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Investigate the gene expression of interleukins 1 and 6 in the testis and sperm characteristics of roosters during puberty

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ABSTRACT

During the puberty period of the rooster, important changes occur in the function of the testes. In addition to changes in the process of spermatogenesis, the expression of various genes such as interleukins (***ILs***) also occurs in the testes. Interleukins are cytokines which are produced by many non-immune cells in non-pathological conditions. *IL-1* and 6 are cytokines that express in sertoli cells, leydig cells and spermatogenic cells which have immune protective role in the process of spermatogenesis. Therefore, the present study was conducted as a completely randomized design with 4 treatments (roosters ages) and 6 replications to investigate the changes of interlukins 1 and 6 gene expression and the changes of sperm characteristics in the tetes of roosters during the puberty. For this purpose, 24 native roosters aged 5, 6, 7, and 8 months were purchased from the native chicken breeding center. After slaughtering and withdrawing the visceral organs of the roosters, semen samples were collected from vasdeferens and the characteristics of the semen were immediately determined. The testicle samples were also transferred to the laboratory to extract the interleukins 1 and 6 mRNA genes and check their expression changes. The results showed that the percentage of motile spermatozoa was the lowest at 5 months and did not change significantly from 6 to 8 months. The percentage of live sperm was lower at 5 and 6 months compared to 7 and 8 months, and at the same time, the semen volume and sperm concentration were the lowest at 5 months and the highest at 8 months. The results of interleukins gene expression also showed that *IL-6* gene was more expressed at 6, 7 and 8 months than at 5 months, while the expression of this gene increased significantly at 7 months compared to 6 months, but *IL-1* gene had no significant expression at these ages. The overall conclusion showed that there is an alignment trend between changes in sperm concentration, semen volume and interleukin-6 gene expression in the testes of roosters during puberty (5-8 months).

Key words: Gene; Interleukin; Rooster; Sperm

INTRODUCTION

The major functions of the testes are spermatogenesis and steroidogenesis. The latter is accomplished by leydig cells localized in the interstitium as compact cell clusters closely associated with blood vessels. Spermatogenesis occurs within the seminiferous tubules (**STs**),

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where sertoli cells, targets of both testosterone and FSH, play a crucial role in germ cell (**GC**) proliferation and differentiation. During the reproductive period of the male bird, the testes undergo many cellular changes. The growth and development of testes is controlled by paracrine and autocrine mechanisms that are directed by hypothalamic-pituitary axis hormones, sex steroids, and cytokines (Thurston and Korn, 2000). The haploid cells of the spermatogenic tissue of the testis produce many antigens during puberty, so the testicular tissue activates several mechanisms to neutralize the action of these antigens in causing inflammation and stimulating immune cells for the success of spermatogenesis. The first mechanism in neutralizing the inflammatory effect of these antigens is the testicular blood barrier, which is created by Sertoli and myoid cells. The second mechanism is the leakage of anti-inflammatory cytokines from immune cells (macrophages, mast cells, dendritic cells, and T cells) and non-immune cells (Leydig, Sertoli, and myoid) located in the testes. Cytokines such as interlukin-1 (***IL-1)***, interlukin-6 (***IL-6),*** and tumor necrosis factor *a* (***TNFa)***, in addition to their anti-inflammatory role, can cause a change in the permeability of the blood-testis barrier, which reduces its protective level. On the other hand, cytokines activate intracellular factors that stimulate cell death in germ cells (Jacobo *et.al.*, 2011).

Cytokines are important factors for stimulating the immune response in various tissues. These compounds are a group of molecular mediators for regulating the activities of immune cells in innate immune responses as well as regulating the physiological activities of various organs such as the testis and epididymis (Anastasiadou *et al.*, 2016). Stimulation of sertoli cells of adult roosters with liposaccharide compounds that are normally present in the cell wall of bacteria caused the expression of various cytokines such as *IL-6* in these cells (Michailidis *et al.*, 2014). In addition to phagocytosis of residual bodies and apoptotic germ cells, sertoli cells also remove antigens resulting from the division of germ cells. During this process, sertoli cells' surface receptors recognize these components and swallow them by changing their membrane structure, which is finally digested by lysozymes in their cytoplasm (Wang and Han, 2019). *IL-6* is a pleiotropic cytokine that is secreted by various cells. Despite the diversity of *IL-6*-producing cells, this chemical messenger acts mainly through a membrane receptor that is expressed only on certain types of cells, including sertoli cells, leydig cells, and spermatogenic cells (Scheller et al., 2011). It seems that the process of phagocytosis of the remaining cytoplasmic bodies in the stage of sperm metamorphosis by sertoli cells causes the activation of interleukins such as *IL-6* and its receptor in the testis (Thurston and Korn, 2000).

Numerous studies have shown that cytokines, in addition to participating in pathological reactions, can be produced without the stimulation of infectious or inflammatory factors and are also effective in physiological processes such as sperm production (Alvez-Silva et al., 2021). Research has also shown that even with increasing age, the expression of interleukin genes in tissues changes; for example, the level of interleukin-6 gene expression was different in the testicles of immature and old mice (Potashnik et al., 2005). Cytokines and testicular growth factors (***IL-1***, ***IL-6,*** and ***TNFa***) affect both the proliferation of germ cells and the secretion of Leydig and sertoli cells. As mentioned before, cytokines and growth factors are also produced by different testicular cells such as sertoli, leydig, myoid, spermatogonia, and even sperm. Cytokine receptors are also present in all types of testicular cells and affect the process of spermatogenesis and steroidogenesis of testicles under the influence of pathological or physiological changes (Huleihel and Lunenfeld, 2004). The development of reproductive organs during puberty depends on the interaction between growth hormones, insulin-like growth factor-1, reproductive hormones, and anti-inflammatory cytokines. It is suggested that cytokines cause changes in feedback mechanisms inhibiting the activity of gonads and initiating their activity during puberty (Casazza *et al.*, 2010). For example, *IL-6* production was significantly higher in boys under 17 years of age (before puberty) than in older (after puberty) ones (Decker *et al.*, 2017). *IL-6* is also one of the secreted proteins of immune cells that play a role in the development of acquired and natural immunity in birds and is expressed in spermatogonial cells in different stages of differentiation, leydig cells, interstitial cells, and myoid cells. Since the secretion of gonadotropins and testosterone mainly influences these cells' activity, the production of *IL-6* in the testicular tissue is under the control of the the reproductive axis (Rodes *et al.*, 2013). *IL-6* expression is also activated by infecting broilers' testis with different *Escherichia coli* strains (Elnagar *et al.*, 2021). It means that pathogens' stimulation of *IL-6* production or physiological changes may influence spermatogonial stem cells in seminiferous tubules (Guazzone *et al.*, 2009). Meanwhile, non-pathological factors such as age changes or sexual maturity can also change the gene expression of immune cells in different tissues (Alvez-Silva et al., 2021). Examining the changes of *IL-6* gene

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expression in the testes of mice during puberty showed that the level of *IL-6* (protein and mRNA) in the testes of immature mice was higher than that of mature mice (Potashnik *et al.*, 2005).

IL-1 is another cytokine that is expressed in the testis and inhibits steroidogenesis in Leydig cells, which indicates the modulating role of this cytokine in LH-dependent testosterone synthesis. Sertoli cells are also able to produce *IL-1*. The expression level of interleukin-1 in these cells is related to the amount of DNA synthesis in spermatogonial cells (Rozwadowska *et al.*, 2001). Therefore, *IL-1* can be a regulatory factor for the activity of seminiferous epithelium. Its expression in Sertoli and Leydig cells depends on the local concentration of FSH and LH and other cytokines (such as TNFα) or growth factors in the testis. Hence, *IL-1* is recognized as another element of the regulatory network necessary to maintain the physiological function of the testis (Heinrich and DeFalco, 2020).

During sexual puberty, with the start of testicular activity, the divisions of spermatogonial stem cells also increase, and simultaneously with the production of more haploid cells, the total amount of antigens produced in the testicle also increases, therefore, it is important to clarify what changes in the gene expression of interleukins occur at the same time as the testicular activity increases during puberty. So far, no research has been recorded in this field to investigate the changes of interleukins 1and 6 gene expression during the puberty period of roosters (5-8 months), so the present study was conducted in order to investigate the semen quality and the gene expression of interleukins-1 and 6 during the puberty period of native roosters.

MATERIALS AND METHODS

This study was conducted in a completely randomized design with 4 treatments and 6 repetitions. For this purpose, the age of the roosters (5, 6, 7, and 8 months old) was considered as experimental treatment, and 6 roosters were also considered for each treatment. During the experiment and at each time of sampling, roosters were randomly selected from a breeding flock related to the Fars Native Poultry Research Center. During the experiment, the roosters of the breeding flock were under the influence of light programs of 12 hours of light and 8 hours of darkness and fed with the ration suggested by that research center (Loohari Yeylaghi *et al.*, 2020). (Table 1).

Table 1. Feed ingredients and chemical composition of the experimental ration

Feed Ingredients (kg of DM)	
Corn	610.7
Barley	4
Wheat bran	100
Hay	10
Soybean meal (44% CP)	219.5
Calcium Carbonate	20
Di Calcium Phosphate	18
NaCl	3.8
Lysin	1
Methionine	1
Vegetable oil	6
Mineral and vitamin supplement	5
Chemical composition	
Metabolizable energy (kcal/kg)	2811.62
Crud protein%	16.64
Lysin%	1
Methionine%	0.5
Calcium%	0.92
Total phosphorous%	0.52
Available Phosphorous%	0.29

In each round of sampling, the roosters were taken to the laboratory and after slaughtering and emptying the visceral organs, semen samples were collected from vasdeferens of roosters and the characteristics of the semen including volume, concentration of sperm in the semen, the percentage of normal and live sperms were evaluated (Meamar and Zamiri, 2010). In brief, to evaluate the characteristics of the semen, the vasdeferens of roosters containing the semen samples were removed from the abdominal area and emptied into a microtube by milking method and kept in the incubator (40˚C) for further evaluations.

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Sperm motility was evaluated using an optical microscopy and with the help of a camera and monitor with the mass motility evaluation method of sperms with the scale of 1 to 10 and then reported as a percentage. In this method a drop of physiologic serum (NaCl 0.9 %) was added to a drop of semen on a warm lamella then the mass motility of the sperms was evaluated under optical microscopy. The concentration of sperm in semen samples was also measured with the help of a hemocytometer slide and a diluting pipette, and the results were reported as the number of sperm per ml of semen. The viability and morphology of sperms were also examined with the help of eosin-nigrosin staining. In this method, one drop of eosin-nigrosin dye was added to one drop of semen sample. Then, the resulting sample was mixed and spread on the main slide and dried with a heater at 40°C. Then, among the 200 counted sperms, those with colored heads were considered dead sperms, and their ratio was reported as a percentage. In the case of normal sperms, after staining, the number of abnormal sperms was counted, and the ratio of their number to the total was reported as a percentage. To calculate the total sperm count, the volume of the semen was multiplied by the sperm concentration, and to calculate the total number of live and normal sperm in each sample, the total sperm count was multiplied by the percentage of live and normal sperm.

To study the changes in the relative abundance of cytokines gene mRNA transcript, total RNA was isolated from testicular cells using an RNX-Plus kit (Sinaclon-IRAN, Catalog number: EX6101) according to the manufacturer's instructions. Then, the concentration and purity of RNA were checked by electrophoresis and spectrophotometry. Then, the RNA solution was treated with DNase I (Cinagen) to remove genomic DNA. The complementary DNA was synthesized by AccuPower® RocketScriptTM RT PreMix kit (Bioneer) and random hexamer primer (Takapozist). Upon completion of the reactions, the final volume was adjusted to 100 µl with RNase-free water before expression analysis and stored at -40°C. To amplify a fragment of the studied genes, using the mRNA sequence in the GenBank database, appropriate primers were designed by Primer 3 plus software (Table 2), and the relative expression of the genes was evaluated using ß-actin gene as reference genes to normalize the data. The mRNA expression of desired genes was quantified using RT-PCR with SYBR Green labeling. Real-time PCR analysis was carried out by CFX96 Real-time PCR System (BioRAD, USA). Each RT-reaction served as a template in a 10 µL PCR reaction containing 4 pmol of each primer and SYBR green master mix (HotTaq EvaGreen qPCR kit, Cinnagen), and REST, 2009, V2.0.13, software was used to analyze gene expression data (Nobakht and Muhaghegh Dolatabadi, 2017).

Table 2. Details of primer sequences used for quantitative real-time PCR.

Gene	primer Sequences	Access number	Annealing temperature (°C)
IL-1β	Forward: cagaaagtgaggctcaacattgcg Reverse: ttgtagcccttgatgcccagtg	DQ393267.1	60
IL-1Ra	Forward: cgcgtgcaaaccaaagtcttcaaat Reverse: caccagctgatcatcgcgca	AJ574909.1	60
IL-6	Forward: aatgacatccagggagaggtttc Reverse: atttctctctcggctgtggtg	HM179640.1	60
β-actin	Forward: ctgtgcccatctatgaaggcta Reverse: atttctctctcggctgtggtg	NM-205518.1	60

IL-1β = Interlukin-1β. IL-1Ra= Interlukin-1 Receptor α. IL-6= Interlukin-6.

Statistical analysis: The analysis of the results of this research was done in the form of a completely random design. For this purpose, the calculations related to the analysis of the transcript frequency of the target genes were performed using the software REST, 2009, V2.0.13. The data related to the characteristics of the sperm were first normalized and then analyzed using the SAS software (SAS. 9.4, U.S.A.) and the PROC GLM procedure. Then, the means were compared using Duncan's test ($P \leq 0.05$).

RESULTS

IL-6 mRNA gene expression: Results of *IL-6* gene expression changes are shown in Figures 1A, B, and C.

The results of figure 1A show that the relative gene expression of *IL-6* at age of 6, 7 and 8 month-roosters increased significantly (P=0.0001) compared to the age of 5 month-roosters.

The results of figure 1B show that *IL-6* gene expression at 7 months increased significantly (P=0.0001) by 51.80 times compared to 6 months. The results also showed that despite the 8.23 -fold

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increase of *IL-6* gene expression at 8 months, this change was not significant (*P*=0.1).
The results of figure 1C show that the level of *IL-6* gene expression at the age of 8 months increased by 0.1590 compared to younger ages, which did not change significantly.

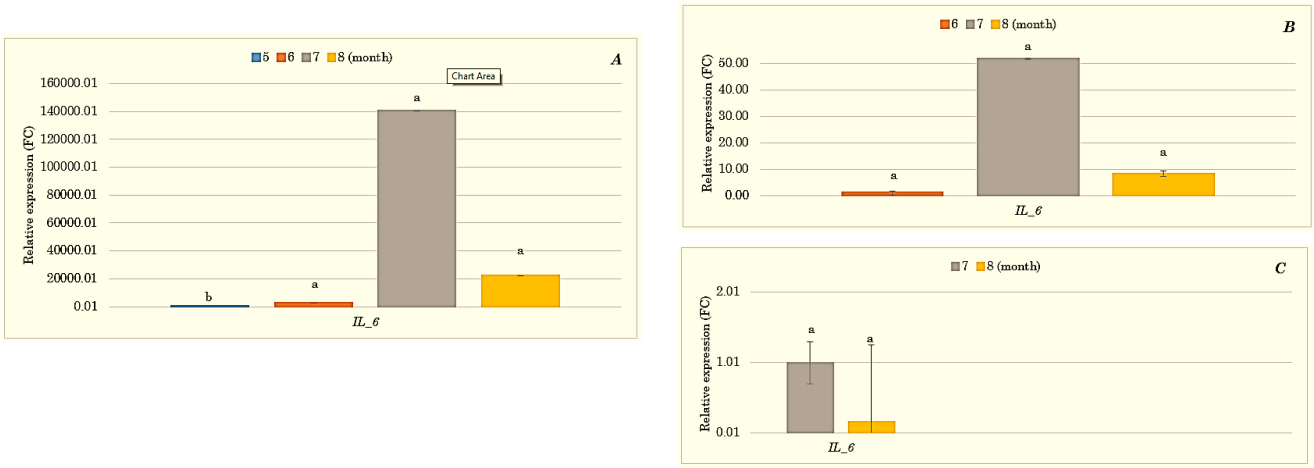

Figure 1. Comparison of relative mRNA expression of IL-6 genes in the testes of roosters: (A) 6-, 7-, and 8-month-old roosters compared to 5-month-old roosters; (B) 7- and 8-month-old roosters compared to 6-month-old roosters; and (C) 8-month-old roosters compared to 7-month-old roosters, with error bars representing the standard error of the mean (SEM) and different superscript letters (a, b, …) indicating significant differences (P ≤ 0.05). FC= Fold change.

IL-1 and IL-1Ra mRNA gene expression: In the present study, the expression of *IL-1* and *IL-1Ra* mRNA genes was also examined. However, despite repeating the test several times to check the relative expression of these genes in the testes of roosters, the mRNAs of these genes were not expressed significantly in the testes of roosters of all ages (5-8 months).
The results of Table 3 show that the percentage of motile sperm in the semen of roosters at 6, 7, and 8 months was significantly higher than that of 5-month-old roosters; in other words, the lowest percentage of motile sperm was observed at 5 months. In Table 3, it can be seen that the percentage of live sperm in the semen of the studied roosters at the ages of 7 and 8 months has increased significantly compared to the ages of 5 and 6 months. The results also showed that the percentage of normal sperm at 7 and 8 months of age was higher compared to 5 and 6 months of age, but there was no significant difference between 7 and 8 months of age and 5 and 6 months of age.
The results also showed that the concentration of sperm in the semen of roosters had an increasing trend from the age of 5 to 8 months, so the sperm concentration was the lowest at the age of 5 months and the highest at the age of 8 months. As sperm concentration, semen volume increased and reached its maximum value at the age of 8 months. Examining the changes in the total number of sperm in the semen also showed that this characteristic reached it's lowest value at the age of 5 months and reached its highest value at the age of 8 months, but no significant difference was observed between the total number of sperm at the age of 6 and 7 months. On the other hand, the total number of live and normal sperm significantly increased with the age of the roosters and reached it's highest value at the age of 8 months.

Table 3. The means (± standard deviation) of semen characteristics of roosters from the age of 5 to 8 months

	Age (month)			
Semen characteristics	5	6	7	8
Sperm motility (%)	$92.81^{b} \pm 3.9$	$98.23^{a} \pm 2.15$	$98.53^{a} \pm 2.12$	$99.53^{a} \pm 0.58$
Normal sperm (%)	$89.5^{b} \pm 2.58$	$91.83^{b} \pm 1.16$	$98.41^{a} \pm 2.05$	$98.83^{a} \pm 1.17$
Live sperm (%)	$86.08^{b} \pm 4.84$	$88.58^{b} \pm 4.32$	$98.25^{a} \pm 1.79$	$98.91^{a} \pm 1.66$
Sperm concentration (× 107 /ml)	$133.76^{d} \pm 3.8$	$147.96^{c} \pm 4.8$	$162.30^{b} \pm 3.6$	$178.93^{a} \pm 2.9$
Semen volume (ml)	$0.33^{d} \pm 0.236$	$0.40^{c} \pm 0.2529$	$0.46^{b} \pm 0.2875$	$0.58^{a} \pm 0.2449$
Total sperm (×107)	$44.18^{c} \pm 4.01$	$58.43^{b} \pm 3.46$	$75.70^{b} \pm 4.26$	$103.99^{a} \pm 5.30$
Total live and normal sperm (×107)	$34.03^{d} \pm 3.87$	$47.50^{c} \pm 3.31$	$71.84^{b} \pm 5.04$	$101.60^{a} \pm 4.13$

Means with different superscript letters are significantly different (*P*≤0.05).

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DISCUSSION

Puberty is a vital transitional process in which a complex set of hormonal and neurological changes leads to physical growth and full sexual maturity (Raivio and Miettinen, 2019). There is a lot of evidence that shows the involvement of growth factors and cytokines in the local mechanisms regulating the process of mitosis and meiosis of testicular tissue stem cells (Gnessi *et al.*, 1997). The mechanisms governing the timing of puberty is a rich field of research that has not been fully explored. However, many exogenous and endogenous factors (nutrition, genes, immune system intervention, and endocrine disruptors) can affect the maturation process (d'Angelo *et al.*, 2021). The evidence of the direct effect of the immune system on the control of reproductive activity by examining the immunohistopathology of rat brain tissue showed that the intracerebral injection of IL-1 caused a decrease in LH secretion through a decrease in GnRH neuron activity in the hypothalamus (Watanabe *et al.*, 2003). The same effects of cytokines on the physiological activity of an organ were observed in other studies. For instance, cytokines promoted angiogenesis and the development of spermatogonia and Leydig cells in the embryonic period and before puberty (Loveland *et al.*, 2017). In the present study, the same results were observed in the expression of IL-6 and gonadal activity in sperm production. Our study indicated that, as the age of the roosters increased during puberty, both the interleukin-6 gene expression and the semen quality of the roosters increased gradually. Other studies (Defalco *et al.*, 2015; Alvez-Silva *et al.*, 2021; Anastasiadou and Michailidis, 2016) also demonstrated that there is a positive correlation between cytokines and gonadal activities. The results of those studies showed that reducing the production of cytokines such as interleukin-6 caused a decrease in the number of spermatogonial stem cells, daily production of spermatids and increasing the expression of IL-6 increased testosterone production and the diameter of the germinal layer in the seminiferous tubules in the testes. Therefore, it can be concluded that in the present study, one of the reasons for the increase in sperm production in the testicles of roosters is the increase in the production of interleukin 6 due to the increase in the expression of this gene during the puberty period of roosters (5 to 8 months old). Of course, it is obvious that the growth and development of the testicles, especially during puberty, are under the control of several main factors, such as the hormones of the hypothalamus-pituitary axis, as well as hormones related to the body's growth and metabolism, such as growth hormone, insulin, etc. It is interesting that cytokines in many tissues, even fat and bone tissue, play a key role in it's growth and development during puberty (Casazza *et al.*, 2010). The results of investigating the expression of IGf-1 gene in the testes of native roosters showed that the expression of this gene and the weight of the testes increased significantly during puberty. (Heshmatipour *et al.*, 2020). So, it can be concluded that one of the reasons for the increasing expression of IL-6 in non-pathological conditions of our study is probably because of the rapid testicular growth during puberty. Other studies were also conducted that cytokines production occur in non-pathological conditions to induce angiogenic and cell growth effects instead of anti-inflammatory action (Loveland et al., 2017). A very important point in the results of the present study was the lower expression of the IL-6 gene at 8 months compared to 7 months (0.1590-fold changes reduction). Previous studies have shown that after puberty, due to the increase in the production of sex hormones, especially testosterone, the production of cytokines is reduced (Casazza *et al.*, 2010). According to the results obtained about the changes in sex hormones of native roosters of the same age in previous research (Loohari *et al.*, 2020), the concentration of testosterone hormone at 8 months was significantly higher than at 7 months, so it seems that one of the reasons for the reduction IL-6 gene expression in 8 months is the increasing testosterone production in the testes of the roosters. Previous studies have shown that testosterone, in addition to its anabolic effects on testicular tissue, simultaneously decreases cytokine production such as IL-1 and IL-6 (Fijak and Meinhardt, 2006). The results of the present study indicated that both IL-1 and its receptor (IL-1Rα) were not expressed in the testes of the roosters during puberty. It seems that the lack of expression of these genes in the testis is because the expression of these genes depends on age and reproductive hormones, especially steroids (Anastasiadou and Michailidis, 2016; Heinrich and De Falco, 2020). In addition, it was shown that the production of interleukins in the testicles, depends on the metabolic rate and the physiological conditions of the body (Martos-Moreno et al., 2006; Zhang *et al.*, 2012).

The results of the present study showed that the quality of the semen of roosters reached its highest level at the end of the puberty period (8 months). In previous studies (Avital-Cohen et.al., 2013), when the bird reaches puberty and simultaneously with the increase in the activity of reproductive hormones, the activity of the spermatogenic layer of the testes also increases and gradually the

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quality of semen, especially the concentration and quality of sperms, also increases. In our study, in addition to the increase in sperm quality during puberty, a simultaneous increase in IL-6 gene expression was also observed. Meanwhile, previous studies (Casazza *et al*., 2010) showed that interleukins can act as a complementary factor to stimulate the growth and maturation of the testicles and increase the activity of spermatogenesis in the testis at young ages (puberty period) as it happened in the present study.

CONCLUSION

The results generally showed that according to the expression changes of the IL-6 gene at the age of puberty in native roosters (5-8 months), the expression of this gene is age-dependent and alters in non-pathological conditions, simultaneously with the high activity of the testes and sperm production during the puberty period.

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CONFLICT OF INTEREST

There was no conflict of interest.

AUTHOR CONTRIBUTIONS

M.M. was responsible for the overall design of the project and supervised the implementation of tests related to sperm evaluation as well as the statistical analysis of all results. J.H. was responsible for consulting in the design and implementation of the project. M.M.D. was responsible for designing and supervising the implementation of gene expression experiments and analyzing their results. R.K. was responsible for preparing laboratory equipment, preparing test samples, and conducting gene expression tests.

DATA AVAILABILITY

The datasets produced and analyzed in the present study are not accessible to the public, however, they can be obtained from the corresponding author upon reasonable request.

ETHICAL CONSIDERATIONS

In this study, all stages of breeding, transportation, sampling, and killing of roosters were carried out following animal rights and Islamic standards, based on the permission (5081852-4000441001) of the postgraduate education department of Yasouj University.

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