




## Original Article

# Role of Fennel (*Foeniculum vulgare*) Seed Powder in Increasing Testosterone and *IGF1* Gene Expression in the Testis of Lamb



Seyed Mohammad Hadi Safaei<sup>1</sup>, Mohammadreza Mohammadabadi<sup>2\*</sup> , Borhan Moradi<sup>1</sup>, Oleksandr Kalashnyk<sup>3</sup>, Nataliia Klopenko<sup>4</sup>, Olena Babenko<sup>4</sup>, Oleksandr Oleksandrovich Borshch<sup>4</sup> and Volodymyr Afanasenko<sup>5</sup>

<sup>1</sup>Department of Animal Science, Faculty of Agriculture, Shiraz University, Shiraz, 7194684334, Iran; <sup>2</sup>Department of Animal Science, Faculty of Agriculture, Shahid Bahonar University of Kerman, Kerman, Iran; <sup>3</sup>Department of Animal Science, Sumy National Agrarian University, Sumy, Ukraine; <sup>4</sup>Department of Animal Science, Bila Tserkva National Agrarian University, Soborna, Bila Tserkva, Kyivska Oblast, Ukraine; <sup>5</sup>National University of Life and Environmental Sciences of Ukraine, Ukraine

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## Abstract

**Background and objectives:** The insulin/insulin-like growth factor (IGF) system has many receptors, signaling pathways, and ligands, making it highly complex. *IGF1* increases the function of germ cells by increasing the synthesis of spermatogonia DNA and slowing down apoptotic cells. One of the most important objectives of this study was to investigate the function of fennel on the expression of the *IGF1* gene in Kermani sheep testes.

**Methods:** The study involved three levels of fennel in the diets (20, 10, and zero g/kg dry matter), and tissue sampling was performed using testis tissue. Samples were rapidly placed in liquid nitrogen before being stored at -80°C. Then, total RNA was extracted and for the *IGF1* gene (target) and *GAPDH* gene (reference), the real-time polymerase chain reaction was applied.

**Results:** The outcomes displayed that increasing levels of fennel in the ration significantly ( $p < 0.05$ ) increases the weight of the testis (0.35 kg and 0.36 kg at the levels of 1% and 2% fennel, respectively) compared to rations without fennel (0.29 kg at the levels of 0%). Association between testosterone and fennel feeding in studied lambs showed that adding fennel to the diet significantly ( $p < 0.05$ ) increases the concentration of blood testosterone (3.5 ng/dL and 4.4 ng/dL at the levels of 1% and 2% fennel, respectively) compared with rations without fennel (1.7 ng/dL). The results show that adding fennel to the diet significantly ( $p < 0.05$ ) increases the amount of *IGF1* gene expression in the testis (2.5 and 2.7 at the levels of 1% and 2% fennel, respectively) compared with rations without fennel (1 at the levels of 0% fennel).

**Conclusions:** Fennel has an affirmative effect on gene expression in the testis and can be added to sheep food rations to progress reproductive functions (by stimulating Leydig cell steroidogenesis, producing more sperm and testosterone, and growing and developing the testis).

**Keywords:** Fennel; *IGF1*; Gene expression; Testis; Testosterone.

**Abbreviations:** cDNA, copy DNA; DM, dry matter; *IGF*, insulin-like growth factor; PCR, polymerase chain reaction; RFU, relative fluorescence units; ROS, reactive oxygen species; ROX, carboxyrhodamine.

\*Correspondence to: Mohammadreza Mohammadabadi, Department of Animal Science, Faculty of Agriculture, Shahid Bahonar University of Kerman, Kerman, Iran. ORCID: <https://orcid.org/0000-0002-1268-3043>. Tel: +98-9133987534, Fax: +98-343 3257231, E-mail: [mrm@uk.ac.ir](mailto:mrm@uk.ac.ir)

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## Introduction

Evolutionary studies have shown that the processes of reproduction, growth, and metabolism are very closely related. In other words, organisms must first reach full maturity to reach the desired level of metabolism to be fertile.<sup>1</sup> Due to the direct connection of usual signaling and regulatory pathways and networks, growth, reproduction, and metabolism are linked.<sup>2</sup> The family of insulin-like growth factors (IGFs), or the insulin/IGF system, affects all organs in the body and regulates growth, reproduction, differentiation, proliferation, and cellular metabolism. The components of this system, by participating in various biochemical processes, connect

the three pathways of metabolic, reproductive, and mitogenic processes.<sup>3</sup> Various factors such as ligand biosynthesis, growth stage, nutrition, and hormonal interactions affect the activity of insulin, *IGF1*, and *IGF2*, and these factors must be coordinated with each other.<sup>1</sup> One of the main and influential factors in testicular growth and proper functioning is the insulin/IGF system.<sup>4</sup>

Even though, the hypothalamic-pituitary-gonadal axis regulates a person's ability to reproduce.<sup>5</sup> When the *IGF1* gene is inactivated in young males, they could become dwarf and infertile, and their serum testosterone levels and sperm production could drop by more than 80%.<sup>6</sup> Postnatal growth, maintaining reproductive function, overall body growth, and body size regulation are among the roles of *IGF1*.<sup>4</sup> The production of *IGF1* in any tissue (in addition to its production in the liver) is very important, as it represents a main task in the growth process of that tissue.<sup>7</sup> Compared to insulin or *IGF2*, the role of *IGF1* in postnatal development is greater and more important.<sup>8</sup> Various studies have shown that the natural activity of *IGF1* and its receptors is necessary for organisms to grow and develop normally.<sup>9–11</sup> Studying the testes of immature mice revealed that *IGF1* was equally present in Sertoli cells and spermatogenic cells by the age of three weeks, but was no longer present in Sertoli cells. Investigating the testes of adult mice showed that *IGF1* was present only in spermatocytes and not in Leydig and Sertoli cells and spermatogonia,<sup>12</sup> while Colón *et al.* showed that the phosphorylated *IGF1* receptor was present in both Sertoli and Leydig cells.<sup>13</sup> Yoon and Roser demonstrated that *IGF1* and *IGF1* receptor expression levels varied from pre-puberty to adulthood.<sup>14</sup> Immunoreactivity in adult stallions was positive and strong for spermatogonia and Leydig cells, weak for spermatocytes, and zero for Sertoli cells.<sup>15</sup> *IGF1* was also shown to motivate Leydig cells for steroidogenesis.<sup>16</sup> These events also depend on the stage of growth and examination of the steroidogenic process of equine Leydig cells during puberty and subsequently in vitro revealed that *IGF1* alone had no effect on the potential of this process. However, in combination with luteinizing hormone, and in a dose-dependent manner, it increased testosterone synthesis in post-pubertal stallions.<sup>14</sup> *IGF1* increases the function of germ cells by increasing the synthesis of spermatogonia DNA and slowing down apoptotic cells.<sup>17</sup> Researchers have shown that in late embryonic and early neonatal life, mouse Sertoli cell proliferation is highly dependent on *IGF1* signaling.<sup>18</sup> *IGF1* signaling also acts as a stimulus for Leydig cell maturation.<sup>19</sup> Treatment of adult and immature mice with *IGF1* in vitro showed a dose-dependent rise in the proliferation of immature Leydig cells and a reduction in the number of Leydig apoptotic cells.<sup>13</sup>

Plants such as fodder plants, peas, and cereals contain phytoestrogens—plant compounds that have a structure similar to animal estrogens and occur in various segments of the seeds and plant.<sup>20,21</sup> Phytoestrogens have been shown to be beneficial and safe for animals: when added to animal feed, they are highly degradable, cannot be stored, and their shelf life in the body is very short.<sup>22</sup> The fennel plant, *Foeniculum vulgare* from the *Umbelliferae* (*Apiaceae*) family, contains important compounds such as anethole, camphene, pinene, fenchone, and phellandrene. The phytoestrogens in fennel have similar effects to synthetic 17-beta estradiol and can be used as an alternative to hypothalamic-pituitary-gonadal axis control.<sup>23</sup>

Considering the use of fennel as an antioxidant, antifungal, antibacterial, anti-tumor, anti-diabetic, anti-thrombotic, anti-cardiovascular hirsute, anti-inflammatory, antioxidant, liver dilator, bronchodilator, acaricide and insecticide, the importance of biomedical and pharmacological applications can be understood.<sup>24,25</sup> One of

the estrogenic active agents of fennel is trans-anethole, which is the most abundant and important compound in fennel oil.<sup>26</sup> Some researchers have shown that fennel has been used in animal rations for a variety of aims, such as reducing the total number of bacteria, improving the oxidative quality of meat, increasing the volume of packed cells, increasing the speed of digestion and growth, increasing the weight and length of the small intestine, increasing hemoglobin and red blood cells, improving feed consumption and its conversion rate, and improving the health of the body.<sup>27–31</sup>

Sheep are one of the most important small ruminants in hot, dry, and desert areas.<sup>32</sup> Therefore, improving their genetic development using all available methods, both quantitatively and qualitatively, is a priority for livestock farmers and researchers in this field.<sup>33</sup> Improving reproductive processes and increasing yield are traditional ways of achieving higher herd yield.<sup>34</sup> Reports indicate that over 50 million sheep are bred in Iran and these sheep are composed of twenty-seven breeds and ecotypes.<sup>35</sup> One of these 27 breeds and ecotypes is the Kermani sheep; this fat-tailed dual-purpose meat-milk sheep with white wool and medium size has an important role in animal husbandry and supplying the needs of nomads and ranchers in this area of Iran.<sup>36,37</sup> Therefore, actualizing the needs of this breed through genetic and non-genetic improvement represents a significant task in the improvement of breeding this sheep and its conservation. In domestic species research, the identification of genes affecting important production traits is an essential area. Studies in the late 1980s revealed that molecular mechanisms including DNA replication, transcription, translation, and even how genes are regulated, are among the most important genetic processes.<sup>38</sup> Investigating the task of fennel on the expression of the *IGF1* gene in Kermani sheep testes was one of the most important objectives of this study.

## Materials and methods

### Animals

In this research, 30 male lambs of the Kermani breed weighing  $27.5 \pm 0.45$  kg (eight months old) were used. Vaccination of all animals was performed using standard vaccines before the start of the experiment. The experiment was carried out in  $1.2 \times 1.5$ -meter pens with free access to food and water at the Animal Science Research and Training Station of Shahid Bahonar University of Kerman, Iran.

### Ethical statement

All procedures related to animals were certified by the Animal Care and Use Committee of Bahonar University (IACUC Protocol #IR2018011) described by the Iranian Council of Animal Care. All guidelines and laws related to animal welfare policies for the animals used in this research were fully followed.

### Feeding and data collection

The animals were fed twice a day with the same rations in terms of protein and energy (Table 1). The diets were grouped into 3 levels of fennel based on g/Kg dry matter (DM) (0, 10 or 1%, and 20 or 2% fennel g/Kg DM). The chemical formula of added fennel to rations was 91% dry matter, 15% crude protein, 87.03% organic matter, 9.76% ether extract, and 12.12 MJ/kg metabolizable energy. To analyze the diet and calculate its components, such as ether extract, nitrogen, ash, and DM, standard methods of AOAC were used.<sup>39</sup> The Van Soest method was applied to determine ash-free neutral detergent fiber (NDFom) and ash-free acid detergent fiber

**Table 1. Ingredients of the diets and fennel used for the studied sheep**

Ingredients of the diets	Amount of fennel seed powder (%)		
	0	1	2
Fennel seed powder (%)	0	1	2
Alfalfa hay (%)	30	30	30
Barley grain (%)	28	27	26
Wheat bran (%)	13	13	13
Wheat straw (%)	10	10	10
Corn grain (%)	9	9	9
Soybean meal (%)	8	8	8
Vitamins E, D, and A (%)	0.6	0.6	0.6
Trace-mineralized salt (%)	0.6	0.6	0.6
Sodium bicarbonate (%)	0.5	0.5	0.5
Limestone (%)	0.3	0.3	0.3

Ingredients of fennel	Amounts	Ingredients of fennel	Amounts
Metabolizable energy	12.12 MJ/kg	Ether extract	9.76%
Crude protein (%)	15	Dry matter	91
Organic matter (%)	87.03		

(ADFom).<sup>40</sup>

One day before slaughtering, at 3 hours after morning feeding, blood samples were collected. Centrifugation (10 min at 6000×rpm) was performed for collected samples and then for further analyses, blood serum samples were frozen at -20 °C. Calculation of testosterone concentration was done using an enzyme-linked immunosorbent assay kit (Stat Fax). It should be noted that a testosterone set (Patangostar-e-Eisar Co, Iran, Lot number: AC13476) was used with this kit. When the study period was over, the investigated animals were slaughtered for sampling and the weight of the testes was then recorded. Tissue sampling (90 samples containing 10 animals × 3 groups × 3 repeats for the tissue) was performed from testis tissue. Before storing the samples at -80 °C, they were quickly located in liquid nitrogen.

#### RNA expression analysis

Total RNA was extracted from the studied tissue (using the step-RNA Reagent kit made by Biobasic Company, Iran) and cDNA was then synthesized using commonly available kits (#K1631, Fermentase Company, Iran). The quality of RNA and cDNA was measured using electrophoresis on agarose gel. The characteristics of the primers used for amplification of two target and reference genes are given in Table 2. The information related to real-time

polymerase chain reaction (PCR) is given in Table 3.

To ensure the amplification of the target fragments, the melting curves for the target gene and the reference gene at the end of the amplification were analyzed by PCR. In addition, the gradient protocol was used to determine the annealing temperature for the *IGF1*; target gene and the *GAPDH*; reference gene. For the evaluation of real-time PCR data, the Pfaffl method was applied.<sup>41</sup>

#### Statistical analysis

The data obtained from this research were analyzed in the mixed procedure with a completely randomized design using statistical analysis system software. The pairwise fixed reallocation randomization test was used to check the normality of the data distribution.<sup>41</sup> In addition, the least significant differences test was used to compare the means ( $p < 0.05$ ).

#### Results

In this research, we studied the effects of different levels of fennel (0, 10, and 20 g/Kg DM) in diets on the weight of the testis of the Kermani sheep breed and the concentration of testosterone and expression of the *IGF1* gene in the testis of the Kermani sheep

**Table 2. The characteristics of the primers used for amplification of *IGF1* target gene and *GAPDH* reference gene**

Gene	Primer	Sequence	accession number in NCBI	Tm	product size
<i>IGF1</i>	forward	5'-ATTACAGCTGCCTGCCCTT-3'	NM_001009774.3	57 °C	265 bp
	reverse	5'-CACATCTGCTTACACCTTACCCG-3'			
<i>GAPDH</i>	forward	5'-ACCACTTTGGCATCGTGGAG-3'	NM_001190390.1	57 °C	76 bp
	reverse	5'-GGCCATCCACAGTCTTCTG-3'			

*IGF1*, insulin-like growth factor 1.

**Table 3.** The information related to Real-Time PCR reactions for *IGF1* target gene and *GAPDH* reference gene

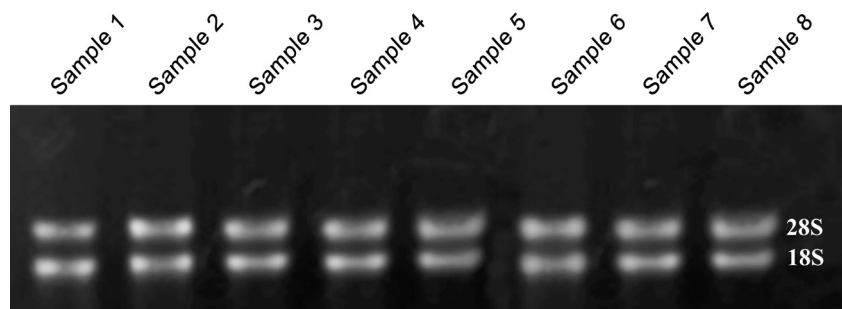
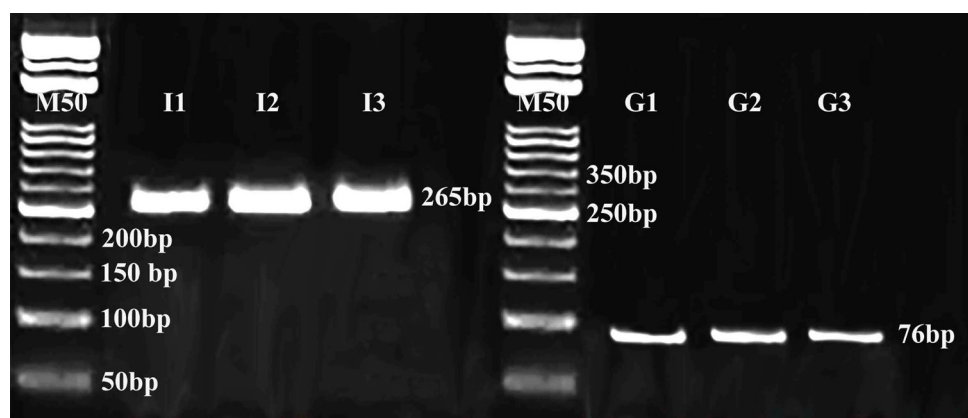
Final volume of each real-time PCR reaction	real-time PCR device	Contents of each real-time PCR reaction tube	Volume of each real-time PCR reaction tube	Program for real-time PCR reactions
15 $\mu$ L	Rotor-Gene Q MDx device (QIAGEN Hilden, Germany)	template cDNA	1.5 $\mu$ L	95 °C for 5 min, followed by a cycle of 95 °C 20 s, 57 °C 30 s and 72 °C 30 s for 38 cycles.
		2X SYBR Green PCR Master Mix (Fermentase Co., Tehran, Iran)	7.5 $\mu$ L	
		ddH <sub>2</sub> O	4.7 $\mu$ L	
		10 $\mu$ M forward and reverse primers	1 $\mu$ L	
		ROX	0.3 $\mu$ L	

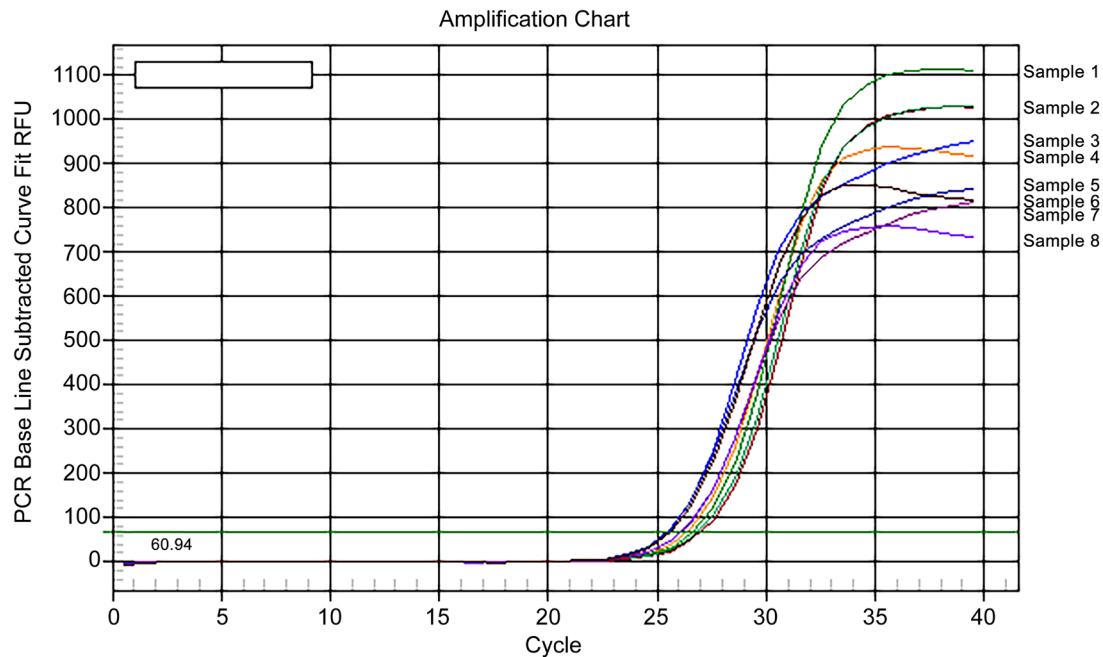
cDNA, copy DNA; *IGF1*, insulin-like growth factor 1; PCR, polymerase chain reaction; ROX, carboxyrhodamine.

breed. The currently obtained results demonstrate that including fennel in the ration significantly ( $p < 0.05$ ) increases the weight of the testis (0.35 kg and 0.36 kg at the levels of 1% and 2% fennel, respectively) in comparison to diets without fennel (0.29 kg at the levels of 0%). Association between testosterone and fennel feeding in studied lambs showed that adding fennel to diets significantly ( $p < 0.05$ ) increases the concentration of blood testosterone (3.5 ng/dL and 4.4 ng/dL at the levels of 1% and 2% fennel, respectively) in comparison to diets without fennel (1.7 ng/dL).

Evaluation of the quality of extracted RNA using electrophore-

sis on agarose gel showed that the total extracted RNA was healthy and complete and had 28S rRNA and 18S rRNA bands (Fig. 1). The observation of a single band on the agarose gel in the range of 265 bp for the *IGF1* gene in the testicular tissue and the presence of one band in the range of 76 bp for the *GAPDH* gene (Fig. 2) confirmed correct amplification of the fragments and the validity of the experiment. The information related to the IGF-I gene amplification curve in the testis is shown in Figure 3. According to the obtained amplification curve, treatments of 1% fennel (10 g/Kg DM) and 2% fennel (20 g/Kg DM) started to amplify in a lower

**Fig. 1.** Eight samples of total RNA extracted from testes of Kermani sheep on agarose gel.**Fig. 2.** Electrophoresis of amplified products for *IGF1* and *GAPDH* genes in the testes of Kermani lambs on agarose gel. Lanes I1-I3 belong to *IGF1* gene, Lanes G1-G3 belong to *GAPDH* gene and M50 is size marker. *IGF1*, insulin-like growth factor 1.



**Fig. 3. PCR amplification curve of *IGF1* gene in testicular tissue of Kermani lambs.** *IGF1*, insulin-like growth factor 1; PCR, polymerase chain reaction; RFU, relative fluorescence units.

cycle than the control (0 g/Kg DM fennel).

The results obtained from the melting curve of the *IGF1* gene showed that the curve produces only one peak at 86 °C. This result indicates the production of a specific product in this reaction.

The obtained results displayed that including fennel in the ration significantly ( $p < 0.05$ ) increases the amount of *IGF1* gene expression (2.5 and 2.7 at the levels of 1% and 2% fennel, respectively) in comparison to diets without fennel (1 at the levels of 0% fennel) in the testis.

### Discussion

The results of our investigation showed that adding fennel seed powder to diets significantly increases the weight of the testis in comparison to diets without fennel. In confirmation of this result, according to Sotoudeh and Yeganeh, a significant increase in fertility and number of eggs, gonad weight, gonad index, fertility, fertilization percentage, and hatching percentage as well as a decrease in average sexual maturity were observed in ornamental fish, Convict cichlid (*Cichlasoma nigrofasciatum*), fed with fennel essential oil.<sup>31</sup> El-Garawani *et al.* reported that the consumption of fennel oil in etoposide-defective rats caused a reduction of sperm defects and increased mature sperm production, which was due to the anti-oxidant properties of the fennel extract.<sup>42</sup> One of the most important and vital factors in optimizing the fertility rate and production of sperm cells is the reduction of reactive oxygen species (ROS), which is possible using plant antioxidants because these antioxidants make ROS formation impossible and, therefore, suppress it.<sup>43,44</sup> Hajalizadeh *et al.* studied the effects of adding fennel (*Foeniculum vulgare*) to the diet of sheep on some characteristics and reported that feeding fennel significantly increases the weight of the testis in comparison to diets without fennel.<sup>23</sup>

The association between testosterone and fennel seed powder in the diets of lambs in our study showed that adding fennel to

diets significantly ( $p < 0.05$ ) increases the concentration of blood testosterone in comparison to diets without fennel. Abbas *et al.* studied how serum sex hormones and fertility in mice and rabbits are affected by *Foeniculum vulgare*.<sup>45</sup> They demonstrated that the use of 2% and 4% *Foeniculum vulgare* in the diet of male rabbits causes a significant increase in serum testosterone levels. One of the compounds of *Foeniculum vulgare* is isoflavones; this compound inhibits the activity of 5 $\alpha$ -reductase and aromatase P450 enzymes. The first enzyme stimulates the conversion of testosterone to 5 $\alpha$ -dihydrotestosterone and the second enzyme converts testosterone to estradiol.<sup>46</sup> Another effect of isoflavones is to increase the synthesis of sex hormone-binding globulin. When sex hormone binding globulin levels rise, it binds to more testosterone, lowering free testosterone levels and promoting its synthesis.<sup>47</sup>

The obtained results demonstrated that the levels of *IGF1* gene expression in the studied sheep testes were high. The results of this study are in line with the results of other researchers who have demonstrated the expression of the *IGF1* gene in the testes of different animals.<sup>12,18,48-51</sup> Testis size and sperm production are directly correlated to the total number of adult Sertoli cells. Pitetti *et al.* showed that growth factors of the insulin family played an essential role in regulating the final number of Sertoli cells, testis size, and daily sperm output.<sup>18</sup> Weller *et al.* investigated the effects of maternal overnutrition on gonadal development and pituitary-gonadal gene expression in cattle fetuses at mid- and late gestation.<sup>49</sup> They demonstrated that *IGF1* gene expression was higher in fetal testes derived from moderate intake than in high (ad libitum) intake cows, irrespective of the day of gestation. Moreover, testicular expression of *IGF1* was higher at day 139 of gestation than at day 199 of gestation and at day 241 of gestation than at day 199 of gestation in both maternal intake groups. As Abd-Elmaksoud-Ahmed explained, the greater expression of *IGF1* at day 139 of gestation compared with day 199 of gestation may be related to the different ages of the fetus, which is correlated to the stage

of testicular development.<sup>52</sup> Additionally, it may be related to the stimulatory role of growth factors during testis enlargement, which increases testis size with further proliferation and differentiation. Similar to this trend, as the pregnancy progresses from 199 to 241 days, when the expression of the *IGF1* gene increases, the growth of the fetus also increases due to the production of more connective tissue. It has also been proven that *IGF1* affects the expression of key testicular steroidogenic enzymes during mouse development before birth.<sup>53</sup>

Müller *et al.* studied *IGF1* gene expression in the testes of prepubertal and adult dogs and concluded that in adult dogs *IGF1* can mediate Leydig cell function and support spermatogenesis.<sup>12</sup> *IGF1* can cause the growth of gonocytes and Leydig and Sertoli cells in the immature testis. It also has positive effects on the function of germ cells, increases spermatogonia DNA synthesis, and reduces the rate of apoptotic cells.<sup>17</sup>

When adult and immature mice were treated with *IGF1* in vitro, the number of Leydig apoptotic cells decreased and the proliferation of immature Leydig cells increased.<sup>13</sup> A study by Peters *et al.* demonstrated that the expression of IGF-binding protein 1 and IGF 1 genes in Sertoli cell tumors and seminomas was lower than in normal testes.<sup>54</sup> Research by Pitetti *et al.* on rodents showed that *IGF1* for the growth of immature Sertoli cells is necessary.<sup>18</sup> If insulin signaling and *IGF1* are lacking in the murine testis, Sertoli cell proliferation in the late embryonic and early neonatal periods decreases. The consequence of this reduction in proliferation in adults will be a decrease in sperm production and testicular weight. Leydig cells, with supervision and control of IGF, manufacture insulin-like hormone 3 and androgens, which are responsible for the masculinization of the testicular descent and urogenital system. Cannarella *et al.* investigated the role of *IGF1* in testicular growth during embryonic development and showed that it increases Sertoli proliferation and germ cells and promotes germ cell differentiation.<sup>55</sup>

Various studies have shown that sperm production and reproductive function of males are regulated by the insulin/IGF signaling pathway in Sertoli and Leydig cells.<sup>1,56,57</sup>

Since, in this study, the inclusion of fennel in the ration enhanced *IGF1* gene expression in the testis and on the other hand, the role of *IGF1* in the stimulation of Leydig cell steroidogenesis, the increased production of sperm and testosterone, and the growth and development of the testis, it can be affirmed that, including fennel in sheep diets can be applied to progress testis function and reproduction of sheep. However, to draw conclusions with 100% certainty, it is necessary to conduct studies with a wider scope.

## Conclusions

Referring to the findings of the present study, it can be affirmed that fennel with a useful effect on the expression of the *IGF1* gene in the testis can be applied to sheep diets to progress reproductive functions (by stimulating Leydig cell steroidogenesis, producing more sperm and testosterone, and growing and developing the testis). Although referring to the findings of the present study, it can be affirmed that fennel can be applied for various goals in sheep breeding, but in future investigations, it would be better to consider various and more complex physiological and epigenetic conditions in order to hold out for a definitive outcome.

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## Conflict of interest

MM has been an editorial board member of *Gene Expression* since September 2022. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Author contributions

Conceptualization, funding acquisition, investigation, methodology, resources, supervision, and experimental validation (MM); data curation (SMHS, BM, and OOB); formal analysis (MM, OB, and VA); project administration (MM, OK, and NK); software (MM and OB); manuscript drafting (MM, OB, SMHS, OK); manuscript review and revise (MM, BM, OOB, VA, NK). All authors read and approved the final manuscript.

## Ethics statement

All procedures related to animals were certified by the Animal Care and Use Committee of Bahonar University (IACUC Protocol #IR2018011) described by the Iranian Council of Animal Care. All guidelines and laws related to animal welfare policies for the animals used in this research were fully followed.

## Data sharing statement

The data sets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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