily transmitted from one avian species to another, and some outbreaks in pheasants were epidemiologically related to the spread of the virus from chickens [6] and feral migratory birds [9]. Considering that viruses emerging from field strains may possess relatively new features, this study was conducted to investigate the clinical manifestations, virus shedding and serological responses following infection with a velogenic chicken isolate of NDV in susceptible and vaccinated pheasants.

Results

Clinical signs

No morbidity or mortality was observed in unchallenged pheasants. The vaccinated challenged birds exhibited watery mucoid feces only in one case, whereas the unvaccinated challenged birds showed anorexia, mild depression and head deviation (Figures 1 and 2). Almost one-fourth of these birds became morbid, but 1 (5%) out of 20 pheasants died at 10 days postinoculation. Clinical symptoms appeared 7 days postinoculation and continued by 12 days after challenge.

Virus shedding

An attempt to detect the virus was made for a period of three weeks, and the results of the PCR are presented in Figure 3. Out of 10 birds in group 2 which were sampled after challenge, 6 cases (60%) shed the NDV through respiratory and/or intestinal tracts from 5 to 14 days postinoculation. The NDV was detected more in tracheal swabs (40%) than in cloacal swabs (30%). The tracheal swabs were virus-positive at 5 and 10 days post-challenge, but the cloacal swabs were virus-positive at 10 and 14 days. The NDV was not detected in samples obtained from the other groups (Table 1).

Serological examination

The antibody response of pheasants to vaccination and or challenge with velogenic NDV is summarized in Table 2. The HI titers of the serum samples of all groups were negative (i.e., $< \text{Log}_2 3$) before vaccination. This status continued in the birds of group 4 by the end of the experiment, whereas a significant seroconversion occurred after vaccination or challenge in the other groups ($p < 0.05$). In group 3, the HI titer increased significantly after vaccination ($p < 0.05$), but its change was not significant from 14 to 28 days post-vaccination ($p > 0.05$). After challenge, the HI titers increased in groups 1 and 2; although its elevation was only significant ($p > 0.05$) in group 2.

Discussion

The NDV continues to be a major threat to the poultry industry. After infection with velogenic NDV, the nonvaccinated birds may die suddenly with no clinical signs and with a death rate of 100% [3], although the severity of the disease observed with any given virus greatly varies depending on host parameters, including species, breed, age and immune status, coinfection with other organisms, environmental and

Figure 1
Depression in a susceptible pheasant chick inoculated with a velogenic chicken isolate of Newcastle disease virus (7 days postinoculation).

Figure 2
Head deviation in a susceptible pheasant chick inoculated with a velogenic chicken isolate of Newcastle disease virus (8 days postinoculation).

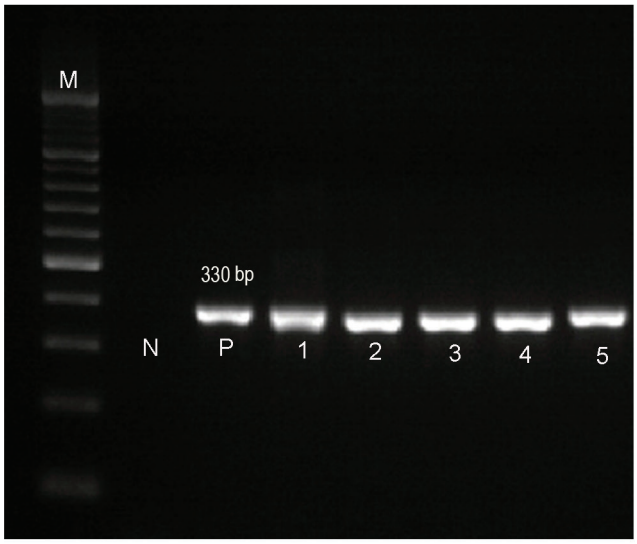

Figure 3
Electrophoresis of RT-PCR product of F gene in pheasants inoculated with a velogenic chicken isolate of Newcastle disease virus; M: ladder (100 bp), N: negative control, P: positive control (330 bp), lanes 1 - 5: positive samples.

Table 1
Virus shedding in pheasants experimentally infected[1] with a velogenic chicken isolate of Newcastle disease virus.

Group	Swab	Days postinoculation[2]					
		0	2	5	10	14	21
Vaccinated and challenged	Tracheal	–	–	–	–	–	
	Cloacal	-	-	-	–	–	
Unvaccinated and challenged	Tracheal	–	-	2	2		
	Cloacal	–	-	–	1	2	
Vaccinated and unchallenged	Tracheal	–	-	–	–	–	
	Cloacal	–	-	–	–	–	
Unvaccinated and unchallenged	Tracheal	–	-	–	–	–	
	Cloacal	–	-	–	–	–	

[1]At 30 days of age, pheasants in vaccinated groups received B1 strain vaccine via eye-drop. Two weeks later, each bird in challenged groups was inoculated through ocular route with 10^5 EID_{50} of viral inoculum.
[2]At each time point, two birds per group were sampled and examined for NDV infection by RT-PCR

Table 2
Haemagglutination-inhibition titers (Log2)[1] in pheasants following vaccination with B1 strain and or challenge with a velogenic chicken isolate of Newcastle disease virus.

Group	Days after vaccination		
	0[2]	14[3]	28[4]
Vaccinated and challenged	1.4 ± 1.06 [c]	4.8 ± 0.71 [b]	5.6 ± 0.74 [b]
Unvaccinated and challenged	1.3 ± 1.04 [c]	1.1 ± 0.83 [c]	7.1 ± 0.88 [a]
Vaccinated and unchallenged	1.3 ± 1.04 [c]	4.5 ± 0.93 [b]	4.8 ± 0.96 [b]
Unvaccinated and unchallenged	1.8 ± 1.04 [c]	1.3 ± 1.05 [c]	0.9 ± 0.83 [c]

[a-c] Values within columns/rows with no common superscripts differ significantly ($p < 0.05$).
[1] Values represent Means ± SE calculated from eight birds per group at each time point.
[2] Thirty days of age, [3] Before challenge, [4] Two weeks after challenge.

nutritional conditions, and route of exposure [1,16]. The virus used in this study was isolated from an outbreak of ND in a vaccinated chicken flock with a high mortality, and was characterized as a velogenic strain based on the sequencing of the F protein cleavage site. However, in this experiment, only one of 20 birds in group 2 died and the other morbid birds recovered rapidly. To our knowledge, there is no information regarding the experimental pathogenicity of NDV for pheasants to be compared with our results. But, the lower severity of the disease observed in pheasants can be associated with their less susceptibility, as well as route of infection (i.e. experimental vs natural) [2,10]. In a similar study performed by Wakamatsu et al. (2006), chickens challenged with velogenic NDV exhibited clinical sings at 2 days after inoculation and experienced a mortality rate of 100%, whereas commercial turkeys developed the clinical disease later (i.e., 6 days after inoculation) with no mortality. [16]. In the ND outbreaks in Italy, Capua et al (2000) reported that chickens and Guinea fowl were the most affected species, followed by pheasants, turkeys and ostriches [17]. In the current study, the vaccination of pheasants with B1 strain did not cause any clinical signs associated with post-vaccinal reactions. Similarly, Schmidt et. al. (2008) didn't find any reactions in pheasants vaccinated via eye-drop with B1, Ulster 2C, or LaSota starins [18]. The general clinical signs, including anorexia, lethargy, and head deviation observed in the unvaccinated challenged birds are the typical of the disease. They were all reported previously in natural infections of pheasants with NDV [6,7,17,19].

RT-PCR is one of the reliable laboratory techniques facilitating a rapid diagnosis by detecting NDV virus in clinical specimens [2]. In the current study, NDV was first detected in tracheal swabs obtained from nonvaccinated challenged pheasants at 5 days postinoculation before the appearance of the clinical signs (i.e., 7 days after inoculation). In our previous work, the Wishard bronze poults experimentally infected with highly virulent NDV of chicken isolate shed the virus 2 days earlier than the exhibition of clinical signs [20]. Wakamatsu et al. (2006) isolated NDV from swab samples of infected turkeys at 2 days postinoculation, whereas the onset of the disease was at 6 days postinoculation [16]. Moreover, in the present study, the tracheal swabs had a higher virus detection rate (4/10), compared to the cloacal swabs (3/10). This is somewhat consistent with the results reported by the previous studies, in which NDV was more frequently isolated from oral swabs than from cloacal swabs [16,21]. The detection or isolation rate of NDV may be influenced by the tropism of the virus. In an experimental study performed by Perozo et al. (2008), VG/GA strain of NDV was detected more in samples obtained from the intestinal tract of broiler chicks, whereas LaSota strain was detected more in samples taken from respiratory tracts [22]. Nevertheless, some studies have demonstrated the lack of sensitivity in detecting the virus in fecal samples, because they contain more extraneous organic material that can interfere with RNA recovery and amplification by PCR, suggesting that tracheal or oropharyngeal swabs are often the specimens of choice [2]. In the present study, virus shedding was not observed in vaccinated challenged pheasants. This is likely to be associated with less sensitivity of virus detection than isolation method, the small number of birds sampled at any time point after challenge, and or less susceptibility of pheasants to NDV in comparison with chickens, however additional studies are needed to clearly explain this finding.

As shown in Table 2, the HI titer in group 4 was lower than $\log_2 3$ before vaccination, and remained constant during the experiment, which can be regarded as being nonspecific [2]. On the other hand, a sudden seroconversion was observed in the other groups, implying the induction of active immunity by vaccinal or challenging virus. In group 3, mean HI titers of 4.5-4.8 (Log_2) were resulted from vaccination, but did not change significantly during 14 to 28 days postvaccination. In a similar study performed by Schmidt et. al. (2008), ocular vaccination of 10-day-old pheasants with B1, Ulster 2C, or LaSota produced mean HI titers of 4.2-5.0 (Log2) at 24 days of age [18]. In group 2, a higher HI titer (7.1) was found after challenge by velogenic NDV. Aldous et al. (2007) reported HI titers of 2^4-2^8 (tested with 8 HAU of antigen) in pheasants suffering from an outbreak of ND [10]. Piacenti et al. (2006) found a significant seroconversion at 10 days

after infection of velogenic NDV in commercial turkeys [23]. In group 1, challenge with velogenic NDV could not make a significant rise in the HI titer, which may be associated with the interference of active antibodies with velogenic NDV. Alexander and Senne (2008) reported that eye-drop vaccination of chickens with Hitchner B1 will result in production of lachrymal IgM, IgG, and IgA due to the replication of virus in the Harderian gland, which could be prevented by the presence of maternal IgG in lachrymal fluid [24]. These findings indicate that serum antibody alteration in pheasants following exposure to NDV is rapid and very similar to that in chickens and turkeys. In infection of chickens with NDV, antibodies usually are detectable in the serum within 6-10 days and reach the peak after 2-4 weeks [22,24].

In conclusion, the results indicated that the velogenic NDV circulating in Iranian chicken flocks has a low pathogenicity for pheasants, and that ocular vaccination with B1 strain along with biosecurity could provide a good protection.

Material and methods

The velogenic NDV used in this [redacted] from a broiler chicken flock in southwest Iran during an outbreak in 2013. Based on nucleotide sequence, the virus was previously characterized as genotype VII (subgenotype VIId), and assigned an accession number of NDa:KP347437 [15]. Initially, the virus was propagated twice in 9-day-old embryonated chicken eggs through inoculation into chorioallantoic sac. The 50% embryo infective dose (EID_{50}) was calculated for the second passage according to the method of Reed and Muench [25], and the harvested allantoic fluid was used as inoculum as specified in the experimental design.

Experimental Design

A total of 80 day-old unsexed pheasant chicks were purchased and randomly assigned into four equal groups. They were housed in cages separately in the Animal Research Unit of Shahid Chamran University of Ahvaz, and received feed and water ad libitum during the experiment. At 30 days of age, when the sera were negative for maternal antibodies in conventional hemagglutination-inhibition (HI) test, the birds in groups 1 and 3 were vaccinated with live B1 strain of NDV via eye drop; but those in groups 2 and 4 were sham-vaccinated with distilled water. Two weeks later, each bird in groups 1 and 2 was inoculated with 100 µL (50 µL/eye) of NDV-infected allantoic fluid containing 10^5 EID_{50} of viral inoculum, whereas the birds in groups 3 and 4 received distilled water by the same route. All birds were daily inspected for clinical manifestations and mortality for three weeks.

Sample collection

Tracheal and cloacal swabs were obtained from two birds per group before inoculation and at 2, 5, 10, 14, and 21 days postinoculation. They were examined for NDV infection by reverse-transcription polymerase chain reaction (RT-PCR). Moreover, eight birds from each group were bled through jugular vein before vaccination and at 14, and 28 days postvaccination. The blood samples were left to coagulate at room temperature for 8 h, and then centrifuged at 2000 rpm for 5 min. The collected sera were stored at -20°C until examined by HI test.

Reverse-transcription polymerase chain reaction

The swab samples were individually placed in microtubes containing 250 µl phosphate-buffered saline (PBS). After removing them from the microtubes, the RNA extraction was performed using the RNX™-Plus Kit (CinaGen, Tehran, Iran) according to the manufacturer's protocol. The isolated RNAs were directly used for the RT-PCR or stored at -70 °C. The partial F gene, including the cleavage site sequence, was amplified using a pair of specific primers. The primer sequences were TT GAT GGC AGG CCT CTT GC and GG AGG ATG TTG GCA GCA TT [14]. The complementary DNA (cDNA) was synthesized using BioNeer RT PreMix kit (BioNeer Corporation, South Korea) according to the manufacturer's instruction. The RT-PCR assay was carried out in a 20 µL reaction volume consisting of 2 µL of 10x PCR buffer, 0.2 µL of 10 mM dNTPs, 1 µL of each primer (20 pmol/ml), 0.2 µL Taq DNA polymerase (5U/ml), 0.6 µL of 50 mM magnesium chloride, 10 µL distilled water, and 5 µL cDNA dilution. The RT-PCR conditions included initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 60 sec, 72°C for 60 sec, and a final extension at 72°C for 10 min. The RT-PCR products with 330 base pairs (bp) were subjected to electrophoresis using 1.5% agarose gel. The NDV-infected allantoic fluid from our previous work was used as positive control, and distilled water was employed as negative control. In addition, a 100-bp DNA marker was used in electrophoresis for determining the RT-PCR product size.

Haemagglutination-inhibition test

The sera obtained by centrifugation of the samples were left in a water bath at 56°C for 30 min. Then, they were assessed for haemagglutination-inhibiting antibodies using 4 HA units of NDV antigen and two-fold serum dilutions as recommended by Thayer and Beard (2008) [26]. The results were expressed as Log2.

Statistical analysis

All data were analyzed in SPSS software (Version 24.0., Armonk, NY: IBM Corp.) using one-way analysis of variance. Differences showing $p<0.05$ were considered statistically significant [27]

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Author Contributions

Conceived and designed the experiment: RAJ, AR, ZB, Consulted: MM, Performed the experiment: RAJ, AR, ZB, RZ, Wrote the paper: RAJ, RZ.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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RESEARCH ARTICLE

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The Effect of resistance training and growth hormone injection on circulating IGF-1 and IGFBP-3 levels in a rat model

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ABSTRACT

Growth hormone has mitotic and anti-apoptotic effects which may increase proliferation and transformation of cells when it is expressed aberrantly. This study investigated the effects of resistance training and growth hormone injection on circulating IGF-1, IGFBP-3 levels and IGF-1/IGFBP-3 ratio in male Wistar rats. Thirty-two male Wistar rats were randomly assigned to a control group (C, n = 8), a resistance training group (RT, n = 8), a growth hormone injection group (GI, n = 8) and a resistance training + growth hormone injection group (RG, n = 8). The resistance training protocol comprised of climbing a ladder (5 days/week, 3 sets/5 reps) while carrying a weight suspended from the tail. The growth hormone (2 mg/kg/day, 5 days/week) was injected before an exercise session. Serum IGF-1, IGFBP-3 levels, and IGF-1/IGFBP-3 ratio were measured after 8 weeks. One-way ANOVA analysis was used for comparison of serum IGF-1 and IGFBP-3 levels between groups. Serum IGF-1 levels and IGF-1/IGFBP-3 ratio significantly decreased, but serum IGFBP-3 levels showed no significant change in the RT group compared to the C group. Also, both serum IGF-1 and IGFBP-3 levels and IGF-1/IGFBP-3 ratio in GI and RG groups significantly increased compared to the other groups. In conclusion, resistance training decreases serum IGF-1 levels and/or IGF-1/IGFBP-3 ratio in normal condition. On the other hand, the growth hormone injection with and without the resistance training increases serum IGF-1 levels and IGF-1/IGFBP-3 ratio which could be noted as a condition with a higher risk of neoplasm.

Keywords

Resistance training; Growth hormone injection, IGF-1, IGFBP-3, Cancer

Abbreviations

GH: growth hormone
IGF-I: insulin-like growth factor type 1
IGF-1R: Insulin-like growth factor 1 receptor
IGFBPs: insulin-like growth factor binding proteins;
IGFBP-1-10: insulin-like growth factor binding protein 1-10
GTPase: guanosine triphosphatase
MAPK: mitogen-activated protein kinases
ERK: extracellular signal-regulated kinases
PI3K: phosphoinositide 3-kinase
AKT (PKB): Protein kinase B
mTOR: mammalian target of rapamycin;

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Introduction

Doping is a common phenomenon in the sports which has become more popular among different groups of people from adolescent and youth to non-elite and elite athletes, as well as non-sporting contexts by individuals who try to improve their appearance (1). Growth hormone (GH) is one of the doping agents that is used considerably for its anabolic and lipolytic properties (1, 2). GH stimulates the liver to produce insulin-like growth factors (IGFs), six common IGF binding proteins (IGFBP1-6) as well as other binding proteins (IGFBP7-10) which are discovered more recently (3). One of the main growth factors is IGF-1, which is an endocrine and auto/paracrine peptide expressed in the majority of cell types (4). GH affects the tissues by IGF-1 which stimulates mitosis and inhibits apoptosis. IGF-1 binds to its tyrosine kinase cell-surface receptor (IGF-1R) and activates [at least] two main signal-transduction pathways: 1) GTPase Ras-Raf-ERK/MAPK pathway and 2) PI3K/AKT pathway. Activation of these pathways stimulates proliferation and inhibits apoptosis which may lead in some conditions to malignant neoplasm (5).

Six types of IGFBPs have been known. IGFBPs act as important agents by attaching to IGFs and protect tissues from their undesirable effects. IGFBP-3 is the most abundant (more than 90%) IGF binding protein in circulation that modulates IGF-1 actions by enhancing and inhibiting IGF-1 bioavailability (6). Also, IGF-1 has an important role in the transformation and proliferation of cancer cells (7-10). In most cases, the elevated IGF-I concentrations are considered beneficial; however, cancer remains a significant exception. Some studies have shown that the increment of circulating IGF-1 levels or IGF-1/IGFBP-3 ratio and decrement of circulating IGFBP-3 levels are associated with higher risks of cancer. High levels of IGF-1significantly increase the risks of colorectal (11), breast (9), and prostate cancers (7, 8). At the same time, researchers determined that IGFBP-3 which binds to IGF-1 seems to neutralize it and reduce the risk of these malignancies (11). Circulating IGF-1 is positively associated with breast cancer risk and this association is not substantially modified by IGFBP-3 (9). In recent studies, high and normal plasma of IGF-1 and low levels of IGFBP-3 were independently associated with a greater risks of prostate cancer (7, 8, 12), premenopausal breast cancer (10, 13), lung cancer (14), bladder cancer (15) and colorectal cancer (16, 17). Therefore, optimizing the levels of IGF-1 and IGFBP-3 might decrease the risk of several cancers.

Physical activity is another potential mediator which influences the GH-IGF-1 axis. The increases, decreases, and no changes in circulating IGF-I and IGFBP-3 levels after both acute and chronic exercises have been reported (18-24), and such equivocal findings prevent definitive conclusions. Moreover, the effects of GH injection with resistance training on circulating IGF-1 and IGFBP-3 levels have not been studied.

Thus, the aim of this study was to examine the effects of 8-week GH administration with and without resistance training on circulating IGF-1 and IGFBP-3 levels in Wistar male rats.

Results

Compared to the control group, serum IGF-1 concentration significantly decreased in RT group ($p = 0.048$), while it significantly increased in GI ($p = 0.001$) and RG ($p = 0.001$) groups (Figure 1). Furthermore, serum IGFBP-3 concentration did not show any significant changes in RT group ($p = 0.93$). In contrast,

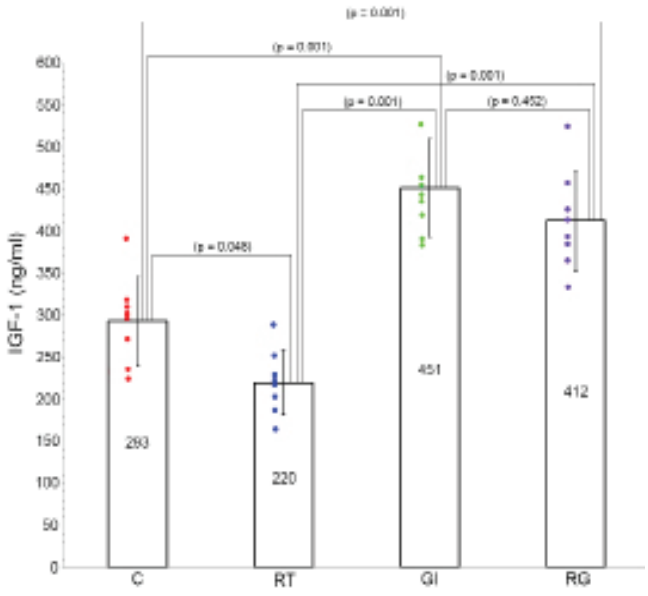

Figure 1
Comparison of circulating IGF-1 levels between control (C) resistance training (RT), growth hormone-injected (GI) and Resistance training + growth hormone-injected (RG) groups. Values are expressed as mean (± SD). Significant differences between groups are shown as $p < 0.05$.

serum IGFBP-3 concentration significantly increased in GI (p = 0.003) and RG (p = 0.011) groups, compared to the control group (Figure 2). In addition, the IGF-1/IGFBP-3 ratio was significantly lower in the RT

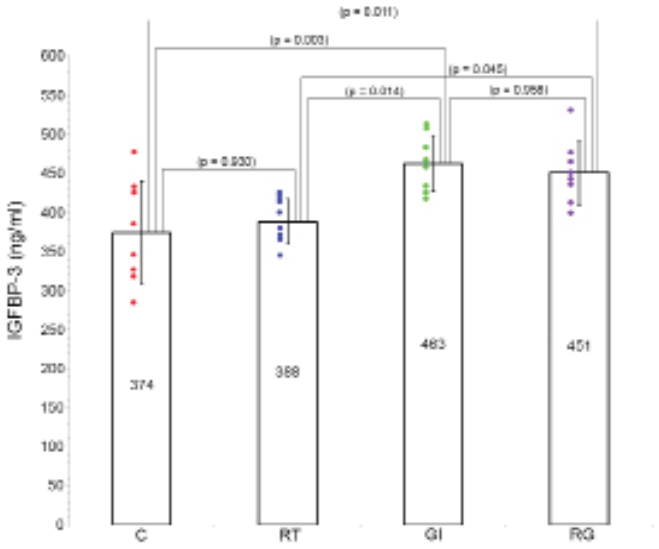

Figure 2
Comparison of circulating IGFBP-3 levels between control (C) resistance training (RT), growth hormone-injected (GI) and resistance training + growth hormone-injected (RG) groups. Values are expressed as mean (± SD). Significant differences between groups are shown as $p < 0.05$.

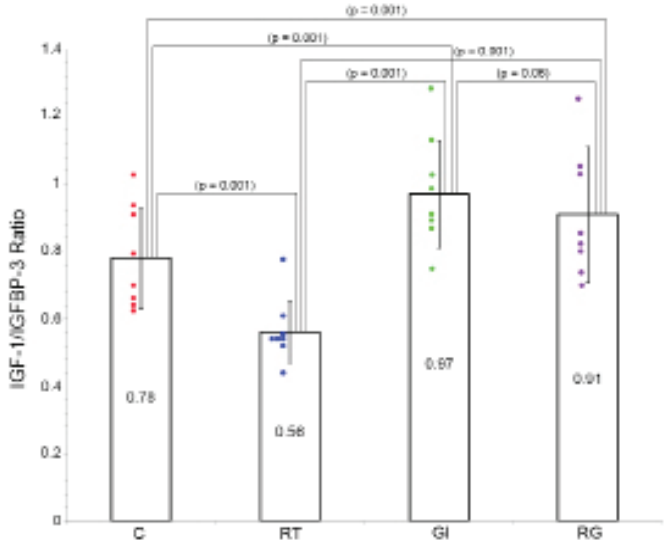

Figure 3
Comparison of circulating IGF-1/ IGFBP-3 ratio between control (C) resistance training (RT), growth hormone-injected (GI) and resistance training + growth hormone-injected (RG) groups. Values are expressed as mean (± SD). Significant differences between groups are shown as $p < 0.05$.

group (p = 0.001). On the contrary, the ratio of IGF-1/IGFBP-3 was higher in both GI (p = 0.001) and RG (p = 0.001) groups compared to other groups (Figure 3).

Discussion

We have examined the response of circulating IGF-1 and IGFBP-3 levels to GH injection with resistance training. The results of our study showed that resistance training reduced serum IGF-1 concentration. Some studies have already reported that acute resistance and endurance exercise increase serum IGF-1 concentration temporarily, which typically returns to baseline after 10 to 15 minutes (20, 23, 25, 26). However, other studies have demonstrated that chronic exercise training causes an increase (18, 21, 27), no change (28, 29), or a decrease (24, 30, 31) in circulating level of IGF-1, which is equivocal. Nindl et al. stated that although local IGF-1 is up-regulated with both acute and chronic exercises, circulating IGF-1 may actually decrease (32). The decrement of circulating IGF-1 in this study might be due to the anabolic role of IGF-1. Decreased circulating levels of IGF-1in response to short-term chronic training (>12 wk), might be related to neuromuscular anabolic adaptations (32). It has been suggested that physical activity might increase the uptake of IGF-1 into peripheral tissues (33)

and central nervous system (34). On the other hand, GH injection caused an increment in IGF-1 with and without resistant training. Recently, a positive correlation between the level of IGF-1 and the risks of several cancers such as colorectal, breast, prostate, lung cancer was found (35). According to cell proliferation and apoptosis inhibitor roles of IGF-1, previous studies have indicated that increased circulating IGF-1 level, decreased circulating IGFBP-3 and/or increased IGF-1/IGFBP-3 ratio might be involved in the commencement of these cancers (5). The consequence of binding IGF-1 to its receptor (IGF-1R) is the phosphorylation of insulin receptor substrate -1 (IRS-1). Subsequently, phosphoinositide 3-kinase (PI3K) is activated and increases phosphatidylinositol 3,4,5-triphosphate (PIP3), which activates the critical protein AKT/PKB through phosphorylation. As a result, the AKT protein stimulates the release of anti-apoptotic protein Bcl-2 from bad and activates protein synthesis through mTOR. All these actions are related to PI3K/AKT pathway of IGF-1 signaling which is ultimately responsible for cell death prevention (5).

Simultaneously, IGF-1 signaling increases cell proliferation through the Ras/MAPK pathway. After IGF-1 binding to IGF-1R and activating IRS protein SHC, GTPase Ras can stimulate Raf. Raf activates a kinase cascade which leads to the activation of mitogen-activated protein kinases (MAPKs), ERK1 and ERK2. Afterward, these MAPKs phosphorylate and activate multiple targets, in particular, the transcription factor ELK1 which increases gene expression and leads to cell growth (5). The imbalance between these pathways may result in the creation of neoplasm.

Secondly, we have found that circulating IGFBP-3 did not show any significant changes after resistance training. In spite of this, GH injection increased circulating IGFBP-3 levels with and without resistance training. IGFBPs are crucial regulators of IGF-1 actions and alter plasma levels of free IGF-I without affecting total IGF-I (4). It has been shown that resistance exercise changes the concentrations of IGFBPs which affects the biological activity of IGF-1 (21). IGFBP-3 is the most abundant protein that carries IGF-1, and lowers the free concentration of IGF-I and protects IGF-I from degradation (36). The results of previous studies are also inconsistent with the effects of exercise training on circulating IGFBP-3 levels (23, 26, 27, 37). Nishida et al. showed that after 6 weeks of aerobic training, circulating IGF-1 levels decreased by 9% and IGFBP-3 did not change significantly (30). Nindl et al. observed a significant increase of IGFBP-3 after resistance exercise and a decrease to the baseline during the next 13 hours (38). Chicharro et al. discussed that 3 weeks of endurance competition did not change circulating IGFBP-3 levels, but it decreased IGF-1 levels (31). These glaring inconsistencies between studies might be due to the type of exercise, training volume, duration of the intervention and age, training background and nutritional status of participants (29, 32).

Finally, in the present study, resistance training decreased IGF-1/IGFBP-3 ratio, whereas GH injection with and without resistance training increased IGF-1/IGFBP-3 ratio. It has been stated that the IGF-1/IGFBP-3 ratio is a biomarker for bioavailability of circulating IGF-1 levels (39). Thus, decreased or increased IGF-1/IGFBP-3 ratio might be related to a lower and higher risk of cancer, respectively.

The results of the present study led us to conclude that resistance training decreases circulating IGF-1 levels and/or IGF-1/IGFBP-3 ratio in normal condition. On the contrary, GH injection with and without resistance training increases circulating IGF-1 levels and/or IGF-1/IGFBP-3 ratio which could be considered as a condition with a higher risk of neoplasm. Further studies are required to extend these results in order to discover the exact resistance training effects on IGF-1 and IGFBP-3 concentration, and to examine the risk of cancer types after GH injection which increases the circulating IGF-1 levels and/or IGF-1/IGFBP-3 ratio.

Material and methods

Animals

Thirty-two Wistar male rats, 12 weeks of age, were purchased from Razi Vaccine and Serum Research Institute of Mashhad. All rats were randomly divided into four groups. Each day, all the animals were injected subcutaneously with either GH or saline (5 times per week for 8 weeks). GH was injected 1 h prior to the exercise. Group C was injected with saline using the same method used for the GH injection groups. Group RT was injected with saline and subjected to resistance training. Group GI was injected with human recombinant growth hormone (Genotropin, Germany) with a dose of 2 mg/kg (BW) 5 days per week. Group RG was injected with human recombinant growth hormone and also subjected to resistance training. All rats were weighed weekly, and the hormone dose was adjusted according to body weight. All rats were housed under controlled conditions and had unlimited access to water and food pellets. The animals' room temperature was maintained at 22 ± 1°C with a 12 h light/dark cycle. Experimental protocols were approved by the Institutional Animal Ethics Committee of Ferdowsi University of Mashhad (Ethic code: IR.MUM.FUM.REC.1396.12).

Resistance training

After the first week of adaptation, training protocol started, rats were trained 5 days a week for the next 8 weeks. Resistance training was accomplished by using a 1 m high ladder with 2 cm grid steps and an 85° grade. In their first week, rats were familiarized with climbing up to the top cage with and without weight on their tails. The weight, in an acryl tube, was attached to the tail with a plastic belt and tapes. Rats started their climbing from the bottom of the ladder and they were forced to climb up to the top by touching and shouting. Training sessions were started with the

intensity at 50% of each rat's bodyweight and increased (10% per week) gradually throughout the eight weeks of the training period. The resistance training consisted of three sets of five repetitions with a one-minute rest interval between the repeats and two minutes between the sets. This procedure was repeated until either the rat finished all three sets of training or failed to climb the entire length of the ladder (40).

Measurement of IGF-1 and IGFBP-3

Food was withheld for 12 h, and GH or exercise was withdrawn 72 h before the rats were anesthetized with 75 mg/Kg ketamine and 25 mg/Kg xylazine and killed. The serum was collected by centrifugation at 3000 RPM for 15 min at 4 °C and then stored at −20 °C until the analysis commenced. Serum IGF-1 and IGFBP-3 levels were measured by ELISA kits (Hangzhou East biopharma, Elisa Kits, CAT.NO: CK-E30653- E91558) according to the manufacturer's protocol with a lower limit of detection of 1.55 ng/ml and 0.93 ng/ml, respectively.

Statistical analysis

The Shapiro-Wilk test was applied to display the normality of data distribution. The mean of circulating IGF-1 and IGFBP-3 levels and IGF-1/IGFBP-3 ratio were compared by One-way ANOVA analysis. The Tukey's test was used post hoc to identify significant differences between groups. $p < 0.05$ was taken to denote statistical significance. Data were analyzed using SPSS software (version 20, SPSS Inc). All data are reported as mean (± SD). Significant differences between groups are shown as $p < 0.05$.

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Author Contributions

BR performed the design and coordinated the study, participated in all of the experiments and wrote the manuscript with support from MM, AR and ZM. MM and AR conceived the idea of research and helped supervise all the stages of the project. AJ contributed to the analysis of the results and to the writing of the manuscript.

Conflict of Interest

We have no conflicts of interest to disclose.

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RESEARCH ARTICLE

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Interactive effects of peripheral and central administration of LPS with inhibition of CRF receptors on food intake in neonatal chicks

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ABSTRACT

Anorexia is a part of the acute phase response (APR). Lipopolysaccharide (LPS) is frequently used to mimic APR and induces anorexia. The mechanism underlying anorexia associated with APR in chicks is not well understood. In the present study, the possible involvement of corticotrophin-releasing factor (CRF) on anorexic effects of LPS in neonatal chicks was investigated. For this aim, different doses of LPS were administrated via both intracerebroventricular (ICV) and intraperitoneal (IP) routes in order to assess its effects on chick's food intake. Subsequently, the effect of ICV injection of astressin, a CRF receptor antagonist, on anorexia induced by ICV and IP administration of LPS was investigated. Food intake was significantly decreased following either central or systemic administration of LPS. ICV co -injection of astressin and LPS significantly diminished anorexic effects of central LPS. However, anorexia induced by peripheral LPS was not attenuated by central injection of astressin. These data indicated that the brain CRF receptors are involved in central LPS-induced anorexia in chicks.

Keywords

Lipopolysaccharide, Acute illness anorexia, Corticotrophin-releasing factor (CRF) receptors, Neonatal chicks

Abbreviations

APR: Acute phase response
CRF: Corticotrophin-releasing factor
LPS: Lipopolysaccharide
COX2: Cyclooxygenase 2
PG: Prostaglandin
EP4: Prostaglandin E2 receptor 4
PVN: Paraventricular nucleus
ICV: Intracerebroventricular
IP: Intraperitoneal

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Introduction

Energy homeostasis mechanisms are complicated and also in part different in animal species, including birds (1, 2). Similar to mammals, under physiological conditions hypothalamic nuclei play a crucial role in chick energy homeostasis (3). Hypothalamic homeostatic functions including appetitive and feeding behavior are extensively affected by immune agents (4, 5). Indeed, infectious challenges initiate acute-phase response (APR), a systemic defense mechanism, which is commonly reflected by immunological, physiological, and behavioral disturbances (6, 7). The behavioral changes are known as "sickness behavior," and are represented by depression, changes in motivational state, fever and the decrease in food intake namely illness anorexia (8). Lipopolysaccharide (LPS), an endotoxin matter of cell surface of gram-negative bacteria, has been widely used as an experimental inflammatory model for evaluation of possible underling mechanism(s) of anorexia in different species (9). LPS motivate expression of pro-inflammatory cytokines and anorexia-related agents, including corticotrophin-releasing factor (CRF) (10, 11). CRF plays an important role in stress responses such as changes in the hypothalamic-pituitary-adrenal axis, autonomic nervous system, immune system-and behavior (12, 13). These actions of CRF are mediated through two receptor subtypes; CRF receptor 1 (CRF1) and CRF receptor 2 (CRF2)(14) .

Several evidences have revealed inhibitory effects of CRF on food intake (15, 16). It has been shown that intracerebroventricular (ICV) administration of CRF inhibits food intake in both mammals and chicks (17, 18). Saito et al (2005) indicated that the inhibitory effect of ghrelin on food intake was mediated by CRF in neonatal chicks (19).

Besides reported anorexic effects of CRF, none is known about the CRF involvement in anorexic effects of LPS in birds. Thus, the present study was conducted to evaluate the effect of ICV injection of Astressin as a nonselective CRF receptor antagonist on anorexic effects of LPS in chicks.

Results

Food intake response to central LPS

Time-course of chick's food intake injected ICV with different doses of LPS is presented in Fig 1. Cumulative food intake started to decrease the appetite of chicks treated with LPS (100 and 1000 ng) 30 min post injection and this suppression continued strongly, so that chicks almost did not consume food until the end of the experiment. However, this suppression was statistically significant form 120 min post injection and thereafter. The central effects of astressin (20µg) and astressin co-injected with LPS (100 ng) on cumulative food intake of birds are represented in Figure 2. Food intake was not decreased by LPS plus astressin (except 240 min post injection), while it was significantly decreased by LPS alone compared to the control 60 to 240 min after injection. Also, astressin or LPS plus astressin, significantly increased food intake compared to the LPS group. All of these results show that anorexia induced by LPS is attenuated by the blockage of CRF receptors.

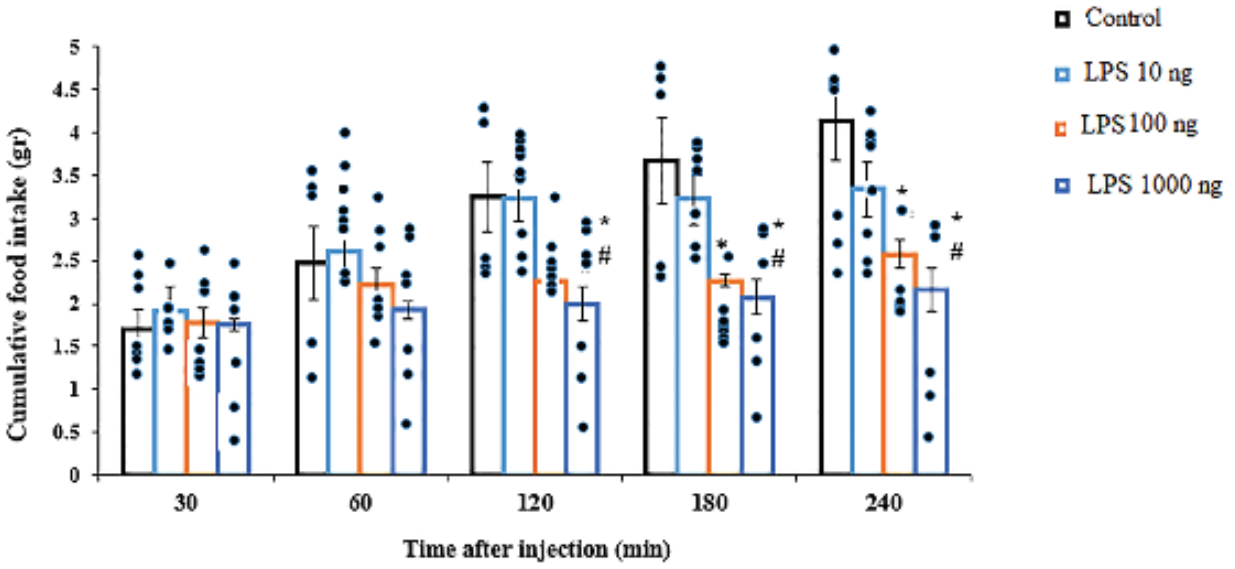

Figure 1
Cumulative food intake following ICV injection of various doses of LPS in chicks. Values correspond to mean ± S.E.M.
* $p < 0.05$ compared to control group
# $p < 0.05$ compared to LPS 10 ng group

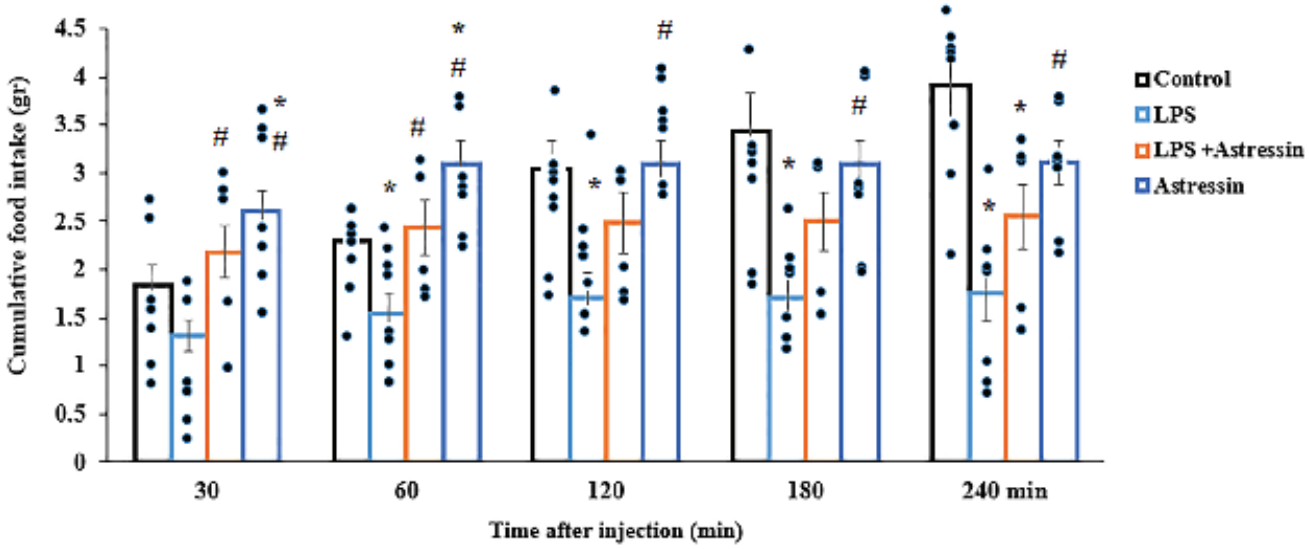

Figure 2
Cumulative food intake following ICV injection of LPS (100 ng) and LPS plus astressin (20 µg) in chicks. Values correspond to mean ± S.E.M.
* $p < 0.05$ compared to control group
# $p < 0.05$ compared to LPS group

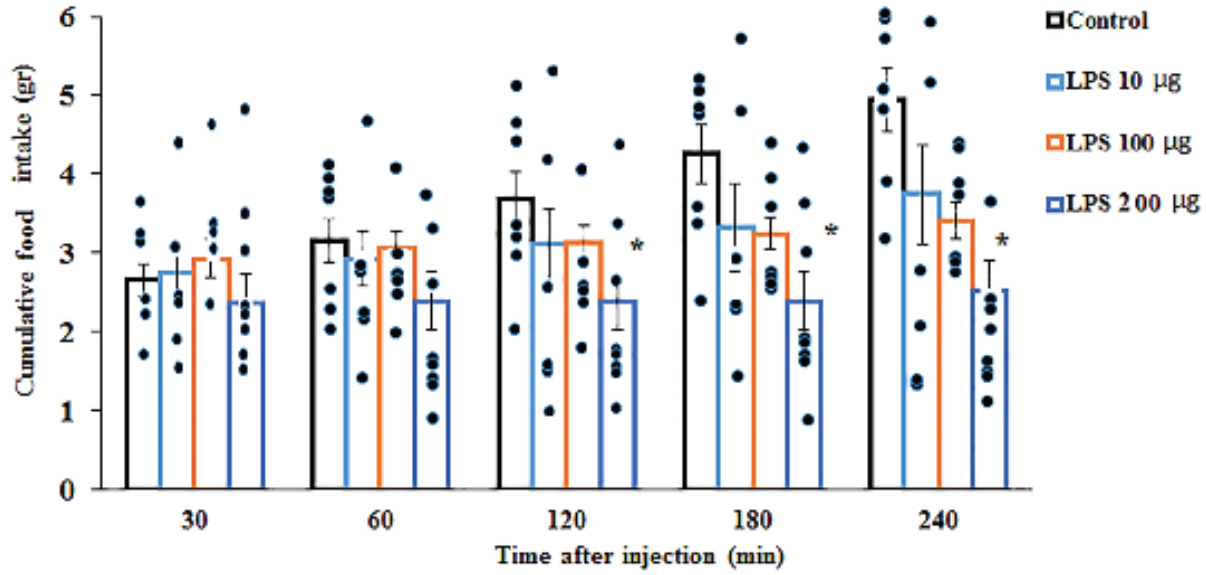

Figure 3
Cumulative food intake following IP injection of LPS in chicks. Values correspond to mean ± S.E.M.
* $p < 0.05$ compared to control group

Food intake response to peripheral LPS

Figure 3 shows the cumulative food intake of birds' injected IP with different doses (10, 100 and 200 µg) of LPS. Food intake tended to decrease by all levels of LPS as a dose dependent manner. However, in chicks with 200 µg LPS, food intake was strongly suppressed so that the birds did not eat nearly until the end of the experiment. However, this suppression was statistically significant from 120 min post injection and thereafter. The anorexia induced by IP injection of LPS was not attenuated by central astressin (both LPS and LPS plus astressin treated groups showed decreased food intake, while there was no significant

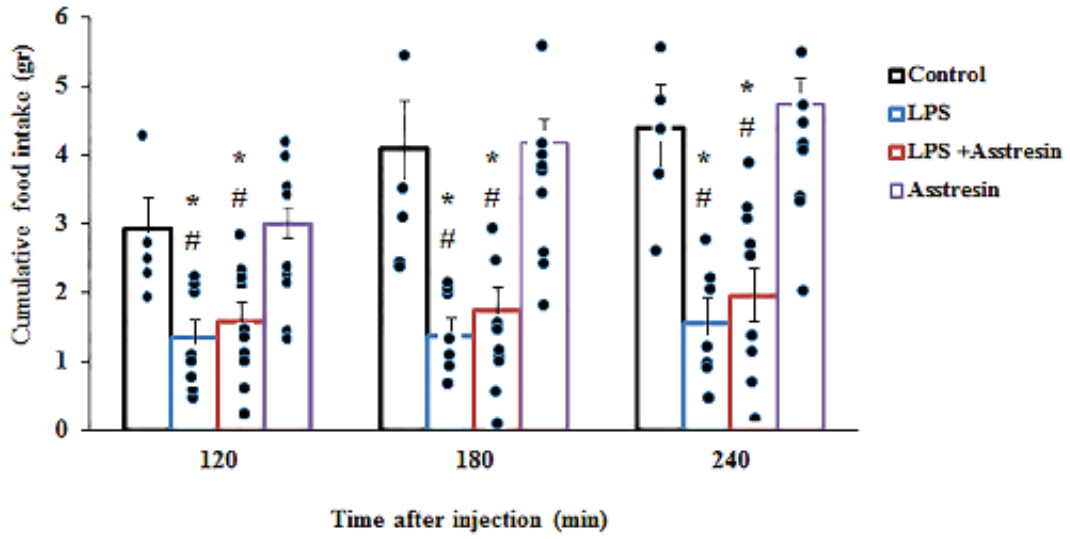

Figure 4
Cumulative food intake following IP injection of either 0 or 200 µg LPS followed by ICV injection of 0 or 20 µg Astressin 90 min later in chicks. Values correspond to mean ± S.E.M.
* $p < 0.05$ compared to control group
# $p < 0.05$ compared to Astressin group

difference between them)(Fig 4).

Discussion

In this study we have hown that peripheral and central administration of LPS could strongly diminish the neonatal chicks' food consumption. The anorexic effects were initiated 30 min after LPS injection and enlarged with increasing doses (Figs 1 and 3). LPS from gram-negative bacterial cell walls are major promoters of the APR and reduced food intake in animals (20). In mammals, many of the physiological effects of LPS via acting on its recognition receptor toll like receptor 4 are mediated by pro-inflammatory cytokines, like interleukin (IL)-1, IL-6 and TNF-α which are released from activated cells of monocyte/macrophage lineage (21-24). Thus, Pro-inflammatory cytokines are major endogenous mediators of the acute illness anorexia. These cytokines activate cyclooxygenase 2 (COX2), an enzyme that facilitates the metabolism of arachidonic acid to prostaglandin (PG) E2 (25). In chickens, in agreement with our results, IP and ICV injections of LPS have been demonstrated to induce hyperthermia and anorexia and to increase corticosterone (26, 27). PGs have also been demonstrated to be involved in LPS-induced hyperthermia and anorexia in chickens (27). Chickens were also injected with indomethacin (a COX2 inhibitor), peripherally or centrally following a challenge with IP injection of LPS. Pretreatment with indomethacin (injected IP but not ICV) significantly attenuated the LPS-induced anorexia (27). In addition, intravenous injection of LPS has been reported to increase plasma PGE2 concentrations in chickens (28). As it was mentioned before, we also investigated the effect of the blockade of CRF receptors by Astressin on anorexia induced by LPS. The results showed that centrally (not peripherally) LPS-induced anorexia is attenuated when the CRF receptors are blocked (Figs 2 and 4). Consistent with this result, it has been demonstrated in chicks that IL-1 and 3 activate stress axis, the key pathway for prostaglandin-induced fever, sickness behavior, and anorexia (29-31). In mammals, both CRF and cortisol influence the central mechanisms involved in the regulation of food intake (32). In chicks, several lines of evidence have shown that many anorectic agents exert their effects via CRF neurons. Indeed, the anorexigenic effects of ghrelin, glucagon like peptide -1, α-melanocyte stimulating hormone, vasoactive intestinal peptide, pituitary adenylate cyclase-activated peptide, glucagon, and cholecystokinin (19, 33-37), are mediated by CRF. Although, mechanisms underlying LPS induced anorexia mediated by CRF is unknown in chicks, there are some indications of this mechanism to be present in mammals. It has

been reported that LPS induces the expression of CRF and prostaglandin E2 receptor 4 (EP4), and activates CRF neurons in the rat PVN (10, 38, 39). Pro-inflammatory cytokines may directly activate CRF neurons within PVN (40). Peripheral injections of LPS or IL-1 β increase COX-2 and microsomal Prostaglandin E synthase-1 expression in blood brain barrier endothelial cells (41-43). PGE2 may directly act on its receptors, EP4 within PVN to release CRF (44). Evidences indicate that PGE2 released in response to LPS (and probably pro-inflammatory cytokines) may also act on serotonergic neurons to elicit anorexia. These evidences suggest that serotonergic neurons expressing EP3 receptors might be activated by PGE2 and project to areas of the hindbrain and forebrain that are involved in the control of food intake (45, 46). Pre-treatment with NS-398, a COX-2 inhibitor, reduced or eliminated LPS-induced c-Fos expression in several brain areas including the raphe complex, a source of serotonergic neurons (47, 48). Serotonergic neurons via their 2C receptors may act on PVN to release CRF (48). Recently, Zendehdel et al reported that pre-treatment with a 2C serotonin receptor antagonist significantly attenuated food intake suppression caused by LPS in chickens (49).

In this study, IP injection of LPS followed by ICV injection of Astressin couldn't attenuate LPS-induced anorexia effects. This discrepancy may be attributed to the difference in peripheral and central pathways of LPS action. In agreement to this, Johnson et al showed that central injection of LPS increases corticosterone plasma levels more than peripheral LPS, indicating that more CRF is released by central LPS (26).

In conclusion, current study revealed that both central and peripheral LPS strongly suppress food intake in chicks 30 min post injection and thereafter. The required amount of LPS for central suppression was about 1000 times lower than that required for peripheral suppression. Our results also identified that the CRF receptors are involved in the anorexic effect of central LPS in chicks. CRF has been shown to be a food intake inhibitor in chicks and many anorexigenic factors act through the CRF pathway in chicks. However, further studies are needed to clarify the CRF receptor subtypes involved in the above mentioned pathway in chicks.

Material and methods

Animals

One-day-old Ross broiler chicks were purchased from a local hatchery (Mahan Chicken Meat Production Complex, Kerman, Iran). All birds were given free access to a commercial feed and water and continuous lighting. The temperature and relative humidity of the animal cage were maintained at 30 ± 1°C and 50 ± 5%, respectively. Animals were placed in individual cages, one day before the experiment. All efforts were made to decrease distress. The principles of working with animals were based on the recommendations of the ethics committee of Kerman University of Medical Sciences, Kerman.

Drugs

LPS from Salmonella typhimurium (Sigma & Aldrich, USA) and Astressin (Tocris Bioscience, UK), a nonselective CRF antagonist, dissolved in sterile 0.85% NaCl plus 0.1% Evance Blue (Sigma & Aldrich, USA). Control animals received drug vehicle. All drugs were freshly prepared on each experimental day.

Microinjections

ICV injection was performed according to Davis et al. method (50). Briefly, the head of the chick was inserted in a straining device which positioned a hole in a plate overlying the skull immediately over the right lateral ventricle. A microsyringe was then inserted into the right lateral ventricle through the hole and infusions were delivered in a total injection volume of 10µl. This method requires no anesthesia and stress level of birds is insignificant (19, 51). At the end of each behavioral test, the animals were killed with intracardiac injection of sodium thiopental and their brain was removed. Validation of drug injection was verified by the presence of Evans blue in the right lateral ventricle. If an injection was not fixed in the correct location, the chicks' data were omitted from the analysis.

Experimental procedure

This study was designed in four experiments. In experiment 1, chicks were given ICV injection of LPS at 0, 10, 100 and 1000 ng. Experiment 2 was conducted to determine the central effects of Astressin, as a CRF receptor antagonist, on LPS-induced change in chicks' food intake. Thus, the birds received ICV injection of LPS at 0 and 100 ng, Astressin at 20 µg and Astressin (20 µg) plus LPS (100 ng). In experiment 3, animals were given an intraperitoneal (IP) injection of LPS at 0, 10, 100 and 200 µg. Experiment 4 was similar to experiment 2 except that the chicks were given IP injection of either 0 or 200 µg LPS, then, 90 min later, they received ICV administration of either 0 or 20 µg Astressin. In all experiments, 6-day-old chicks were deprived of food for 3 h prior to injections in order to motivate and coordinate feeding. Cumulative food intake was measured at 30 to 240 min post injection. 9 -12 chicks were used for each experimental group.

Data analysis

Data was presented as means ± SEM. The results were evaluated statistically using ANOVA (IBM*SPSS statistics* version 23) followed by a post hoc Duncan's new multiple rang test (MRT). Differences were considered statistically significant when $p < 0.05$.

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Author Contributions

Author contributions: Designed the experiments: H.J., M.A. Performed the experiments: M.S., A.SH. Analyzed the data: M.Y., Research space and equipment: M.A., Wrote the paper: R.K.

Conflict of Interest

None.

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The effect of two Iranian viper snake; *vipera albicornuta (zanjani)* and *vipera latifii (lattifii)* venoms on the viability of rat bone marrow mesenchymal stem cells *in vitro* and *in vivo*

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ABSTRACT

Bone marrow is one of the main sources of mesenchymal stem cells (MSCs), they are considered as one of the most promising types of stem cells for re-generative medicine. Snake venom is a complex mixture of different compounds which have potential pharmacological properties and may affect MSCs. The aim of this study was to investigate the effect of two Iranian vipers; vipera albicornuta and vipera latifii crude venoms on the viability of MScs in-vivo and in-vitro. MSCs were isolated from rat bone marrow and transferred into flasks with standard culture medium, and maintained in carbogen (95% oxygen and 5% carbon dioxide) at 37°C. The adherent MSCs were attached to the bottom of the flask, and spread to form monolayer in 4-5 days. The cells in in-vitro tests were treated with different concentrations (1, 2, 3, 4 and 5 µg/100µl) of mentioned venoms for 24, 48 and 72 hours. The cells in in-vivo experiment only treated with v. Latifii venom at the concentration 1µg/100µl and time intervals as in-vitro tests. The cell viability in in-vitro experiment was assessed using MTT assay. The optical absorption of each well was measured using an ELISA reader at 570 nm. The results of in-vitro experiments showed that maximum cell viability was observed at concentrations of 1 and 2 µg/100µl of v. albicornuta and v. latifii venoms after 48 and 72 hours respectively. The results of in-vivo experiment showed that the cells treated with v. Latifii venom for 72 hours in situ have the highest proliferation rate after passages three, four and five in comparison to control. The results of this study showed that, the v. albicornuta and v. latifii venoms can affect the confluence and viability of the MSCs.

Keywords

Mesenchymal stem cells, Venom, Viper snake, Cell culture, Bone marrow

Abbreviations

MSCs: Mesenchymal stem cells
NGF: Nerve growth factor
rBM-MSCs: Rat bone marrow-mesenchymal stem cells
V: vipera
EVs: Extracellular vehicles

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Introduction

MSCs are known as self-renewing, multipotent progenitor cells, capable of differentiating into mesodermal linages, including adipocytes, osteocytes, and chondrocytes (1). They were initially discovered in bone marrow (2) and then were isolated from almost every type of tissue (3,4) and because of their ease of isolation and ex vivo expansion, MSCs are known as promising therapeutic tools for the treatment of various diseases (5). In human medicine, MSCs are used for their anti-inflammatory properties and ability to aid in tissue and bone regeneration (6). Due to the advantages of MSCs compared to other cells for therapeutic applications, the techniques for MSC proliferation and differentiation are needed. In stem cell therapy, extensive use of synthetic substances, recombinant cytokines and growth factors could be a contributing factor in increased side effects and toxicity. Therefore, search for alternative natural products to be used as growth factors and other effective components in stem cell therapy is important (7). Venomous animals possess a novel source of pharmacologically effective substances including metallic cations, carbohydrates, nucleosides, biogenic amines and lipids and other components in their venoms (8,9). Among them, snake venoms are a cocktail of diverse molecules, biologically active compounds, and a combination of many different proteins and enzymes with a variety of pharmacological effects and valuable therapeutic potential (8,10,11). A number of studies have revealed the ability for snake venom toxins to be used as a diagnostic tool, and that they possess possible therapeutic properties and great potential for the development of lead compounds for new drugs (12,13). Previous studies have reported that nerve growth factors (NGFs) isolated from Chinese cobra snake venom, exert potential effects on the chondrogenic differentiation of bone marrow MSCs (BMSCs) and cartilage regeneration *in vitro* and *in vivo* (14,15). Direct addition of the snake venom phospholipase A2 is effective in the enhancement of the PMA-induced HL-60 cell differentiation to macrophages (16). Surveys such as that conducted by Liu et al., showed that vascular endothelial growth factor (VEGF) dependent mechanisms, stimulate the balance between osteoblast and adipocyte differentiation in BMSCs (17).

Gasparotto and colleagues used a new fibrin sealant (FS), derived from *Crotalus durissus terrificus* snake venom to evaluate the *in vitro* growth and cell viability of MSCs (25). The new fibrin sealant was a three-dimensional scaffold that maintained cells survival without promoting their differentiation (18). Kouchesfahani et al., showed that applying honey bee venom (BV), which consisted of mellitin, phospholipase A2, apamin and other bioactive ingredients, with retinoic acid has an effect on mouse P19 cell proliferation and differentiation to neurons (19).

The objective of this research is to determine the possible effects of two Iranian snakes, *v. albicornuta (zanjani)* and *v. latifii (latifii)* venoms on the viability rate of MSCs derived from rat bone marrow *in vitro* and *in vivo*.

Results

Vipera Latifii venom effect on viability of BMSCs in vitro

BMSCs were treated with various concentrations of *v. latifii* venom (1, 2, 3, 4, 5 µg/100 µl) *in vitro*, and the effect on cell viability was analyzed using the MTT assay. The maximum cell viability and proliferation at

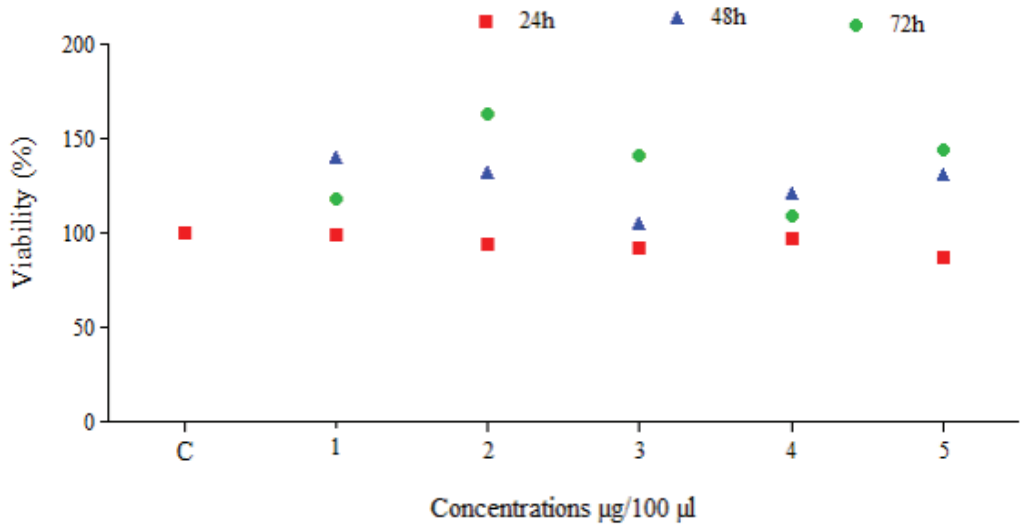

Figure 1
Viability (%) by vipera latifii snake venom at different concentrations (1, 2, 3, 4, 5 µg/100 µl), at 24, 48 and 72 hours

different concentrations was observed after 72 hours (Figure 1). The viability differences between 24 and 72 hours was significant (p= 0.015) at concentration of 2 µg/100µl, while it was not as significant between 48 and 72 hours. In addition, viability differences between different concentrations was not significant (Figure1).

Vipera albicornuta (zanjani) venom effect on viability of BMSCs in vitro

BMSCs were treated with various concentrations of *v. albicornuta (zanjani)* venom (1, 2, 3, 4, 5 µg/100µl) *in vitro*, and MTT assay and Post Hoc test were used to evaluate the viability of BMS cells. The maximum cell viability at different concentrations was observed after 48 hours (Figure 2). At concentration of 1 µg/100µl the viability differences between 24 and 48 and also between 48 and 72 hours was significant (p= 0.046) and (p= 0.014), respectively. At concentration of 3 µg/100µl the viability differences only between 48 and 72 hours was significant (p= 0.039) (Figure 2).

The measured viability showed that among different concentrations there is no significant differences at 24, 48 and 72 hours (Figure 2).

Comparison between vipera latifii and vipera albicornuta (zanjani) snake venom effects on viability of BMSCs at 72 hours

At concentration of 2 and 3µg/100µl the viability differences between *v. latifii* and *v. albicornuta* venoms was significant (p= 0.004) and (p= 0.014), respectively (Figure 3).

Preliminary results of the effect of Vipera Latifii venom on rat bone marrow

The quality of extracted MSCs were evaluated by the doubling time in cell culture. Cells were grown to 80 to 90% confluency as determined by visual assessment using phase contrast microscopy every 24

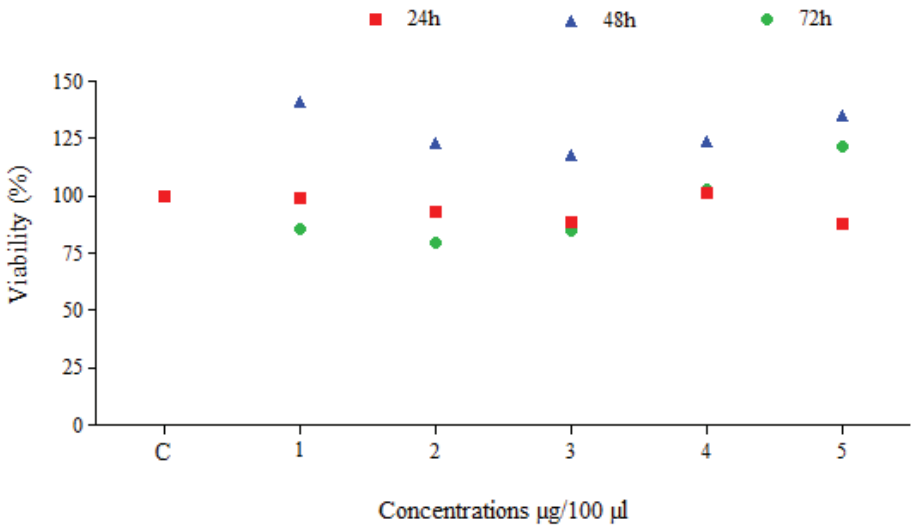

Figure 2
Viability (%) by vipera albicornuta (zanjani) snake venom at different concentrations (1, 2, 3, 4, 5 µg/100 µl) at 24, 48 and 72 hours

hours. Cells were harvested and subcultured at 1:2 ratio. The results showed that the cells treated with venom for 72 hours *in situ* have the highest proliferation rate after passages three, four and five in comparison to control, while cells treated for 24 hours with venom *in situ* were growing more slowly in comparison to control (Figure 4).

The cells at different passages in control group were reached to confluency in relatively the same time interval, while the cells in treatment groups showed higher rate of proliferation and growth. As shown in figure 4, the rate of cell proliferation increased after third passage at 48 and 72 hours.

Discussion

We sought to evaluate the effects of two Iranian viper snake venoms on MSCs and this study demonstrated that these venoms are able to affect cell viability *in vitro* and *in vivo*. The results of this study showed that MSCs cells viability increased after incubation *in vitro* with media containing venoms. For *v. latifii* snake venom, the highest viability was observed after 72 hours incubation at concentration of 2 µg/100 µl

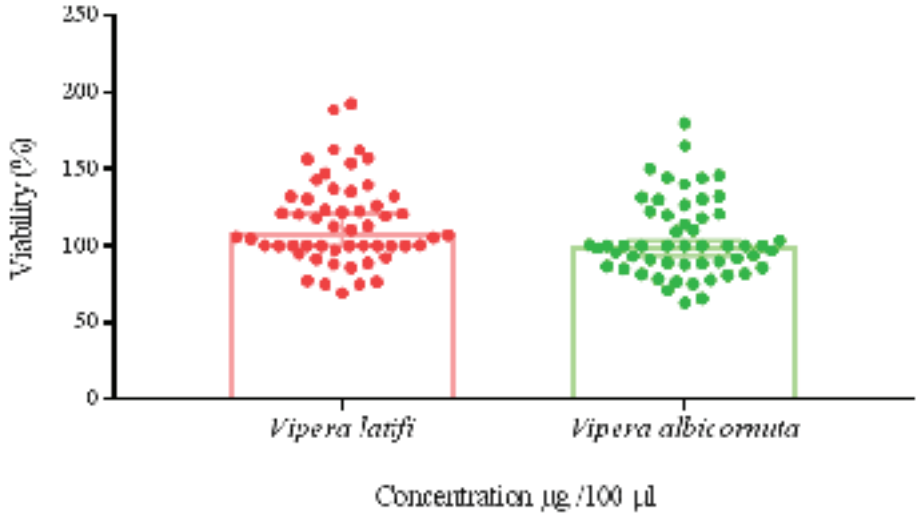

Figure 3
Viability (%) by vipera latifii and vipera albicornuta (zanjani) snake

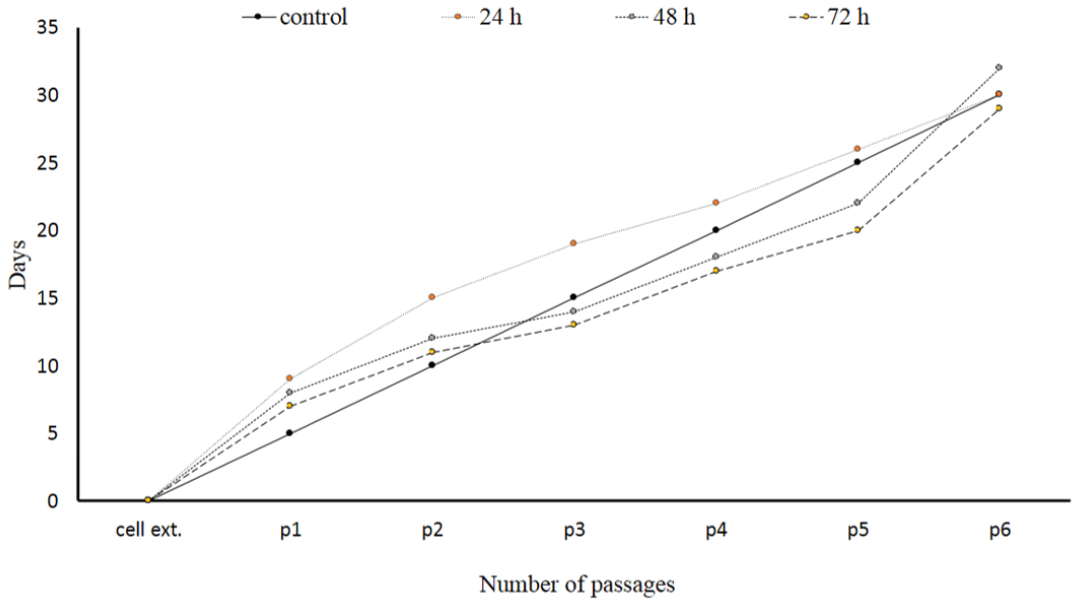

Figure 4
The time interval between passages of bone marrow derived MSCs treated with *vipera latifii* venom at 1µg/100 µl.

venom with 63% increased compare to control, and the lowest viability was after 24 hours (Figure 1). For *v. albicornuta (zanjani)* snake venom, the maximum viability occurred at 48 hours of treatment at concentration of 1 µg/100 µl venom with 40% increased compared to control (Figure 2). The observed effects seems to be more dependent to time than concentration as there was not significant differences among different concentrations at 24, 48 and 72 hours.

It was found that *in vivo*, the doubling rate of the cells after being treated with *latifii* venom was the highest in the 72 hours treatment group (Figure 4).

Considering the effects of endogenous factors and injected venom interactions, MSC viability may differ from that of *in vitro*. It is important to recognize that this study is the first study of its kind to investigate the effects of snake venom on stem cells *in situ*. Although the exact mechanisms by which these venoms affected the viability of MSCs are not clear, several points can be considered in an effort to explain this phenomenon. For example, in 2009, Chen and colleagues showed that initial expansion of MSC cells is influenced by the culture parameters such as media, cell density and even culture flasks (20). Therefore one possible conclusion is that any factors which change the media composition like venoms, may can affect cell viability. It has been shown that MSCs behavior such as tissue repair, immunomodulation, differentiation and proliferation may be influenced by their secretion products (paracrine factors) including soluble factors and extracellular vehicles (EVs) which participate in formation of a favorable microenvironment (21). Some of EV's are secreted after cell apoptosis, some during stress or metabolic changes, and some are secreted continuously (22–26). It is possible that after dissolving within this specific microenvironment, the individual components of the complex venoms undergo a biochemical change which could result in an alteration of MSC viability. Furthermore, growth factors are often added to stimulate the differentiation and improve cell proliferation. Several reports indicate that all snake venoms are a rich source of growth factors such as NGF (27). In 2017, Lu and colleagues isolated NGF from venom of Chinese cobra snake which induced the BMSCs to differentiate into the chondrogenic lineage *in vitro* and *in vivo* (14). Therefore, another possibility can be that these snake venoms possess several growth factors that stimulate MSCs proliferation and viability.

In 2002 Liu et al., reported that bee venom (BV) inhibited cell proliferation and induced K1735M2 mouse melanoma cells differentiation *in vitro*. In 2015, Jung et al. reported that bee venom has neuroprotective effects against rotenone-induced cell death in NSC34 motor neuron cells and pre-treatment of these cells with bee venom significantly enhanced cell viability. In 2010, Kouchesfahani and colleagues reported that honey bee venom induce differentiation of cholinergic neurons. They also showed that applying a combination of bee venom with retinoic acid (RA) has an additive effect on cell differentiation and proliferation. They concluded that phospholipase A2 (PLA2), the most abundant component of bee venom play an important role in the differentiation of cholinergic neuron in P19 cell line. Previously Nakashima et al. have suggested that PLA2 of BV plays an important role in cell differentiation and induced neurite outgrowth in PC12 cells (28) . Also in 2005, Mora and colleagues reported that snake venom Lys49 PLA2 homologues at lower concentrations, caused cell proliferation (29). To the best of authors' knowledge, no systematic publication exists to address the exact presence of PLA2 in *vipera latifii* and *vipera albicornuta* snake venoms. Although, it has been explored in prior studies that (PLA2) is one of the most abundant protein families found in vipera venoms (8,30). This suggest that possible (PLA2) of *vipera latifii* and *vipera albicornuta* venom treated the viability of rat MSCs the way the bee venom (PLA2) treated.

Conclusions

This is the first study investigating the effect of vipera snake venoms on MSCs *in vitro* and *in vivo*. In this study, direct injection of venom into bone marrow raised this possibility that venom may react to bone marrow composition *in situ,* that in turn affected the rate of cell growth and viability. The present results showed that the cells that were treated with *vipera latifii* venom in bone marrow for 72 hours can grow noticeably faster when they transfer to normal culture media.

It is beyond the scope of this study to examine the effect of venom peptide and enzyme compounds separately on MSCs. It is undisputable that venoms have varieties of pharmacologically active compounds and therefore various biological effects. The venoms somehow affect the viability of MSC and altered the survival and proliferation rate of rat bone marrow MSCs *in vitro* and *in vivo*. However, their fundamental and detailed molecular mechanisms is not clear. In addition, the bone marrow can be considered as a suitable model to investigate venom and similar compound effects on viability of stem cells *in situ*, however more research on this topic needs to be undertaken to explore the effect of individual components of these venoms and their mechanisms.

Material and methods

Chemicals

All chemicals including: Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin and amphotericin B, phosphate-buffered saline (PBS), fetal bovine serum (FBS), chloroform, ketamine, xylazine and, MTT powder were obtained from Sigma (Germany) unless otherwise stated. Reagents were prepared and stored according to the manufacturer's guidelines. Non chemical material were; insulin syringes, 75 cm^2 culture flasks and, 96-well plates.

Venoms

The *vipera albicornuta* and *vipera latifii* venoms were generously provided by Exhibition of Animal Sciences Development, Tehran, Iran.

Animals

Five Sprague-Dawley female rats (1-2 month old) were purchased from Animal House of Mashhad University of Medical Sciences. The rats were housed in the Animal Center of Faculty of Veterinary Medicine in a standard animal facility and maintained on a 12-hour light/dark cycle and were allowed food and water ad libitum. It should be noted that all animal experiments were approved by Animal Care and Use Committee of Ferdowsi University of Mashhad.

Isolation and culture of MSCs

One rat was succumbed with chloroform, its legs cleaned with warm water and shaved, afterwards the skin was swabbed with 70% alcohol. With two small surgical incisions, its two femurs and tibias were removed and transferred to the laminar hood. The muscles around the bones were removed, and washed 3-5 times with 1x phosphate-buffered saline (PBS). Bones were sterilized with 70% ethanol, washed by Dulbecco's Modified Eagle's Medium (DMEM) and epiphysis of the bone was separated by scissors. The bone marrows were then flushed out using DMEM, supplemented with 10% (v/v) FBS, 1% penicillin/streptomycin and 0.1 % Amphotericin B. The medium was flushed into the bone using an insulin syringe. Cell suspension was collected and centrifugated at 200g for 5 minutes. Supernatant was removed and cell pellet was re-suspended with 1ml of culture medium. Finally, the released cells were collected into 75 cm2 culture flasks with 10 ml DMEM culture medium. The cells cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2. Cells were allowed to attach for 4-5 days, the non-adherent cell population was removed and the culture medium was replaced with fresh pre-warmed culture medium. MSCs were attached to the bottom of the flask, due to their adhesion properties.

In-vitro study design

The cells were used after three passages, seeded into 96-well plates at a density of 0.05×10^6 cells/well. The cells were divided into six groups that included one control and five test groups. 150 µl of fresh pre-warmed culture media was added to each well of all groups. After 24 hours, the medium was aspirated and the test groups were treated with 200 µl phosphate-buffered saline (PBS) containing different concentrations (1, 2, 3, 4, and 5µg/100 µl) of v. albicornuta and v. latifii venoms while only 200 µl PBS was added to the control group. After one hour venom/PBS solutions were removed and all groups prepared for MTT test. It should be noted that, the selected venom concentrations were based on our previous research (unpublished results), on determination of the LD0, LD50 and LD100 of these venoms. Based on that experiment we chose the concentrations lower than LD50.

MTT Assay

Cell viability was assessed via a quantitative colorimetric assay using MTT (27). Five mg MTT powder was dissolved in 1ml PBS and the solution was sterilized using 0.2 µm filter. After 24, 48 and 72 hours, 20 µl MTT solution was added to each of 96-well containing the cultured cells in the culture medium and incubated for 4 hours at 37°C. The culture medium was carefully aspirated and 150 µl DMSO solution was added to each well. Finally, the optical absorption of each well was measured using an ELISA reader at 570 nm.

In-vivo experiments

Three rats were anesthetized with a combination of 60 mg/kg ketamine and 6 mg/kg xylazine (IP route) and one rat used as control. The animals were kept in a dorsal recumbent position, the anterior face of their thighs shaved, and the area disinfected with 70% ethanol. For intra-femoral injection, a minor surgery under anesthesia was performed. By opening the femur skin, the greater trochanter of the proximal femur was exposed. Five hundred microliters of *v. latifii* venom at concentration of 1 µg/100 µl was injected into the bone marrow of the right femur by using a 22 gauge metallic needle and then the animals returned to their cages. Their food and water consumption were normal and they were in healthy condition. MSCs were extracted after 24, 48, and 72 hours from the bone marrow of these rats. The cells were counted in each passage. To determine doubling time, when the cells reached to 90% confluency, they were cultured to sixth passages.

Statistical analysis

The Kruskal Wallis nonparametric test was used to compare medians among groups (n=3 per group) and Dunn's multiple comparison test was used to compare two groups in terms of viability. Values of p lower than 0.05 were considered statistically significant.

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Author Contributions

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

Conflict of Interest

The authors declare that there is no conflict of interest.

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RESEARCH ARTICLE

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Antibacterial effect of *Lavandula stoechas* and *Origanum majorana* essential oils against *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Escherichia coli*

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ABSTRACT

This research examined the antimicrobial effect of *Lavandula stoechas* (lavender) and *Origanum majorana* (marjoram) essential oil against three pathogens: *Staphylococcus aureus, Streptococcus agalactiae*, and *Escherichia coli*. Gas chromatography-mass spectrometry (GC/MS) analysis revealed that the main components of the lavender and marjoram oils were 17-Pentatriacontene, Linalyl acetate, Eucalyptol, linalool and 3-Cyclohexene-1-ol,4-methyl-1-(1-methylethyl)-,(R)-, α-terpineol, P-cymene, respectively. Broth dilution testing was performed using autoclaved whole milk instead of broth to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oils alone and in combination. In addition, time-kill assay of lavender and marjoram oils were determined in milk up to 24 h. MIC values ranged from 3.12 - 4.37% v/v and MBC between 6.25 - 8.75% v/v for the lavender. The MIC and MBC of the marjoram ranged from 0.62 - 1.87% v/v and 1.25 - 3.75% v/v, respectively. The MIC ranged from 2.5 - 5% v/v and MBC between 5 - 10% v/v for lavender + marjoram combination. In time-kill assays, the presence of lavender and marjoram oils at a sub-MIC concentration significantly reduced the bacterial population in 4, 10 and 24 h. Generally, essential oil of marjoram had greater antibacterial activity than lavender against all mastitis-causing pathogens tested and has the potential to be evaluated as an alternative or adjunct to antibiotics in the treatment of bovine mastitis.

Keywords

Antibacterial activity, Lavender, Marjoram, Organic farm

Abbreviations

MIC: minimum inhibitory concentration
MBC: minimum bactericidal concentration
GC/MS: gas chromatography/ mass spectrometry

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Introduction

Treatment of bacterial diseases are often encountered problems of increase in drug resistance and side effects of conventional medication [1]. In this context, natural products have a key role in discovery of alternative drugs [2]. Secondary metabolites of medicinal and aromatic plants present key candidates for discovering antimicrobial agents to fight against numerous microbial diseases.

Essential oils (EOs) have antibacterial, anti-fungal and antiviral activity and have been studied for finding new antimicrobial compounds, alternatives to cure microbial diseases [3]. Essential oils show antibacterial effects, therefore, the study of EOs antibacterial effects against bacterial agents is justifiable [4].

Lavandula is a medicinal plant from the family of *Lamiaceae* which is traditionally utilized to overcome diseases [1]. Antimicrobial [1,5,6] and antioxidant [7] activities of *L. stoechas* EO have demonstrated in several studies.

Origanum majorana (marjoram) from the *Lamiaceae* family has been used in traditional and folklore medicines for many disorders of gastrointestinal, respiratory, cardiac, and nervous system. Chemical constituents such as monoterpene hydrocarbons, oxygenated monoterpenes, and phenolic compounds have been isolated from marjoram essential oil. In pharmacological studies of marjoram, antibacterial, antifungal, antiprotozoal, and antioxidant activities have been reported in modern medicine [8].

Different diseases are caused by *Staphylococcus aureus, Streptococcus agalactiae*, and *Escherichia coli*. Mastitis is a common and important disease that can be produced by all these bacteria. The main causative agents of bovine mastitis are bacteria such as *Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Klebsiella pneumonia* [9] and Coagulase-negative staphylococci (CNS) [10].

There is an increasing need for new antibacterial agents to treat and control bovine mastitis, so we investigated the antimicrobial activity of these EOs in milk instead of synthetic laboratory medium for future application as an intra-mammary infusion in cows. Bacteria must survive and replicate in mammary gland to induce an infection [11]. Moreover, albumin, starch, and fat of milk can potentially interact with the antimicrobial constituents and reduce bioavailability of EOs [12]. Therefore, in the present study, milk was selected as the in vitro model for evaluating the antibacterial effect of lavender and marjoram EO for mastitis treatment. The antibacterial activity of EOs was determined on *Staphylococcus aureus (S. aureus), Streptococcus agalactiae (S. agalactiae)*, and *Escherichia coli (E. coli).*

Results

Chemical composition of the essential oils

GC/MS analysis revealed that the main components of the lavender and marjoram oils were 17-Pentatriacontene (42.15%), linalyl acetate (26.82%), eucalyptol (18.87%), linalool (5.7%), and 3-Cyclohexene-1-ol,4-methyl-1-(1-methylethyl)-,(R)-(44.84%), α-terpineol (6.83%), P-cymene (6.75%), respectively. (Tables 1 and 2).

MIC and MBC

The MIC and MBC of lavender and marjoram EOs on the mastitis bacteria are shown in Table 3. Although lavender, marjoram, and marjoram + laven-

Table 1
Chemical composition (relative % of peak area) of essential oil of lavender determined by GC-MS analysis.

RT	Compound	%
3.635	17-Pentatriacontene	42.15
4.117	1R-α-Pinene	0.73
5.909	m-Cymene	1.73
6.024	D-Limonen	2.4
6.099	Eucalyptol	18.87
7.667	Linalool	5.7
9.765	p-Menth-1-en-4-ol, (R)-(-)-	1.11
11.577	Linalyl acetat	26.82
12.433	Lavandulol acetate	0.48

RT: Retention time on HP-5MS column in minutes

Table 2
Chemical composition (relative % of peak area) of essential oil of marjoram determined by GC-MS analysis.

RT	Compound	%
4.11	Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-	1.39
4.83	Sabinen	1.5
5.739	Terpinolen	1.63
5.909	P-Cymene	6.75
6.011	D-Limonene	1.55
6.086	Eucalyptol	2.58
6.676	γ-Terpinene	4.96
6.961	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1α,2α,5α)-	2.17
7.667	Linalool	5.12
7.722	Terpineol, cis-β-	4.58
8.312	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-	1.97
8.76	4-Isopropyl-1-methylcyclohex-2-enol	1.41
9.792	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	44.84
10.145	α-Terpineol	6.83
11.544	Linalyl acetate	3.45
12.209	trans-Ascaridol glycol	1.5
12.677	1,4-dihydroxy-p-menth-2-ene	1.46
13.438	4,4-Dimethylpent-2-enal	1.46
15.97	Caryophyllene	2.03
19.758	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aα,4aα,7β,7aβ,7bα)]-	1.42
19.873	Caryophyllene oxide	1.38

RT: Retention on HP-5MS column in minutes

Table 3
MIC and MBC of lavender and marjoram essential oils against *Staphylococcus aureus, Streptococcus agalactiae,* and *Escherichia coli*

	Bacterium	MBC (%V/V)	MIC (%V/V)
Lavender	*Escherichia coli*	6.25	3.12
	Staphylococcus aureus	8.75	4.37
	Streptococcus agalactiae	7.50	3.75
Marjoram	*Escherichia coli*	3.12	1.56
	Staphylococcus aureus	1.25	0.62
	Streptococcus agalactiae	3.75	1.87
Lavender + Marjoram (1:1)	*Escherichia coli*	5	2.5
	Staphylococcus aureus	10	5
	Streptococcus agalactiae	10	5

RT: Retention on HP-5MS column in minutes

der essential oils displayed antibacterial effects, marjoram oil was the most effective against the bacteria. Marjoram had the lowest MIC and MBC for the three pathogens compared to the other two essential oils.

Bactericidal kinetics of the oils

The bactericidal kinetics of lavender and marjoram against mastitis bacteria in milk were shown in Figures 1, 2, 3. The initial population of bacteria in the control and treatment groups for the three bacteria was approximately 6.0 log10 cfu/ml. The population of bacteria reached about 12 log10 cfu/ml in the control group during the 24 h incubation period. The presence of lavender and marjoram at a sub-MIC concentration significantly reduced the bacterial population to 3.59 to 4.68 log10 cfu/ml in 4, 10 and 24 h except for lavender that insignificantly decreased *E. coli* population to 5.31 log10 cfu/ml in 4 h. However, the time-kill assay and MIC and MBC experiments showed the antibacterial effect of lavender and marjoram in milk.

Discussion

Bovine Mastitis is an important disease in organic farms due to its prevalence and lack of efficient cures for that. There is a growing need for organic antibacterials that can be used in dairy farms. A growing need is for organic antibacterials, which can be used in organic dairy farms, that do not emerge antibiotic resistance. It seems that the possibility of drug resistance decreases when a dairy herd transits from conventional to organic status [13].

The broth dilution method is frequently used to study antimicrobial efficacy of different essential oils [14], but milk was used instead of broth in the present study to mimic the udder environment. Moreover, hydrophobic properties of lipids and other lipophilic molecules of milk may reduce the antibacterial activity of essential oils on mastitis-causing bacteria [15].

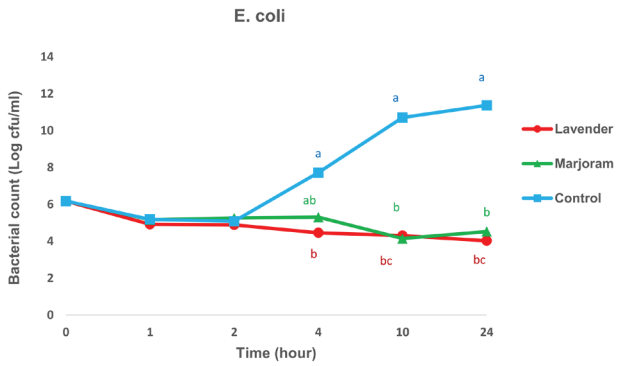

Figure1
Survival curve of E. coli in milk containing 0% (control, blue) and sub-MIC concentration
of essential oil of lavender (red) and marjoram (green).
[a-c]Values that are significantly ($P < 0.05$) different within the same time are indicated by different letters.

This work has demonstrated that the major components of the lavender EO were 17-Pentatriacontene, linalyl acetate, eucalyptol, and linalool. Main components of lavender were reported to be fenchone, camphor, and terpineol from Morocco [1] and fenchone, eucalyptol, and camphor from Spain [7]. Ashghari et al. reported camphor, 1,8 cineol, linalool, borneol and Mashak et al. found 1,8 cineol, borneol, camphor, linalool as main components of lavender EO in Iran [16,17]. Some of the aforementioned compounds were absent in our study that was in agreement with Carrasco et al. who declared that the main components of lavender had high variability even in the common constituents, i.e., camphor (0–49%) and fenchone (0–66%) [7]. This shows that study of biochemotypes existing in the different locations is important.

In the present study, main constituents of marjoram were 3-cyclohexene-1-ol,4-methyl-1-(1-methylethyl)-(R)-, P-cymene and linalool. Hajlaoui et al. reported that the Tunisian marjoram EO mainly consisted of terpinene-4-ol, followed by cis-sabinene hydrate, g-terpinene, and P-cymene [18]. The main components of the Venezuelan Andes marjoram EO were reported cis-sabinene hydrate, terpinene-4-ol, g-terpinene, a-terpineol, trans-sabinene hydrate, linalool acetate and a-terpinene [19]. In a study, terpinolene-4-ol, γ-terpinene, and α-terpinene were reported as the main constituents of marjoram EO in Iran [20]. Factors such as species, location of herb, growth stages, climatic conditions, distillation conditions, and the analyzed part of the plant are involved in variation of lavender and marjoram EO components [18].

Lavender and marjoram oil had antibacterial activity against the bacteria in the present study which are prevalent bacteria on organic farms [13]. Antibacterial effects of Lavender and marjoram oil has been reported against *E. coli* [1,18,19] and *S. aureus* [1,8,21,22]. Thus we hypothesized that lavender and marjoram oil are effective antibacterial agents against pathogens causing bovine mastitis. In the current study, we showed that lavender and marjoram oil could kill all tested bacteria. The MIC of marjoram oil for all tested bacteria obtained was lower than that of lavender and lavender + marjoram. In general, marjoram oil exhibited stronger activity than did lavender and lavender + marjoram oil. In agreement with our results, Dadalioglu and Evrendilek reported that Spanish lavender essential oils had a weak antibacterial effect [5].

In this research, MIC and MBC of lavender were 3.12% and 6.25% for *E. coli* and 4.37% and 8.75% for *S. aureus*, respectively. Also, MIC and MBC of marjoram were 0.62% and 1.25% for *E. coli* and 1.56% and 3.2% for *S. aureus*, respectively. Different values have reported for MIC and MBC of lavender and marjo-

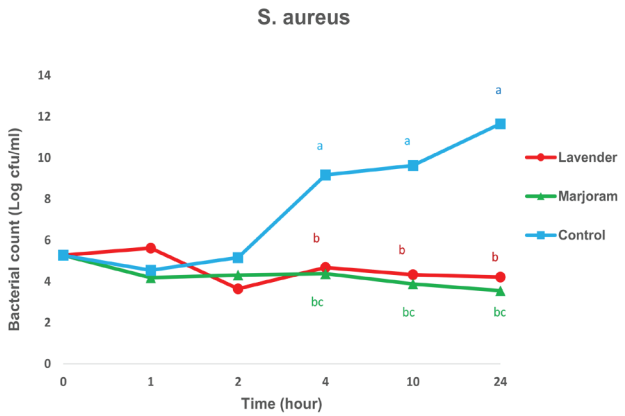

Figure 2
Survival curve of S. aureus in milk containing 0% (control, blue) and sub-MIC concentration of essential oil of lavender (red) and marjoram (green).
[a-c]Values that are significantly ($P < 0.05$) different within the same time are indicated by different letters.

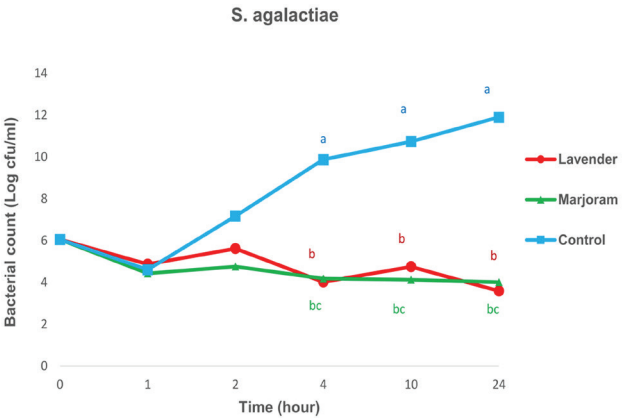

Figure 3
Survival curve of S. agalactiae in milk containing 0% (control, blue) and sub-MIC concentration of essential oil of lavender (red) and marjoram (green).
[a-c]Values that are significantly ($P < 0.05$) different within the same time are indicated by different letters.

ram. Bouyahya et al. and Gayatri et al. obtained MIC values of 0.5% and 0.25% against *E. coli* for lavender and 0.5-2% and 0.5% against *S. aureus*, respectively [1,23]. MIC and MBC values of 7.8% and 15.6% against *E. coli* and 1.9% and 7.8% against *S. aureus* for marjoram have reported, respectively [18]. In another study, MBC value of marjoram for *S. aureus* was obtained 0.25% v/v [24]. The different values of MIC and MBC in various studies might be because of the variable components of EOs and susceptibility of strain.

Antimicrobial effects of EO is not due to one specific mechanism because there are several different chemical groups in the structure of EO. Hydrophobicity of essential oils or their components helps them to target the cell membranes of bacteria that contain lipid. This property increases the permeability of membranes, thus contents of cell leak [12]. An outer membrane is present in Gram-negative bacteria that prevent penetration of essential oils into cells. Moreover, periplasmic extracellular enzymes might deactivate anti-microbial components of essential oil [25]. Thus we expected that essential oils to be more effective against Gram-positive *S. agalactiae* and *S. aureus* than Gram-negative *E. coli* but, MIC and MBC of S. aureus and S. agalactiae was higher than E. coli in all treatments except marjoram that MIC and MBC of *S. aureus* were lower than *E. coli*. Our results from the MIC and MBC indicated that the most sensitive microorganism against lavender and lavender + marjoram was *E. coli* and against marjoram was *S. aureus*. In agreement with our findings, in a previous study, MIC of lavender EO was higher against *S. aureus* than *E. coli* [1]. Similar to our finding, Tunisian marjoram EO showed a higher MIC and MBC against *E. coli* than *S. aureus* [18]. In contrast, MIC of marjoram against *S. aureus* was one fold upper than that of *E. coli* [26]. Essential oils of Plant potentially have several antimicrobial constituents. Comparing the findings of different researches is difficult because they use different bacterial strains, test methods, and source of antimicrobial samples. The great variability of composition of the essential oil can be attributed to the extraction method of the EO, geographical region, variety, and plant age [27].

We further carried out a time-kill curve set of tests, to ascertain time of inhibition or killing these pathogens. Lavender and marjoram oil displayed a bacteriostatic effect in the first 2 h and bactericidal effect between 4 and 24 h. Lavender and marjoram oil at sub-MIC caused a ~2.0 log10 cfu/ml reduction of *S. aureus, S. agalactiae* and *E. coli* within 24 h (Figures 1, 2, and 3).

Mullen et al. reported the antimicrobial effect of an herbal intra-mammary product on mastitis-causing bacteria and declared that the antibacterial effect of the formula might be due to *Thymus vulgaris* (thyme) [28].

In conclusion, essential oil of marjoram had greater antibacterial activity than lavender on the mastitis-causing bacteria (*S. aureus, S. agalactiae,* and *E. coli*). Results of this research showed that marjoram EO might be effective as an alternative or adjunct to antibiotic therapy to control bovine mastitis. However, further in vivo tests are needed to evaluate the efficiency on treatment of bovine mastitis and potential side effects on the mammary gland tissue.

Material and methods

Essential oils

Lavender and marjoram EO were purchased from Barij Essence Pharmaceutical Company, Kashan, Iran and Giah Essence

Agro-Industry & Phytopharm Company, Gorgan, Iran, respectively.

Analysis of Chemical Composition of the Essential Oils

GC/MS analysis was performed using an Agilent 7890B gas chromatograph coupled to a mass detector (Model 5977A, Agilent Technologies, USA) and a HP-5MS capillary column (phenyl methyl siloxane, 30 m × 0.25 mm ID 0.25 um, Agilent Technologies). The temperature of injector was 270°C, and the temperature of oven was raised from 60°C (0 min) to 200°C by a rate of 5°C /min. The analysis was performed using helium as a carrier gas while the flow rate was adjusted to 1 mL/min and injection volume (1ul). The interface temperature was set at 280°C and mass range was 35 - 500 m/z.

Bacterial strain

The activity of the EOs was tested toward three major mastitis bacteria including *Staphylococcus aureus* (ATCC 9144), *Streptococcus agalactiae* (ATCC 13813), and *Escherichia coli* (ATCC 25922). These bacteria were obtained as a lyophilized culture from Persian Type Culture Collection, Tehran, Iran (PTCC). The lyophilized cultures were grown twice in tubes containing 10 ml of Tryptic Soy Broth (TSB) (Biolife, Milano, Italy) at 37°C for 18 - 20 h (overnight). Afterwards, cultures were diluted with sterile glycerin (1:5) and stored in micro tubes at - 20°C for our research. To obtain fresh bacteria, it was cultured twice in TSB at 37°C for 20 h followed by streaking on Tryptic Soy Agar (TSA) (Biolife, Milano, Italy) slants and incubation under the same conditions. The cultures were stored at 4°C and sub-cultured monthly [29].

Preparation of Inoculum

Cells were transferred from working cultures to tubes of TSB and incubated at 35 °C for 18 h to obtain Bacterial inoculum. Next subcultures were performed and incubated at 35°C for 18 h. A spectrophotometer (Libra S12, Biochrom Ltd., Cambridge, London) was used to set the bacterial broth cultures to optical density (OD) of 0.1 at 600 nm, and a cell concentration of 2.4×10^{11} cfu/ml for *E. coli*, 3.4×10^{10} for *S. aureus* and 1.64×10^{11} for *S. agalactiae* were obtained. The number of cells in the suspensions was estimated by duplicate plating from tenfold serial dilutions on TSA and counting the colonies after 24 h incubation at 35°C [29]. The working inoculum was prepared by 1: 500 dilutions of the primary inoculum.

Milk Preparation

Milk without antibiotic residues was collected and then autoclaved for 15 min at 121°C.

Determination of MIC and MBC

Essential oils were diluted (1:1) in dimethylsulphoxide (DMSO, Sigma, Germany) and filter sterilized. This dilution was used in the antibacterial analysis. Herbal oils alone or in combination (1:1) were tested using a modified protocol for broth dilution testing according to the Clinical & Laboratory Standards Institute (CLSI) instruction [30]. Whole autoclaved milk was used as the growth medium. Twofold serial dilutions of the oil dilution were performed for the determination of MIC. Treatments were added to milk and were vortexed. Total volume of test vials was 1 mL. Then 100 µl of inoculum of each bacterium was inoculated into each tube. The vials again were vortexed and incubated at 37°C for 24 h. Eight 10-fold dilutions were prepared using sterile 0.85% saline solution. The samples were plated on a TSA plate and incubated at 37°C for 24 h for enumeration of inoculated bacteria. The MBC was defined as the lowest concentration without visible growth and subsequent concentration was taken as the MIC. Milk was cultured alone as a negative control to ensure that autoclaing was successful. Milk + bacteria were included as positive control to document growth of bacteria in milk. Vehicle control was DMSO to evaluate possible antibacterial effect of this solvent.

The occurrence of synergism/antagonism in antibacterial action among the EOs of lavender and marjoram was evaluated against mastitis pathogens. For this purpose, the essential oils were mixed volume to volume (1:1).

Bactericidal kinetics of the oils

Sterile milk was inoculated the sub-MIC of EOs with each pathogen in the same way as the above MIC tests. Control samples were inoculated milk without EO. Bacterial populations were counted at 1, 2, 4, 10, and 24 h of incubation at 37°C for 24 h on TSA plates. Each treatment was done in duplicate. Time-kill curves were constructed by plotting log10 cfu/ml against time (hour).

Statistical analysis

All tests were carried out in duplicate. The data were evaluated by analysis of variance (ANOVA) and the Tukey's test using the SPSS 18 statistical software (IBM Corp., Armonk, NY, USA) at $p < 0.05$ statistical level.

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Author Contributions

Conceived and designed the experiments: RR, JBK. Performed the experiments: SN. Analyzed the data: RR, FBB. Research space and equipment: RR, JBK, FBB. Contributed reagents/materials/analysis tools: RR, SN. Wrote the paper: RR.

Conflict of Interest

The authors declare that there is no conflict of interest regarding to publication of this paper.

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Effect of different levels of milkweed (*Calotropis persica*) seed powder on the growth parameters, immunity and gut microbiota of *Oncorhynchus mykiss*

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ABSTRACT

An 8-week feeding experiment was conducted to investigate the inclusion potential of five levels of *Calotropis persica* seed powder (CSP) (0, 10, 20, 30, 40 and 50 g/kg of the basal diet) in rainbow trout (*Oncorhynchus mykiss*) diet, in a completely randomized design. To perform the test, 3600 fries (11.5 ± 3.64 g) were treated for 56 days. According to the results, the increase in milkweed seed powder up to 40 g/kg, resulted in a significant increase in specific growth rate and intestinal lactic acid bacteria count compared to the control ($p < 0.05$). The best results of survival rate, feed conversion ratio, hepatosomatic and gastrosomatic indices were achieved in the treatments receiving 20, 30, 40, and 50 g/kg CSP ($p < 0.05$). Antibacterial activity of skin mucus, lysozyme and alkaline phosphatase showed the highest level in the 40 g/kg treatment ($p < 0.05$). Based on the results, the inclusion of 40 g/kg milkweed seed powder caused positive health effects and could be a suitable herbal feed additive in the rainbow trout diet.

Keywords

Calotropis persica, growth, immunity, gut microbiota, Oncorhynchus mykiss

Abbreviations

CSP: *Calotropis persica* seed powder
FCR: feed conversion ratio
HIS: hepatosomatic index
GaSI: gastro-somatic index
SGR: specific growth rate
ALP: alkaline phosphatase
PTCC: Persian Type Culture Collection
RaRBC: rabbit red blood cells
OD: optical density
CFU: colony forming unit
TVC: total viable count
LAB: lactic acid bacteria
ANOVA: analysis of variance

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Introduction

Despite the positive effects of hormones, antibiotics, vitamins and several other chemicals in the aquaculture industry, their residual and other side effects restrict their use in aquaculture operations (1). Thus, various alternatives have been proposed, including the use of probiotics, prebiotics, synbiotics, herbal medicines, etc. For reasons such as the availability, affordability and accessibility of herbal medicines, they have attracted much attention to aquaculture research, due to the advantages of herbal additives on growth performance, immunity, and appetite promotion (2), inhibitory and/or stimulatory effects in reproduction (3) and their antimicrobial properties (4). The review of the current literature indicates that there is a lot of information about the application of famous herbs such as garlic, rosemary, onion, peppermint, etc. (5) in aquaculture, while other plants with medicinal properties such as milkweed (*Calotropis spp.*) are less studied.

Giant milkweed (*Calotropis persica*) is a wild plant belonging to the *Asclepiadaceae* family (6). It grows in dry habitats (150–1000 mm precipitation per year) (7) and is native of West, North and East Africa, Arabian Peninsula, Southern Asia, and Indochina up to Malaysia (8). The various components of *Calotropis spp.* have different medical and industrial properties reported in literature (9–14). The plant is a rich source of many bioactive compounds which are of medicinal and industrial importance, for example Mohanraj et al, 2009 showed that the leaf extracts of *C. procera* have anti HIV-1 and antimicrobial activity. Other researchers used the latex protein of *C. procera* to prevent the septic shock due to lethal infection by *Salmonella enterica* (9). Also, the antifungal properties of the *C. gigantea* extract are used to treat *Fusarium mangiferae* and floral malformation in mango (12). For industrial applications of *C. gigantea*, oil extraction from seed and biodiesel production can be mentioned (11).

Some researchers have shown that the antibacterial, anti-fungal and anti-viral properties of different parts of *Calotropis Spp.* can be usefully exploited in aquaculture. Recently, *C. persica* ethyl acetate leaf extract was used against shrimp and fish pathogens. Results showed that the extract efficaciously suppressed the bacterial pathogens *Pseudomonas aeruginosa*, *Vibrio harvey*i and *Aeromonas hydrophila*; the fungi *Fusarium Sp.*, and the virus causing the white Spot Syndrome (WSS).

Although previous studies have examined the phytochemical power of different parts of the plant such as leaves, roots, shoots, flowers, fruits and latex, there is a lack of information regarding *C. persica* seed application. Therefore, this study aimed at investigating the effects of *C. persica* seed powder on growth performance, skin mucus immunity, antibacterial activity and gut microbiota, of rainbow trout (*Oncorhynchus mykiss*).

Results

Growth performance

The results of growth performance, survival rate and physiological parameters of *O. mykiss* at the end of the 56 days experiment are shown in Table 1. The results revealed that the dietary administration of different levels of CSP significantly improved SGR, FCR, HSI and survival rate compared to the control ($p < 0.05$). The statistical analysis of the results also indicate that, during the trial, the incremental trend in

Table 1
Weight (Mean ± SD), Condition factor, Specific Growth Rate, Survival Rate, Feed Conversion Ratio, Hepato-Somatic Index, Gastero-Somatic Index of *O. mykiss* fed diets containing different levels of *C. Persica* Seed Powder for 56 days (n = 3) ($p < 0.05$).

	Different levels of dietary CSP (%)					
	0	10	20	30	40	50
Initial weight (g)	11.40 ± 3.65	11.50 ± 3.64	11.60 ± 3.64	11.70 ± 3.66	11.40 ± 3.64	11.80 ± 3.70
Final weight (g)	33.50 ± 4.97 ^{a}	37.30 ± 3.04 ^{b}	37.30 ± 4.05 ^{b}	41.70 ± 2.18 ^{c}	42.80 ± 3.00 ^{cd}	36.70 ± 5.60 ^{b}
Condition Factor (%)	1.18 ± 0.42	1.17 ± 0.73	1.19 ± 0.52	1.22 ± 0.31	1.24 ± 0.40	1.29 ± 0.32
SGR (%/day)	1.92 ± 0.23 ^{a}	2.12 ± 0.22 ^{c}	2.09 ± 0.23 ^{c}	2.27 ± 0.23 ^{d}	2.36 ± 0.30 ^{e}	2.03 ± 0.20 ^{b}
Survival Rate (%)	82.00 ± 8.66 ^{a}	90.70 ± 8.14 ^{c}	91.70 ± 7.91 ^{e}	90.10 ± 8.06 ^{d}	89.40 ± 8.57 ^{c}	86.80 ± 9.12 ^{b}
FCR	1.71 ± 0.21 ^{c}	1.49 ± 0.10 ^{b}	1.31 ± 0.10 ^{a}	1.38 ± 0.11 ^{ab}	1.47 ± 0.10 ^{b}	1.92 ± 0.11 ^{d}
HIS (%)	1.43 ± 0.20 ^{b}	1.48 ± 0.21 ^{d}	1.45 ± 0.21 ^{c}	1.50 ± 0.20 ^{e}	1.48 ± 0.22 ^{d}	1.37 ± 0.20 ^{a}
GSI (%)	10.13 ± 1.32 ^{a}	11.35 ± 1.37 ^{b}	12.60 ± 1.55 ^{c}	13.41 ± 1.48 ^{d}	14.38 ± 1.90 ^{e}	15.24 ± 1.83 ^{f}

Different superscripts within a row indicate significant differences at $p < 0.05$.
SGR: specific growth rate; FCR; Feed conversion ratio; HIS; hepato-somatic index; GaSI; gastero-somatic index *C. Persica* Seed Powder (CSP)

GaSI paralleled the amount of CSP in the diet. On the other hand, SGR and HSI decreased significantly at 50 g/kg CSP level of inclusion in the diet. ($p < 0.05$). Furthermore, the least FCR was observed in fish receiving 20 g/kg CSP ($p < 0.05$).

Skin mucus immunological parameters

Evaluation of the skin mucus parameters (lysozyme activity, ALP activity and dissolved protein content) showed that dietary administration of CSP at any level of inclusion, significantly improved lysozyme activity and ALP activity and dissolved protein compared to the control diet group (Table 2) ($p < 0.05$).

Skin mucus antibacterial activity

The skin mucus of rainbow trout (*O. mykiss*) showed significant antibacterial activity ($p < 0.05$) against *Y. ruckeri*, *A. hydrophila*, *S. iniae*, *S. faecium* and *M. luteus* in all treatments as shown in Figure 1. The highest growth inhibition zone for all tested bacteria was observed in the skin mucus of fish receiving 40 g/kg CSP for 56 days ($p < 0.05$). The results also showed that the highest and lowest antibacterial activity of skin mucus was observed in *A. hydrophila* and *S. faecium*, respectively ($p < 0.05$).

Intestinal bacteria analysis

Table 3 shows the results of Total Viable Count (TVC) and lactic Acid Bacteria (LAB) levels in the intestine of rainbow trout (*O. mykiss*) after 56 days of feeding on different levels of CSP. The count of the intestinal microbiota was reported as colony forming

Table 2
Skin mucus dissolved protein (Mean ± SD), alkaline phosphatase activity and lysozyme activity of *O. mykiss* fed with different levels of CSP in the diet for 56 days (n=3) ($p < 0.05$).

	Different levels of dietary CSP (%)					
	0	10	20	30	40	50
DP (µg/ml)	0.48 ± 0.01 ^{a}	0.50 ± 0.03 ^{a}	0.96 ± 0.02 ^{b}	0.63 ± 0.01 ^{c}	0.67 ± 0.02 ^{d}	0.71 ± 0.02 ^{e}
ALP (µg/ml)	0.58 ± 0.03 ^{a}	0.96 ± 0.02 ^{b}	0.96 ± 0.02 ^{b}	1.51 ± 0.03 ^{de}	1.55 ± 0.03 ^{e}	1.48 ± 0.01 ^{d}
Lysozyme (µg/ml)	1.19 ± 0.08 ^{a}	1.43 ± 0.07 ^{b}	0.96 ± 0.02 ^{b}	2.16 ± 0.03 ^{d}	2.74 ± 0.05 ^{f}	2.28±0.03 ^{e}

Different superscripts (a-f) in each column show significant differences at $p < 0.05$.
DP: dissolved protein; ALP: alkaline phosphatase

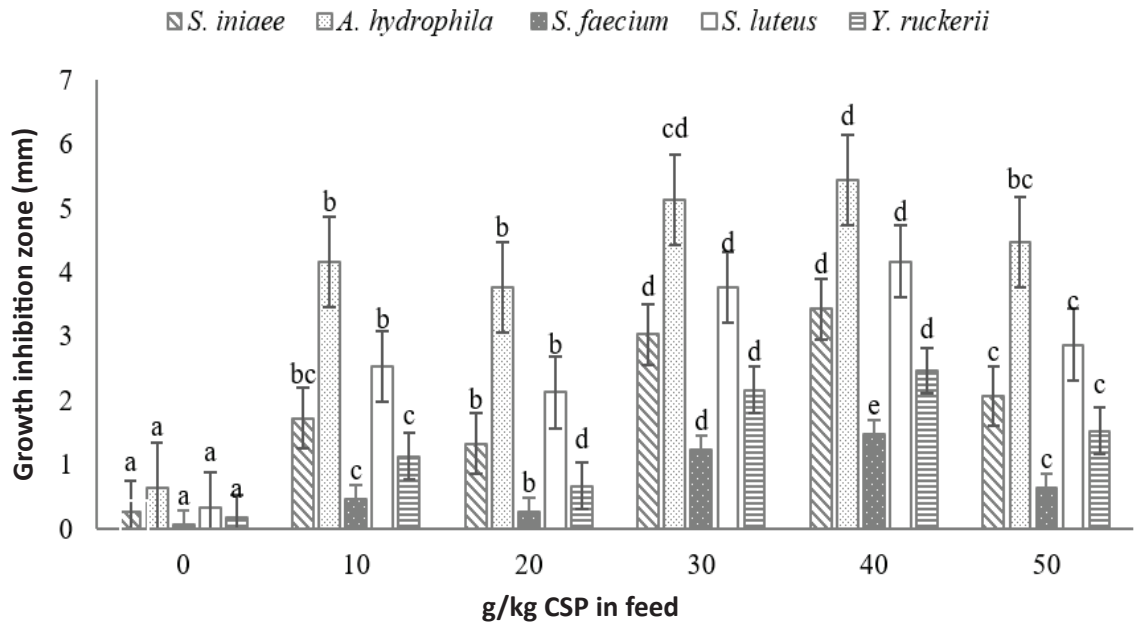

Figure 1
The mean (± SD) of bactericidal activity of juvenile *O. mykiss* fed with different levels of CSP in the diet for 56 days using diffusion disk plates on agar. Bars with different letters are significantly different ($p < 0.05$) against each strain bacteria at three replicates.

units per gram of intestinal sample (CFU/g). The results indicate that a significant increase in TVC and LAB was observed in the intestinal microbiota of CSP-treated fish, compared to the control ($p < 0.05$). The highest TVC and LAB were shown in the diet containing 40 g/kg ($p < 0.05$). The ratio of LAB count to the total viable aerobic bacteria count reached the highest value in the diet containing 40 g/kg and decreased significantly in the diet containing 50 g/kg ($p < 0.05$).

Table 3
TVC (Mean ±SD; $\times 10^6$), LAB ($\times 10^4$) and the ratio of the LAB to TVC ($\times 10^3$) in the intestine of *O. mykiss* fed with diets containing different levels of *C. persica* seed powder (CSP) for 56 days (n = 3) ($p < 0.05$).

	Different levels of dietary CSP (%)					
	0	10	20	30	40	50
TVC (CFU)	4.85 ± 0.49	4.83 ± 0.49	4.75 ± 0.48	4.75 ± 0.48	4.66 ± 0.49	4.79 ± 0.51
LAB (CFU)	3.20 ± 0.35 ^{a}	4.70 ± 0.35 ^{c}	5.00 ± 0.29 ^{c}	5.30 ± 0.31^{cd}	6.10 ± 0.52 ^{e}	3.40 ± 0.39 ^{b}
LAB/TVC	6.60 ± 0.58 ^{a}	9.70 ± 1.00 ^{b}	10.50 ± 1.00 ^{bc}	11.40 ± 0.99 ^{d}	12.70 ± 0.99 ^{d}	6.9 ± 0.52 ^{a}

Different superscripts within a row indicate significant differences at $p < 0.05$.
TVC: Total viable count of aerobic heterotrophic bacteria; LAB: Lactic acid bacteria

Discussion

In recent decades, special attention has been paid to the role of intestinal bacteria regarding the regulation of growth and reproduction in aquatic animals (15). The bacterial communities in the gastrointestinal tract increase the digestibility of proteins, lipids and carbohydrates of the diet by partaking in digestive enzymes (amylase, protease and lipase) secretion (16). LAB are among the main beneficial gut bacteria, and include more than 50 species. These bacteria use carbohydrates as energy and produce lactic acid (16, 17). Some indigestible feed ingredients such as fibers could stimulate lactic acid bacteria propagation and play symbiotic roles (18).

In this study, the inclusion of CSP in the diet of rainbow trout, brought about a significant improvement in growth performance, survival rate and nutritional indices. The findings were similar to that reported by Jayapracas et al. (1997), who investigated the effects of different concentrations of Livol powder in Rohu (Labeo rohita) feed for 112 days. The results indicated that after receiving 2 mg/kg Livol powder, the fish showed the highest SGR and FCR. In the current study, the LAB count increased as a result of the CSP inclusion in the diet, which may suggest that the high fiber content in CSP may contribute to LAB increase. LAB are believed to positively affect growth, FCR and survival rate (19, 20). Therefore, the enhancement of these parameters, may be related to the LAB count increase. However, further studies are needed.

Fish skin mucus is well known to be the first barrier of defense (21), since it contains different immune proteins such as complements, lysozyme, immunoglobulins, protease and lectins (22). Previous studies have demonstrated that fish skin immunity can be improved through the administration of different additives to their diet such as vitamins (23), probiotics (24), prebiotics (25), synbiotics (26) and herbal components (2,27,28). Based on the available information, no studies were found on the effects of CSP on either fish or shellfish skin mucus immunity. The results of the current study indicated that the antibacterial activity in rainbow trout mucus against *S. iniaee*, *A. hydrophila*, *S. faecium*, *S. luteus*, and *Y. ruckerii* increased along with the dietary CSP level of inclusion. However, the mucosal antibacterial activity against all five strains of bacteria decreased with the increment of CSP levels in the diet from 40 to 50 g/kg, an outcome that may indicate that the overdose of CSP supplement can have detrimental effects. Similar results were obtained from other researches (29) which surveyed the effects of Peppermint (*Mentha piperita*) on the skin antibacterial activity of rainbow trout against *Y. ruckeri*. The results presented here also showed that the activity of the immune proteins and total protein content in the skin mucus increased significantly along with CSP presence in the diet. Similar results have been reported in relation to the use of other herbal substances such as garlic (5,30), onion (2,31), peppermint (32), etc., which confirm the increase in the level of blood and mucosal immunity in fish and crustaceans. Given that mucosal lysozyme plays an active role in bactericidal activity, it is likely that mucosal antibacterial activity would be enhanced by stimulated mucus activity (33). This is because the immune proteins in mucus, such as lysozyme and protease, play a vital role in destroying the walls of invasive bacteria (34). The physiological and nutritional status of an aquatic organism plays a significant role in skin mucus immunity such as bactericidal activity (33). In recent studies, it has been found that differ-

ent parts of milkweed leaf powder (35) latex (36), and seed oil (37) have antibacterial activity. Consequently, it can be assumed that including CSP in the diet causes the increase of the activity of skin mucus compounds (lysozymes, proteases, immunoglobulins, etc). However, the role of gut lactic acid bacteria (LAB) increase on improving mucosal immunity cannot be ignored.

Conclusions

In conclusion, this study we demonstrated the suitability of *C. persica* seed powder as feed additive in rainbow trout *O. mykiss* diet. The best level of inclusion was found to be 40 g/kg CSP added to the diet, capable of improving the mucosal immune responses, the gut microbiota composition and the growth performances.

Material and methods

Fish preparation

A total of 360 healthy rainbow trout [*O. mykiss* (Walbaum 1792)] (average weight 11.50 ± 3.64 g), were obtained from a local fish supplier in Mashhad, (Khorasan Razavi, Iran) and maintained at a density of 200 individuals per cement tank (1 m^3). The fish were acclimatized to the laboratory conditions and kept under observation for clinical health for 14 days prior to starting the experiment. The physico-chemical parameters of water, such as temperature (16.15 ± 3.5˚C), dissolved oxygen (7.90 ± 0.14 ppm) and pH (7.1 ± 0.5), were maintained in accordance with the standard values for rainbow trout culture. All experiments were done according to FUM animal ethics.

CSP preparation

C. persica produces lots of seeds which are flat and brown, and have tufts at one end. Seeds used in this study was obtained from nature (Kerman, Jiroft, Iran). After removing tufts, seeds were dried in an oven at 50°C for 24 h and then ground into powder before adding them to the diets (38).

Diet preparation

A control diet (45.03 g/kg BW, crude protein; 18.40 g/kg, crude lipid; 18.80 J kg, crude energy) was developed by WUFFDA (Windows User-Friendly Feed Formulation; University of Georgia, Georgia, USA) software (39) (Table 4). To prepare the experimental diets, *C. persica* seed powder (CSP) at the inclusion levels of 0, 10, 20, 30, 40 and 50 g/kg for basal diet was employed. CSP was replaced with Carboxymethyl cellulose (CMC). Diets were isonitrogenous, isoenergetic. Feed ingredients were converted into a uniform paste by adding water. Thereafter, the dough was passed through a meat grinder with 2 mm diameter holes (40). The obtained "spaghett" were cut into pellets and dried at 30°C for 24 h (41), and stored at 4°C until use. The experiment was carried out in the form of a completely randomized design. Each diet was fed to three replicate tanks for 56 days and rainbow trouts were fed 2% of their body weight thrice daily.

Evaluation of growth performance and survival rate

The experimental fish were weighted at the end of the trial on a digital balance with the accuracy of 0.01 g. Survival rate, growth parameters, feed conversion ratio (FCR), hepatosomatic index (HSI) and Gastro-Somatic Index (GaSI) were calculated as follows (41);

Specific Growth Rate (SGR) = [(Ln final weight (g)–Ln initial weight (g))/experiment days] ×100

Feed Conversion Ratio (FCR) = (Feed consumed (g)/Weight gain (g))

Table 4
Composition (g/kg dry matter) of the control diet fed *O. mykiss*

Ingredient	g/kg (dry-weight basis)
Fish meal	45
CSP	0
Soybean meal	8
Wheat flour	4.5
Corn gluten	5.6
Corn starch	10.3
Fish oil	9
Canola oil	9
Choline chloride	0.1
Vitamin C (stay)	0.5
Vitamin premix1	1.5
Mineral premix2	1
Dicalcium phosphate	0.5%
Carboxymethyl cellulose	5
Chemical composition of control diet	
Dry matter	870.30
Crude protein (%)	45.03
Crude lipid (%)	18.40
Crude energy (jkg-1)	18.80
Chemical composition of CSP	
Dry matter (%)	95.71
Crude protein (%)	4.75
Crude lipid (%)	20.11
Crude fiber (%)	38.57
Ash (%)	5.04
Nitrogen-free extract	27.29
P (%)	1.54
Ca (%)	2.35
Crude energy (jkg-1)	1.98

Mineral premix contains (mg/kg) Mg, 100; Zn, 60; Fe, 40; Cu, 5; Co, 0.1; I, 0.1; Antioxidant (BHT), 100. 2.Vitamin premix contains (mg kg-1) E, 30; K, 3; Thiamine, 2; Riboflavin, 7; Pyridoxine, 3; Pantothenic acid, 18; Niacin, 40; Folacin, 1.5; Choline, 600; Biotin, 0.7 and Cyanocobalamin, 0.02 (provided from kimia rosh© Gorgan, Iran).

Condition factor = (Final weight (g)/Total lenght^3 (cm)) ×100
Survival rate (%) = (Final individual numbers/Initial individual numbers) ×100
Hepato-Somatic Index (HSI) = (Liver weight (g)/Total body weight (g)) ×100
Gastro-Somatic Index (GaSI) = (Gut weight (g)/ Total body weight (g)) ×100

Chemical analyses

Crude protein (Kjeldahl method), crude fat (Soxhlet method), gross energy (parr; electric bomb), crude fiber, ash (electric furnace at 550°C for 6 h), phosphorus (titration with vanadium molybdate) and nitrogen-free extract contents of CSP and control diet (Table 1) were measured according to the standard methods (42).

Skin mucus immunological parameters

At the end of the experiment, the skin mucus samples were collected according to the protocol described by Ross et al. (2000) with some modifications, to monitor antibacterial activity. Briefly, anesthetized fish (clove powder (5 mg/l)) were placed for 2 min in individual plastic bags containing 5 ml of 50 mM NaCl (30 fish per treatment, one by one without pooling). Thereafter, the mucus samples were centrifuged at 1500×g for 10 min at 4°C and the supernatant was stored at -80°C until use.

Protein concentration was determined according to the method of Lowry et al. (1951), using bovine serum albumin as a standard. The absorbance was read using a spectrophotometer (Biochrom, Libra S12) at 750 nm. Skin mucus alkaline phosphatase (ALP) activity was estimated using Pars Azmoun (Pars Azmoon Co, Iran) commercial kit according to the manufacturer's protocol. Lysozyme activity was determined based on the lysis of the lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus*, according to the protocol described by Demers (43). Alternative complement activity was assayed by using rabbit red blood cells (RaRBC) (44) and based on the guidance released by Neissi et al. (45).

Skin mucus antibacterial activity

The skin mucus antibacterial activity of *O. mykiss* fed CSP was checked out against two gram-negative fish pathogens, *Yersinia ruckeri* PTCC 1888 and *Aeromonas hydrophila* ATCC 7966 and three gram-positive bacteria *Streptococcus iniae* PTCC 1887, *Streptococcus faecium* ATCC 19434 and *Micrococcus luteus* PTCC 1169, using the disk diffusion method (46). 0.1 ml of each bacteria (1 ×10^5 CFU ml/1;OD600) was mixed with the nutrient agar medium (Merck, Germany). Paper discs (6 mm diameter) were inoculated with 150 ml of the mucus sample and kept for 20 min to allow the mucus placed on the medium to be absorbed. Plates were incubated for 24 h at 37°C. Finally, the diameter of the inhibition zone was measured by Image J (1.45s) software.

Intestinal bacterial analysis

At the end of the experiment, the fish were anesthetized with clove powder (5 mg/l), disinfected with 70% ethanol and the whole gut was removed (47). Enumeration of the total viable count (TVC) of heterotrophic bacteria and *lactobacillus* bacteria (LAB) in the intestine was assessed using 1 g of posterior intestine sample. The samples were homogenized in 9 ml normal sterile saline solution (0.90% w/v of NaCl) and dilutions prepared to 10^8. Then, 0.1 ml of the saline solution was spread over duplicate plates of plate count agar (for total heterotrophic bacteria) and MRS agar (for LAB). The plates were incubated at room temperature for 72 h. The bacterial colonies were counted in each sample based on the colony forming unit (CFU/g) (colony count × dilution-1= CFU / g intestine) (48).

Statistical analysis

All percentage data were transformed using the arcsine method. Levene's test was used to confirm the homogeneity of variance, while the Kolmogorov-Smirnov test was used to determine the normality of data (Zar, 1999). The data were analyzed using one-way ANOVA and Duncan's multiple range test was applied to determine any significant differences among the treatments ($p < 0.05$).

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Author Contributions

Conceived and designed the experiments: O.S., Performed the experiments: O.S., Analyzed the data: H.A.M., Research space and equipment: O.S., H.A.M., Contributed reagents/materials/analysis tools: O.S., M.P., Wrote the paper: H.A.M.

Conflict of Interest

The authors declare that they have no competing interests.

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The effects of extenders containing proline and glutamine on oxidative stress and motion parameters of stallion semen during cold storage

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ABSTRACT

This study examined the effects of skim-milk based extenders supplemented with proline and glutamine on motility, lipid peroxidation and enzymatic antioxidant status of cooled-stored equine sperm and determined the role of seminal plasma as well. The semen was collected with artificial vagina. In experiment 1, native semen was diluted in skim-milk based extender containing 5mM glutamine and 3mM proline, stored at 5°C and analyzed at 4, 24 and 48 hours storage for motion parameters. In experiment 2, semen was centrifugated, sperm pellet resuspended in the extenders and stored at 5°C for 4 hours to determine motion parameters. The level of catalase, glutathione peroxidase activity and malondialdehyde formation was determined for all samples at 4 hours. Glutamine and proline significantly preserved the percentage of motile sperm (76.5 ± 2.7 and 79.4 ± 1 vs 69 ± 1.4), increased the progressive motility of cold-stored semen (66.1 ± 2.5 and 73.7 ± 2.9 vs 56.2 ± 1.4), increased catalase activity, and decreased malondialdehyde. However these effects were disappeared after seminal plasma removal. We conclude that glutamine and proline would amplify the antioxidant activity of equine cold-stored semen and preserve its motility. This effect seems to be related to interactions with seminal plasma.

Keywords

glutamine, proline, cold storage, stallion, semen, antioxidant activity

Abbreviations

SP: seminal plasma
MDA: malondyaldehyde
CAT: catalase
GPX: glutathione peroxidase

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Introduction

The use of artificial insemination in world quine industry eliminates the detrimental effects of animal transportation and transmission of diseases, as well as providing more opportunities for breeders to make use of genetically superior stallions [1]. The techniques of cooling and transportation of semen, as well as cryopreservation allows artificial insemination to take place, irrespective of the location and availability of stallions. However, it is obvious that the fertility rate for artificial insemination is lower than that for natural service [2].

Oxidative stress is an important factor associated with the decline in fertility of stored semen. During refrigerating storage, the number of viable sperms decreases [3], fertility rate declines [4], and damaging intracellular reactive oxygen species (ROS) is found to be accumulated [5, 6]. Semen is composed of spermatozoa and seminal plasma, endowed with enzymatic and non-enzymatic defense mechanisms against oxidative stress [7]. Seminal plasma is a complex mixture of organic and inorganic substances with controversial effects on sperm properties [8, 9], and is thought to influence the fertility outcome of the cooled-shipped semen. Some studies have revealed that seminal plasma exerts deleterious effects on sperm during cooling transportation, and that the separation of seminal plasma reduces those effects and maximizes post-cooling motility [10, 11]. Removal of seminal plasma has been described to result in increased percentage of motile sperm and membrane and chromatin intactness, both during refrigerated storage, as well as delayed cryopreservation [12-15]. Nevertheless, some studies propose that removal of seminal plasma eliminates the potential protective capacity provided by the SP [16, 17]. This potential can be related to antioxidant properties and ROS scavenging capability of seminal plasma, which can even be enhanced through interacting with semen extenders [18]. However, centrifugation of cooled sperm is thought to be detrimental to sperm motility [13, 19]. The equipment for seminal plasma removal is not always available at the location of semen collection. In cases where farm is far from laboratory, semen must be cooled-shipped and it is necessary to preserve equine semen in extender for several hours without removing seminal plasma. Hence, during transportation, sperms are exposed to seminal plasma. In these cases the modification of semen extender composition has been proposed as an alternative to separation of SP [20]. The protection of cooled equine semen against oxidative stress and loss of motility might be improved by adding some specific components to extenders.

Some amino acids are known to be involved in protecting several types of animal cells against hypothermia [21-23]. The amino acid proline can preserve membrane structure of sperm cells [24], reduces LPO, protects against free radical damages, inhibits intracellular ice formation and influences the motility and velocity [25, 26]. Likewise, amino acid glutamine is known to play a regulatory role in several cell-specific processes [27, 28]. Based on these information, glutamine and proline have been proposed as efficient additives in semen extenders to function as cryoprotectant in several species [24, 27-31]. However, the results of studies considering these amino acids as cryoprotectant have been controversial in terms of improving either the maintenance of motility or the fertility of cooled semen.

The hypothesis of this study was that addition of proline and glutamine to extender could improve the longevity of stallion sperm motility during cold storage. The study was designed to investigate whether the skim-milk based extender supplemented with proline and glutamine could preserve stallion semen for 48 hours of cold transportation. Moreover, the effects of seminal plasma on the enzymatic antioxidant status of extended semen in the presence of these amino acids were evaluated. It will be determined whether the inclusion of glutamine and proline in the extender in the presence and absence of seminal plasma, have any effect on the motility, lipid peroxidation, and enzymatic antioxidant status of stallion sperm during storage at 5°C. Such studies can help breeders to optimize protocols for the transportation of stallion's spermatozoa, which must be maintained for long intervals.

Results

Experiment 1

Mean percent motile and progressive sperm motility of raw semen at the time of collection were 79.5 ± 1.2 and 73.2 ± 1.5, respectively. Effects of semen treatments on the percentage of motile sperm and progressive motility of stallion semen stored up to 48 hours are presented in Table 1. In each group of extenders, (E1, E2, and E3) all the parameters at 4 hours storage were significantly different relative to 24 and 48 hours storage ($p < 0.05$). At 4 hours storage, the percent motile sperm of E2 (76.5 ± 2.7) and E3 (79.4 ± 1.9) were significantly higher than E1 (69 ± 1.4) ($p < 0.05$). The progressive motility of E2 (66.1 ± 2.5) and E3 (73.7 ± 2.9) were significantly higher than E1 (56.2 ± 1.4) ($p < 0.05$). At 24 and 48 hours storage, percent motile sperm and progressive motility of E2 an E3 were significantly higher than E1 ($p < 0.05$) (Table 1). At 4 hours storage, the MAD, BCF and STR of E2 and E3 were significantly decreased compared to E1. The VCL and LIN of E3 were significantly decreased compared

Table 1
The Effects of aminoacids on the percentage of motile sperms and progressive motility of stallion semen cooled to 5°C up to 48 hours.

		Extenders		
		E1	E2	E3
Motion parameters	Time (h)			
Percent motile sperm (%)	4	69 ± 1.4^{a1}	76.5 ± 2.7 ^{a2}	79.4 ± 1.9^{a2}
	24	20 ± 0.9^{b1}	33.9 ± 1.2^{b2}	40.7 ± 0^{b2}
	48	11.2 ± 0.7^{b1}	30.2 ± 0.8^{b2}	34 ± 0.3^{b2}
Progressive motility (%)	4	56.2 ± 1.4^{a1}	66.1 ± 2.5^{2a}	73.7 ± 2.9^{a2}
	24	10.8 ± 1^{b1}	15.8 ± 1.2^{b}	21.5 ± 0 ^{b2}
	48	10.1 ± 0.4^{b1}	14.1 ± 1.8^{b2}	13.9 ± 2.2 ^{b2}

Different numbers in each row and different letters in each column are indicative of statistically significant differences at $p < 0.05$. E1: control extender; E2: the control extender supplemented with 5 mM glutamine, E3: the control extender supplemented with 3 mM proline. Values are mean percentages ± SEM of ten split ejaculates (2 ejaculates x 5 stallions). The control extender contained: INRA82 +2% egg yolk + 2 5% (v/v) glycerol.

to E1 and E2. At 24 and 48 hours storage, differences in kinematic parameters between three groups were not statistically significant (Table 2).

Experiment 2

As shown in Table 3, after 4 hours storage, the percent motile sperm and progressive motility of equine spermatozoa in E4 were not different with E5 and E6. At 48 hours of storage the motility parameters of E4, E5 and E6 were reduced to near zero.

The results shown in Table 4 indicate that the content of MDA is significantly different in E4 (0.7 ± 0.08 nmol/mg protein) relative to E5 (0.18 ± 0.08 nmol/mg protein) and E6 (0.39 ± 0.04 nmol/mg protein) ($P < 0.05$). The MDA of E4 is also significantly higher than E2 (0.35 ± 0.09 nmol/mg protein) and E3 (0.3 ± 0.03 nmol/mg protein). There is no difference between MDA content of E1 with E2, E3 and E4. The activity of GPX remained unchanged in all groups. The activity of CAT enzyme was significantly different between E1 (35.4 ± 7.8 KU/ mg protein), E2 (166.2 ± 21 KU/ mg protein) and E3 (154.6 ± 60 KU/ mg protein). Differences between E4, E5, and E6 were not statistically significant (Table 4).

Discussion

In the present study, the effects of glutamine and proline on motion and kinematic parameters, as well as lipid peroxidation and antioxidant capacity of cooled stallion semen, and their relationship to seminal plasma were tested. The results of experiments 1 and 2 revealed that addition of proline and glutamine significantly preserved the motion parameters of cooled stallion semen over time. In the absence of SP, none of the motion parameters were affected by two used amino acids.

The positive effects of glutamine and proline on stallion sperm subjected to cryopreservation have been reported [27, 30, 32]. The present study revealed that these two amino acids improved the motion parameters of cold-stored native semen, but in groups lacking seminal plasma, motility was unaffected. It was previously proven that in the absence of seminal plasma, addition of extenders composed of nonfat, dried skim milk solids and glucose provide adequate support for the preservation of spermatozoa motility [10]. Our study showed compensatory effect for proline and glutamine in preservation of equine spermatozoa motility in the presence of seminal plasma.

Although the reduction in spermatozoa1 motion parameters in extended stallion semen after 24 hours of cold storage has previously been reported [11], the role of seminal plasma in motion characteristics of sperm in different studies is contradictory. The reduction [9], improvement [10, 11, 33] and no change [34] in spermatozoa1 motility after seminal plasma removal have been reported. Two studies [35, 36] demonstrated no effect for SP during cooling of the stallion semen up to 12 and 24 hours, respectively. Whereas another study [12] speculated that long exposure to seminal plasma in cooled stallion sperm may be responsible for the irreversible loss of motility and DNA degradation. It is thought that centrifugation for SP removal eliminates decapacitating factors in SP [37], resulting in premature capacitation [38]. This has been related to the protective roles of some components of seminal plasma, which is variable in different stallions [39]. The results of Moor et al., indicated that prolongation of the semen incubation time causes undesirable effects, probably due to the presence of seminal

Table 2
Kinematic parameters of stallion semen extended in the control extender (E1), and extenders supplemented with 5 mM glutamine (E2) and 3 mM proline (E3), stored at 5°C for 48 hours.

		Extenders		
		E1	E2	E3
Kinematic parameters	Time (h)			
VCL (µm/s)	T4	77.8 ± 2.3 ^{a1}	73.2 ± 13.5 ^{a1}	69.3 ± 3.2 ^{a2}
	T24	20.7 ± .5 ^{b}	23 ± 2.3 ^{b}	22.8 ± 0 ^{b}
	T48	18.9 ± .5 ^{b}	20.3 ± .5 ^{b}	19.2 ± .8 ^{b}
VSL (µm/s)	T4	41.6 ± 2 ^{a1}	40 ± 7.8 ^{a1}	39.3 ± 1.3 ^{a1}
	T24	6 ± .1 ^{b}	5.9 ± .4 ^{b}	6 ± 0 ^{b}
	T48	4.4 ± .3 ^{b}	5 ± .7 ^{b}	5.2 ± .6 ^{b}
VAP (µm/s)	T4	47.5 ± 2 ^{a1}	46.8 ± 8.9 ^{a1}	46.4 ± 1.4 ^{a1}
	T24	10.2 ± .3 ^{b}	9.9 ± .4 ^{b}	10.8 ± 0 ^{b}
	T48	8.3 ± .4 ^{b}	9.4 ± .6 ^{b}	8.6 ± .5 ^{b}
MAD (°)	T4	46.1 ± 4.1 ^{a1}	24.4 ± 6 ^{a2}	22.8 ± 1.8 ^{a2}
	T24	2.5 ± .2 ^{b}	2.6 ± .1 ^{b}	2.5 ± 0 ^{b}
	T48	2.3 ± .1 ^{b}	2.5 ± .1 ^{b}	3.2 ± .5 ^{b}
ALH (µm)	T4	3.1 ± .1 ^{a1}	3.2 ± .3 ^{a1}	3.1 ± .1 ^{a1}
	T24	1.6 ± .1 ^{b}	1.7 ± .1 ^{b}	1.7 ± 0 ^{b}
	T48	1.5 ± .04 ^{b}	1.6 ± .06 ^{b}	1.6 ± 0.1 ^{b}
BCF (Hz)	T4	1.5 ± .1 ^{a1}	7 ± .2 ^{a2}	.8 ± .05 ^{a2}
	T24	.03 ± 0 ^{b}	.03 ± 0 ^{b}	.02 ± 0 ^{b}
	T48	.02 ± 0 ^{b}	.02 ± 0 ^{b}	.03 ± .02 ^{b}
LIN (%)	T4	48.4 ± 1.6 ^{a1}	49.2 ± 2.4 ^{a1}	43.9 ± 1.1 ^{a2}
	T24	24.2 ± .7 ^{b}	19.5 ± 0 ^{b}	24.3 ± 0 ^{b}
	T48	18.6 ± .8 ^{b}	23.7 ± 3.$^{5\,b}$	23.8 ± 2.9 ^{b}
WOB (%)	T4	59.2 ± 1.4 ^{a1}	62.4 ± 2.1 ^{a1}	57 ± 2.1 ^{a1}
	T24	40.5 ± 2.7 ^{b}	34.1 ± .9 ^{b}	41.8 ± 0 ^{b}
	T48	33.3 ± 1.1 ^{b}	40.6 ± 2.9 ^{b}	37.2 ± 2.3 ^{b}
STR	T4	74.2 ± 4.4 ^{a1}	69.9 ± 1.3 ^{a2}	63.3 ± .7 ^{a2}
	T24	39.2 ± .9 ^{b}	37.7 ± 2.6 ^{b}	42.3 ± 0 ^{b}
	T48	32.9 ± 1.3 ^{b}	39.4 ± 4.2 ^{b}	38.3 ± 3.$^{3\,b}$

Different numbers in each row and different letters in each column are indicative of statistically significant differences at $p < 0.05$. Values are mean percentages ± SEM of ten split ejaculates.

Table 3
The effects of SP and extenders supplemented with amino acids on motion parameters of 4 hours cold storage of equine sperm at 5°C.

	Extenders					
	E1	E2	E3	E4	E5	E6
Motion parameters						
Percent motile sperm (%)	69 ± 1.4[a]	76.5 ± 2.7[b]	79.4 ± 1.9[b]	71.54 ± 1.8[a]	71.6 ± 3.2[a]	72.23 ± 3.4[a]
Progressive motility (%)	56.2 ± 1.4[a]	66.1 ± 2.5[b]	73.7 ± 2.9[b]	73± 2 ± 0.0[b]	72.2 ± 4.3[b]	73.6 ± 3.2[b]

Different subscriptions: Significant difference between control and supplemented groups at $p < 0.05$ in each row. Values are mean percentages ± SEM of ten split ejaculates (2 ejaculates x 5 stallions).

Table 4
Effects of control extenders supplemented with aminoacids on lipid peroxidation and antioxidant activity of cooled stallion semen and spermatozoa.

	Extenders					
	E1	E2	E3	E4	E5	E6
parameters						
MDA (nmol/mg protein)	.49 ± 0.1[a,b]	0.35 ± 0.09[b]	0.3 ± 0.03[b]	0.7 ± 0.08[a]	0.18 ± 0.08[b]	0.39 ± 0.04[b]
GPX (IU/mgr protein)	2713 ± 28	3116 ± 225	2731 ± 124	3286 ± 378	2471 ± 40	3053 ± 357
Catalase (KU/ mgr protein)	35.4 ± 7.8[a]	166.2 ± 21[b]	154.6 ± 60b	74.5 ± 65	65 ± 9.6	73 ± 27

Different subscriptions: Significant difference between control and supplemented groups at $p < 0.05$ in each row. Values are mean ± SEM of ten split ejaculates (2 ejaculates ×5 stallions). the levels of malondialdehyde formation (MDA) as an indicator of lipid peroxidation and the activity of glutathione peroxidase (GPx), catalase (CAT) and Superoxide Dismutase (SOD) as indicators of antioxidant activity are presented.

plasma [40]. Our results revealed that the decrease in motion parameters of stallion sperm during cold storage was compensated by addition of extenders containing aminoacids. and this positive effect of aminoacids became evident only in the presence of seminal plasma. It seems that the interaction between proline and glutamine with seminal plasma is responsible for such compensatory effects.

Based on our results, glutamine and proline enhanced CAT activity in the presence of SP. This is when the motility of sperm is improved. Interestingly, MDA was decreased in groups containing aminoacids irrelevant to seminal plasma. Seminal plasma contains organic and inorganic substances such as high levels of enzymatic and non-enzymatic antioxidants. It is indicative of an important role that SP plays in the preservation of spermatozoa against degradation caused by ROS [41, 42]. However, It has been demonstrated that the interactions of an enzyme or a protein of bulbourethral origin in SP [43] with egg yolk [44] or milk [20] of extenders is detrimental to sperm. On the other hand, stallion seminal plasma is characterized by high sodium concentration, which is thought to induce spontaneous lipid peroxidation of the sperm membrane, resulting in decreased membrane fluidity and fertilizing capacity [45, 46]. Thus, it is expected that high concentration of seminal plasma would be deleterious to sperm cells subjected to cooling and storage [33, 34]. In order to solve this problem and to maximize post-cooling motility, separation of seminal plasma and addition of semen extender have been suggested [10, 11]. The single semen extender induces a compensatory effect on non-enzymatic antioxidant activity after seminal plasma removal. This is due to the composition of the extender, which shows enough antioxidant activities for semen protection against oxidative stress [47]. On the other hand removal of seminal plasma eliminates the potential protective capacity provided by SP [16], and centrifugation may not be easily available in the field as well, so the modification of extender composition to prevent enzymatic reactions might be more acceptable for cooling storage instead of SP removal.

Several studies have shown that supplementation of semen extenders with glutamine and proline can improve total sperm motility of cooled ram sperm, protect sperm cells against free-radical-induced damage, and provide cryoprotection to ram sperm by minimizing lipid peroxidation both at pre-freeze and post-thaw semen [48]. Rudolph [49] stated that proline may interact with phospholipid bilayers and stabilize the membrane structures during freezing. Similarly, in this study, inclusion of glutamine and proline to the extender reduced malondyaldehyde for-

mation, which was concomitant with positive changes in motion parameters, which shows that lipid peroxidation was inhibited by proline and glutamine during cooling storage. This was confirmed by another study showing that the positive role of proline for the motility of freeze-thawed stallion spermatozoa [30]. In the same way, Pagl [50] explained the dysfunctional role of plasma membrane and mitochondrial membrane in the loss of cooled-stored sperm motility.

We also found that CAT activity, as well as motion parameters increase in cooled semen in the presence of proline and glutamine with SP. Bucak [23] hypothesized an antioxidative effect for glutamine through increasing CAT activity, and Baumber [51] demonstrated that CAT enzyme prevents negative effects of ROS on motility and DNA fragmentation of equine sperm. On the other hand, Kancofer [18] proposed that the interactions among components of extenders and antioxidative enzymes of seminal plasma boost the antioxidative/oxidative capacity of stallion seminal plasma. This means that some factors in extenders influence activities of antioxidant enzymes present in seminal plasma. In addition, amino acids in combination with glycerol affect calcium ATPase of sperm cells [52], as well as the phosphate groups of sperm plasma membrane phospholipids for protection against thermal shock [53]. In this research, we found that removal of seminal plasma significantly conversed the effect of glutamine and proline on antioxidant enzymes. Therefore, it appears that these aminoacids need a certain amount of seminal plasma for enhancing enzymatic antioxidative capacity during cooling storage, which preserve motion parameters of cold-stored sperm as well.

It is concluded that the skim-milk extender containing glutamine and proline amplify the antioxidant activity of equine cold-stored semen, as well as motility preservation. This effect seems to be related to interactions with seminal plasma.

Material and methods

Semen collection

Five fertile stallions aged between 4-9 years were used in this study. They were on a routine semen collection schedule and fed balanced diet. Semen samples were collected using artificial vagina, and two ejaculates were obtained from each stallion. They had already been included in previous semen cryopreservation program, and had been shown to have good semen freezability [30].

Reagents

Unless otherwise indicated, all reagents used in the experiments were obtained from Sigma-Aldrich Company (St. Louis, MO).

Preparation of extenders and semen

Table 5
Osmotic pressure and pH values of the extender media used for cooling storage of semen samples.

parameters	Osmotic pressure (mOsm/kg)	pH
BM	6.8	336
BM+ 5 mM glutamine	6.8	338
BM+ 3 mM proline	6.8	337

BM: INRA82 medium+ 20mM HEPES/L + 2% centrifuged egg yolk and 2.5%, v/v glycerol

All extender media were derived from basal medium (BM), composed of INRA 82 medium (0.5 L saline solution: 25 g glucose, 1.5 g lactose, 1.5 g raffinose, 0.25 g sodium citrate dihydrate, 0.41 g potassium citrate, 50,000 IU penicillin, 50 mg gentamycin and 0.5 L skim milk) reaching final concentration of 2% (v/v) centrifuged egg yolk, 20 mM HEPES and 2.5% v/v glycerol (pH: 6.8) [32].

Aliquots of BM extender were supplemented with different concentrations of proline and glutamine (Merck corporation, Germany): 0 (E1 and E4 as control group), 5 mM glutamine (E2 and E5) and 3 mM proline (E3 and E6) (pH: 6.6- 6.8). Osmotic pressure of the extender media was measured with an automatic osmometer (OSMOMETER 800c l, SLAMED, Germany) (Table 5).

A total of ten Ejaculates were collected using a Missouri-model artificial vagina. After removing the gel fraction from the ejaculate by filtration through gauze, the gel-free portion of the ejaculate was evaluated for volume and progressive motility, as well as measuring spermatozoa concentration by a hemocytometer. One part of the raw semen was diluted 1:1 by adding: basal medium as the control (E1), basal medium containing 5mM glutamine (E2) and basal medium containing 3mM proline (E3). Then, it was cooled to 5°C, and stored for 48 hours.

The other part of native semen was submitted to centrifugation for seminal plasma removal, and spermatozoa were resuspended to a concentration of 25×10^6 motile sperms/ml in three types of extenders: basal medium as the control (E4), basal medium containing 5mM glutamine (E5), and basal medium containing 3mM proline (E6). Then, it was cooled to 5°C and stored for about 4 hours.

Experimental design

In Experiment 1, routine semen analysis was performed in cooled semen E1, E2 and E3 at 4, 24 and 48 hours storage at 5°C.

In experiment 2, changes in malondyaldehyde (MDA) formation (TBARS concentration as an indicator of lipid peroxidation), enzymatic antioxidant activities of catalase and glutathione peroxidase as well as CASA parameters for all 6 groups were monitored in cooled semen samples after 4 hours storage.

Evaluation of Semen Motion and kinematic parameters

Semen quality analysis was performed using the CASA system (HFT CASA- Houshmand Fanavar- Tehran- Iran) to evaluate the sperm motion parameters. Sperm samples (10 mL) were diluted in 0.5 mL of phosphate-buffered saline (PBS) and subjected to CASA. Five field images (minimum of 500 cells) were randomly selected and analyzed for the following parameters: motile sperm and progressively motile sperm percentages, as well as sperm kinematic parameters, including amplitude of lateral head displacement (ALH), beat cross frequency (BCF), curveilinear velocity

(VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN = VSL/VCL), mean angular displacement (MAD), wobble (WOB= VAP/VCL) and straightness (STR = VSL/VAP).

Biochemical analysis

Biochemical assays were performed on the sperm samples homogenized by an ultrasonic homogenizer (FAPAN, Iran); then, total protein level of sperm was measured using a Bio-Rad protein assay kit (Bio Rad, Hercules, CA, USA). The levels of MDA, GPX and CAT were measured in protein content of sperm cells.

Concentration of malondialdehyde, as indices of the LPO, was determined colorimetrically using the method of Buege & Aust [54]. Briefly, 0.1 mL of sperm homogenate was treated with 2 mL of TBA–TCA–HCl reagent, placed in water bath for 15 min, cooled, centrifugated and then the absorbance of supernatant was measured against reference blank at 535 nm (Unico 2100, United Products Instruments, Inc., Dayton, NJ, USA). Concentrations were calculated using an extinction coefficient of 1.56×10^5 mol/L/cm. The MDA concentration was expressed in nmol/ml.

Activity of glutathione peroxidase was estimated according to the method of Lawrence & Burk [55], in which the reaction mixture containing potassium phosphate buffer, EDTA, sodium azide, NADPH, glutathione reductase and reduced glutathione was incubated for 5 min at 25°C, and after adding H_2O_2 and sperm homogenate, the absorbance changes at 340 nm was monitored for 1 min. One unit of GPX activity was reported as µmol NADPH consumed per min per mg sperm protein, using the appropriate molar absorptive coefficient for NADPH (6220 mol/L/cm).

Activity of sperm catalase was assayed according to the method of Goth [56]. Briefly, sperm homogenate samples were incubated in the reaction mixture composed of potassium phosphate buffer and H_2O_2, and 60 seconds later ammonium molybdate solution was added to terminate the reaction. The absorbance of the yellow color of this complex was measured at 405 nm. One unit of catalase activity was defined as the amount of enzyme that catalyzes the decomposition of 1 µmol of hydrogen peroxide per minute.

Statistical Analysis

Data were expressed as the mean ± SEM. Statistical analysis was carried out using SPSS 14.0 (SPSS, Chicago, IL, USA). The normality and homogeneity of variables were confirmed using Shapiro–Wilk and Levene's tests, respectively. One-way analysis of variance followed by Tukey's post hoc test was performed to compare the differences between groups. The repeated measure analysis of variance was performed for compression of indices during the cold storage period. The differences were considered significant at $p < 0.05$.

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Author Contributions

Conceived and designed the experiments: ND. Performed the experiments: ND, AK. Analyzed the data: AK, AA. Research space and equipment: ND, AK, AA. Wrote the paper: ND.

Conflict of Interest

We wish to confirm that there is no known conflict of interest associated with this publication.

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Abstracts (in Persian)

شیوع تومورهای پوستی سگ از شیراز، ایران

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چکیده

مطالعه حاضر بر روی ۴۲ سگ با تشخیص هیستوپاتولوژیک تومور پوستی که از آوریل ۲۰۱۲ تا دسامبر ۲۰۱۷ به درمانگاه دامپزشکی دانشگاه شیراز ارجاع شده بود، انجام شد. همه موارد بجز تومورهای غدد پستان مورد بررسی قرار گرفت. نوع تومور از نظر هیستوپاتولوژی، شیوع، جنس، سن، نژاد و توزیع محل تومورها توصیف شد. علاوه بر این، مطالعات منتشر شده بر روی تومورهای پوستی سگ از سایر مناطق جغرافیایی مورد بررسی قرار گرفت و با نتایج مطالعه حاضر مقایسه شد. از نظر هیستوپاتولوژی، ۱۵ نوع تومور مختلف تشخیص داده شد. شیوع تومورهای اپیتلیالی، مزانشیمی، ملانوسیتیک و لنفوهیستیوسیتیک به ترتیب ۹/۶۱، ۷/۳۵، ۴/۲ و صفر درصد بود. شایع-ترین تومورها آدنوم غدد چربی (۴۲/۲۱ ٪)، کارسینوم سلول سنگفرشی (۹/۱۱ ٪) و لیپوم (۹/۱۱ ٪) بودند. فراوانی این تومورها بیش از سایر تحقیقات بود. اگرچه توضیح واضحی برای این تفاوت های جغرافیایی وجود ندارد، اما ممکن است مکان جغرافیایی، اثرات محیطی، جمعیت مورد مطالعه و نژاد موثر باشد. تریر شایع ترین نوع سگ در این مطالعه (۴/۳۴ ٪) بود. یافته های حاضر در مورد سن سگ های مبتلا به تومورهای پوستی مختلف و مکان های آناتومیکی نشان می دهد که تفاوت قابل توجهی در این پارامترهای مهم، بین سگ های ایرانی و سایر نقاط جهان وجود ندارد. این اطلاعات پیش از این درباره جمعیت سگ های ایران منتشر نشده است.

واژگان کلیدی

تومورهای پوستی، سگ، هیستوپاتولوژی، ایران

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عفونت تجربی قرقاول ها با ویروس حاد نیوکاسل جدا شده از ماکیان گوشتی

رمضانعلی جعفری[1]، آناهیتا رضایی[2]، زهرا برومند[1]، منصور میاحی[1]، رضا زارع[3]

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چکیده

بیماری نیوکاسل یک عفونت واگیردار در بسیاری از گونه های پرندگان است که خسارت زیادی به صنعت طیور دنیا وارد می کند. این مطالعه با هدف تعیین چهره بالینی، دفع ویروس و پاسخ ایمنی به دنبال آلودگی با ویروس حاد نیوکاسل جدا شده از ماکیان گوشتی در قرقاول های حساس و واکسینه انجام گرفت. هشتاد جوجه قرقاول یکروزه به ۴ گروه تقسیم شدند. در ۳۰ روزگی، گروه های ۱ و ۳ با واکسن B1 به روش قطره چشمی واکسینه شدند. دو هفته بعد، هر پرنده در گروه های ۱ و ۲ با 105EID50 (number 5 have to be in superscript) ویروس نیوکاسل در حجم ۱۰۰ میکرولیتر (۵۰ میکرولیتر در هر چشم) چالش شد. تمام گروه ها برای ۳ هفته زیر نظر بودند. نمونه های سوآب در مقاطع زمانی مختلف تهیه و از نظر آلودگی به ویروس نیوکاسل به وسیله واکنش زنجیره ای پلیمراز معکوس (RT-PCR) بررسی گردیدند. پاسخ ایمنی نیز با آزمایش ممانعت از هماگلوتیناسیون بررسی شد. قرقاول های گروه ۱ فقط در یک مورد مدفوع موکوسی آبکی داشتند. قرقاول های گروه ۲ نشانه های بی اشتهایی، افسردگی و انحراف سر را بروز دادند. از ۲۰ پرنده در گروه ۲، یک مورد (۵ درصد) تلف شد. بر اساس RT-PCR، دفع ویروس نیوکاسل فقط در قرقاول های غیرواکسینه و در روزهای ۵ تا ۱۴ بعد از چالش مشاهده گردید. میزان ردیابی ویروس به وسیله سوآب نای (۴۰ درصد) بیش تر از سوآب کلواک (۳۰ درصد) بود. تغییرات سرولوژیک در قرقاول های آلوده نیز معنی دار بود. نتیجه گیری می شود که ویروس حاد نیوکاسل موجود در گله های ماکیان ایران، بیماری زایی کمی برای قرقاول ها دارد و واکسن قطره چشمی B1 می تواند محافظت خوبی ایجاد کند.

واژگان کلیدی

پاسخ ایمنی، بیماری نیوکاسل، قرقاول، دفع ویروس

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اثر تمرین مقاومتی و تزریق هورمون رشد بر سطوح در گردش IGF–۱ و IGFBP–۳ در مدل موش های صحرایی

بهنام روزبه[۱]، مهتاب معظمی[۲]، امیر رشیدلمیر[۲]، زهرا موسوی[۳]، علی جوادمنش[۴]

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۳ گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران
۴ گروه علوم حیوانی، دانشکده کشاورزی، دانشگاه فردوسی مشهد، مشهد، ایران

چکیده

هورمون رشد اثرات میتوژنیک و آنتی- آپوپتوزیس دارد و زمانی که بیش از اندازه بیان شود ممکن است تکثیر و تغییر شکل سلولی را افزایش دهد. این مطالعه به بررسی اثرات تمرین مقاومتی و تزریق هورمون رشد بر سطوح در گردش IGF-1 و IGFBP-3 و نسبت IGF-1/IGFBP-3 در موش های صحرایی نر پرداخته است. ۳۲ موش صحرایی نر به طور تصادفی به گروه کنترل (n=۸)، گروه تمرین مقاومتی (n=۸)، گروه تزریق هورمون رشد (n=۸) و گروه تمرین مقاومتی+ تزریق هورمون رشد (n=۸) اختصاص داده شدند. برنامه تمرین مقاومتی شامل صعود از نردبان (پنج روز در هفته، سه ست، پنج تکرار) با حمل یک وزنه آویزان به دم، بود. هورمون رشد (2mg/kg/day, 5days/week) یک ساعت قبل از جلسه تمرین تزریق شد. سطوح IGF-1 و IGFBP-3 سرمی و نسبت IGF-1/IGFBP-3 پس از هشت هفته اندازه گیری شدند. آزمون تجزیه و تحلیل واریانس یک طرفه (ANOVA) برای مقایسه سطوح سرمی IGF-1 وIGFBP-3 بین گروه ها استفاده شد. سطح سرمی IGF-1 و نسبت IGF-1/IGFBP-3 به طور معنی داری در گروه تمرین مقاومتی در مقایسه با گروه کنترل کاهش یافت، اما سطح سرمی IGFBP-3 تغییر معنی داری را نشان نداد. همچنین، سطوح سرمی IGF-1 وIGFBP-3 و نسبت IGF-1/IGFBP-3 در گروه تزریق هورمون رشد و گروه تمرین + تزریق در مقایسه با گروه های دیگر به طور معنی داری افزایش یافت. در نتیجه، تمرین مقاومتی سطوح سرمی IGF-1 و نسبت IGF-1/IGFBP-3 را در شرایط طبیعی، کاهش می دهد. از طرف دیگر، تزریق هورمون رشد با و بدون تمرین مقاومتی سطوح سرمی IGF-1 و نسبت IGF-1/IGFBP-3 را افزایش می دهد که می تواند به عنوان شرایطی با خطر بیشتر نئوپلاسم در نظر گرفته شود.

واژگان کلیدی

تمرین مقاومتی، تزریق هورمون رشد، IGF-1، IGFBP-3، سرطان

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Abstracts (in Persian)

نقش گیرنده فاکتور آزادکننده کورتیکوتروپین (CRF) بر اثرات بی اشتهایی القا شده با LPS در جوجه های نوزاد

حسین جنیدی[1]، مهدی عباس نژاد[2]، محدثه سلطان نژاد[1]، عبدالحمید شریفی مهر[2]، راضیه کوشکی[2]، منوچهر یوسفی[2]، مبین آقاپور[2]

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چکیده

بی اشتهایی یک قسمت پاسخ فاز حاد (APR) است. لیپوساکارید (LPS) به شکل متداول برای تقلید و القا APR استفاده می شود. مکانیسم بی اشتهایی همراه با APR در جوجه شناخته نشده است. در مطالعه حاضر درگیری احتمالی فاکتور آزادکننده کورتیکوتروپین(CRF) بر اثرات بی اشتهایی القا شده با LPS در جوجه های نوزاد مورد بررسی قرار گرفت. برای این هدف، دوزهای متفاوت LPS به شکل مرکزی و محیطی برای بررسی آثار آن بر مصرف غذا توسط جوجه اعمال شد. سپس اثرات تزریق درون بطنی astressin به عنوان آنتاگونیست گیرنده CRF بر بی اشتهایی القا شده با LPS بررسی گردید. مصرف غذا به دنبال تزریق مرکزی و محیطی LPS کاهش یافت. پیش درمان با astressin توانست اثرات تزریق مرکزی LPS را کاهش دهد. نتایج حاضر نشان می دهد گیرنده CRF در بی اشتهایی القا شده با LPS درگیر می باشد.

واژگان کلیدی

لیپوساکارید، بی اشتهایی عصبی حاد، گیرنده های فاکتور آزاد کننده کورتیکوتروپین، جوجه های نوزاد

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Abstracts (in Persian)

اثر زهر مارهای افعی ایرانی، زنجانی و لطیفی بر زنده مانی سلو ل های بنیادی مزانشیمی استخراج شده از مغز استخوان رت بصورت داخل و برون تنی

فاطمه سلامی[1]، فاطمه یونسی سلطانی[1]، امین توسلی[2]، بهروز فتحی[1]

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چکیده

مغزاستخوان یک منبع اصلی سلولهای بنیادی مزانشیمی (MSCs) است. آنها بدلیل ویژگی های خاص خود قادرند سلول های بالغ بافتی خاص، تولید کنند. سلولهای بنیادی مزانشیمی یکی از نویدبخش ترین، انواع سلول های بنیادی برای پزشکی بازساختی درنظر گرفته می شوند. زهرمار یک ترکیب پیچیده با مواد مختلف با خصوصیات فارماکولوژیک است. هدف از این تحقیق، بررسی اثر زهرمارهای افعی آلبیکورنوتا vipera albicornuta (زنجانی) و افعی لطیفی vipera latifii (لطیفی) بر رفتار سلول های بنیادی مزانشیمی در محیط کشت بود. سلول های بنیادی مزانشیمی ازمغز استخوان رت استخراج شدند و به فلاسک های حاوی محیط کشت، شامل ۱۰ در صد سرم جنین گاوی، ۱ در صد پنی سیلین-استرپتومایسین و ۰.۱ درصد آمفو تریسین بی، انتقال یافتند و در انکوباتر با دمای ۳۷ درجه سانتی -گراد و رطوبت ۹۵ درصد و منواکسید کربن ۵ درصد قرار گرفتند. سلول ها بعد از ۴ الی ۵ روز به کف فلاسک ها چسبیدند. سلول ها با غلظت های مختلف (۱، ۲، ۳، ۴، ۵ µg/100µl) از زهر های نام برده به مدت ۲۴، ۴۸ و ۷۲ ساعت in vitro و in vivo تیمار شدند. سلول ها در شرایط in vivo تنها با زهرمار افعی لطیفی در غلظت ۱µg/۱۰۰µl و فواصل زمانی مشابه آزمایش in vitro تیمار شدند. زنده مانی سلولی با استفاده از تستMTT با الایزاریدر در ۵۷۰ nm برآورد شد. بیشترین زنده مانی سلولی در غلظت ۱µl/۱۰۰µg و ۲ برای زهر مار زنجانی و لطیفی به ترتیب بعد از ۴۸ و ۷۲ ساعت بود. نتایج آزمایش in vivo نشان داد که سلول های تیمار شده با زهر افعی لطیفی برای ۷۲ ساعت در شرایط in situ بالاترین نرخ تکثیر را بعد از پاساژهای ۳ و۴و۵ در مقایسه با کنترل داشتند. زهر مارهای زنجانی و لطیفی می توانند برتراکم و رفتار سلول های بنیادی مزانشیمی اثر بگذارند.

واژگان کلیدی

سلول های بنیادی مزانشیمی، زهر، مار افعی، کشت سلول، مغز استخوان

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تاثیر ضد باکتریایی اسانس اسطوخودوس و مرزنجوش بر استافیلوکوک آرئوس، استرپتوکوک آگالاکتیه و اشریشیاکلی

سمیرا نوری[1]، رضا راه چمنی[2]، جواد بیات کوهسار[2]، فاطمه بحری بیناباج[2]

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۲گروه علوم دامی، دانشکده کشاورزی و منابع طبیعی، دانشگاه گنبد کاووس، گنبد کاووس، ایران

چکیده

در این مطالعه اثر ضد باکتریایی اسانس اسطوخودوس و مرزنجوش بر سه باکتری بیماریزا شامل استافیلوکوک آرئوس، استرپتوکوک آگالاکتیه و اشریشیاکلی بررسی شد. آنالیز اسانس ها به روشGC/MS نشان داد که مهمترین ترکیبات اسطوخودوس 17-pentatriac-ontene، linalyl acetate، eucalyptol، linalool و مرزنجوش α-terpineol، 3-Cyclohexen-1-ol,4-methyl-1-(1-methylethyl)-(R)- و P-cymene بودند. روش رقت سازی لوله ای با استفاده از شیر کامل اتوکلاو شده به جای محیط کشت مایع برای تعیین حداقل غلظت مهاری و حداقل غلظت کشندگی اسانس ها به تنهایی و در ترکیب با هم استفاده شد. علاوه بر آن منحنی رشد باکتری ها در حضور اسانس ها و در محیط شیر تا ۲۴ ساعت رسم شد. محدوده حداقل غلظت مهاری و حداقل غلظت کشندگی به ترتیب برای اسطوخودوس ۳/۱۲-۴/۳۷ ٪، ۶/۲۵-۸/۷۵ ٪، مرزنجوش۱/۷۸-۰/۶۲ ٪، ۱/۲۵-۳/۷۵ ٪ و ترکیب آنها ۵-۲/۵٪، ۱۰-۵٪ بود. از نظر منحنی رشد، اسانس اسطوخودوس و مرزنجوش باعث کاهش معنی دار تعداد باکتری ها در ساعت های ۴، ۱۰ و ۲۴ شدند. در مجموع اسانس مرزنجوش اثر ضدباکتریایی قوی تری نسبت به اسطوخودوس علیه سه باکتری مورد مطالعه داشت و می تواند در مطالعات آینده به عنوان آنتی بیوتیک یا به همراه آنتی بیوتیک در درمان ورم پستان گاو مورد بررسی قرار گیرد.

واژگان کلیدی

اثر ضد باکتریایی، اسطوخودوس، دامداری ارگانیک، مرزنجوش

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تاثیر استفاده از سطوح مختلف پودر بذر استبرق (*Calotropis persica*) بر شاخص های رشد، ایمنی و میکروبیوتای روده ماهی قزل آلای رنگین کمان (*Oncorhynchus mykiss*)

حمیدرضا احمد نیای مطلق[1]، امید صفری[1]، مارینا پائولسی[2]

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۲ گروه علوم و تکنولوژی، دانشگاه سانیو، بنونتو، ایتالیا

چکیده

مطالعه ای به مدت هشت هفته، جهت بررسی امکان استفاده از شش سطح (۰، ۱۰، ۲۰، ۳۰، ۴۰ و ۵۰ گرم بر کیلوگرم جیره پایه) پودر بذر استبرق (*Calotropis persica*) در رژیم غذایی ماهی قزل آلای رنگین کمان (*Oncorhynchus mykiss*)، در قالب طرح کاملا تصادفی انجام شد. برای انجام آزمایش، ۳۶۰۰ بچه ماهی (۳/۴ ± ۱۱/ ۵ گرم) به مدت ۵۶ روز تحت تیمار قرار گرفتند. بر اساس نتایج، افزایش پودر دانه استبرق تا ۴۰ گرم در کیلوگرم باعث افزایش قابل توجهی در نرخ رشد ویژه و تعداد باکتری های اسید لاکتیک روده در مقایسه با شاهد شد (P <۰/۰۵). بهترین نتایج مربوط به بقاء، ضریب تبدیل غذایی، شاخص های گنادی و روده ای در تیمارهای ۲۰، ۳۰، ۴۰ و ۵۰ گرم درکیلوگرم در روز (P <۰/۰۵) به دست آمد. فعالیت آنتی باکتریال موکوس پوست، لیزوزیم و آلکالین فسفاتاز بالاترین سطح را در تیمار ۴۰ گرم در کیلوگرم نشان داد (P <۰/۰۵). بر اساس نتایج بدست آمده، پودر دانه استبرق به میزان ۴۰ گرم بر کیلوگرم باعث بروز تأثیرات مثبت ایمنی شده و می تواند افزودنی گیاهی مناسبی در رژیم غذایی ماهی قزل آلای رنگین کمان باشد.

واژگان کلیدی

Calotropis persica، رشد، ایمنی، میکروبوتای روده، *Oncorhynchus mykiss*

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Abstracts (in Persian)

تاثیر محیط نگهدارنده حاوی گلوتامین و پرولین بر روی استرس اکسیداتیو و پارامترهای حرکتی منی نریان در زمان نگهداری به صورت سرد شده

نجمه داودیان[1]، علی کدیور[2]، ابراهیم احمدی[1]

۱ پژوهشکده فناوری جنین دام، دانشگاه شهرکرد، شهرکرد، ایران
۲گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه شهرکرد، شهرکرد، ایران

چکیده

این مطالعه اثرات محیط نگهدارنده غنی شده با اسیدآمینه های پرولین و گلوتامین را بر تحرک اسپرم، پراکسیداسیون لیپیدی و وضعیت آنتی اکسیدانی منی سرد شده اسب مورد بررسی قرار داد و همچنین نقش پلاسمای منی را نیز مشخص کرد. منی با واژن مصنوعی جمع آوری شده و با محیط نگهدارنده حاوی ۵ میلی مول گلوتامین و ۳ میلی مول پرولین مخلوط شده و در دمای ۵ درجه سانتیگراد نگهداری شدند و در آزمایش اول، پارامترهای حرکتی در زمان های ۴، ۲۴ و ۴۸ ساعت مورد آنالیز قرار گرفت. در آزمایش دوم، پلت حاصل از سانتریفیوژ منی ، در محیط های پایه و محتوی آمینو اسیدها مخلوط شده و پس از چهار ساعت نگهداری در دمای ۵ درجه سانتی گراد، مورد ارزیابی پارامترهای حرکتی قرار گرفت. سطح فعالیت کاتالاز، گلوتاتیون پراکسیداز و تشکیل مالون دی آلدهید برای تمامی نمونه ها در زمان ۴ ساعت سردسازی اندازه گیری شد. نتایج نشان داد که گلوتامین و پرولین بطور مشخصی باعث حفظ پارامترهای حرکتی اسپرم نریان شدند، فعالیت کاتالاز را افزایش داده و تولید مالون دی آلدهید را کاهش دادند. با این وجود پس از حذف سمینال پلاسما، این اثرات آمینواسیدها نیز مشاهده نشد. نتیجه گیری می شود که گلوتامین و پرولین فعالیت آنتی اکسیدانی منی سردشده اسب را تقویت کرده و تحرک اسپرم را حفظ می کنند و به نظر می رسد این نقش به اثرات متقابل با پلاسمای منی ارتباط داشته باشد.

واژگان کلیدی

پر، فعالیت آنتی اکسیدان، سیمن نریان، سردسازی، تامین

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IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

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Acknowledgements

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Discussion should include the answer to the question proposed in the introduction and emphasize 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.

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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 Jan;62(1):112-6.

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

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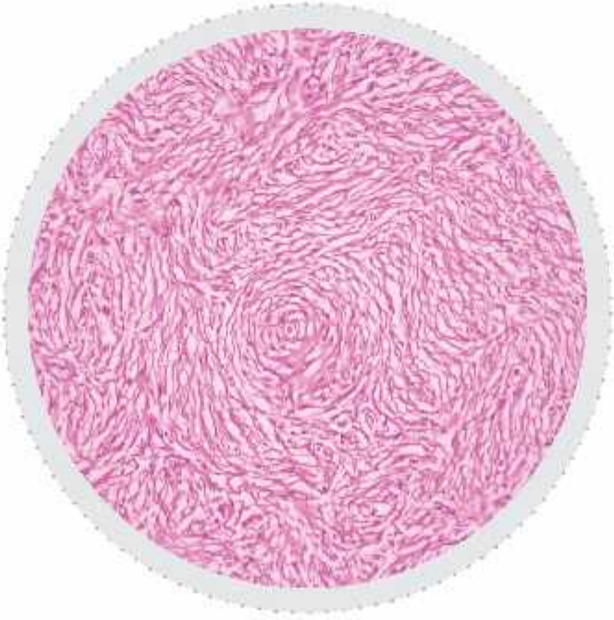

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