



Original Article



Effect of Fennel (*Foeniculum Vulgare*) Seed Powder Consumption on Insulin-like Growth Factor 1 Gene Expression in the Liver Tissue of Growing Lambs

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Abstract

Background and objectives: Today, medicinal plants and phytobiotics that contain flavonoids, alkaloids, and essential oils are widely used in animal feed. These additives have many benefits, including increasing liver function and preventing certain diseases. The liver is one of the organs that plays a key role in insulin-mediated regulation of metabolism as well as in glucose, whole-body, and lipid homeostasis. The aim of this study was to investigate the effect of fennel (*Foeniculum vulgare*) seed powder consumption on insulin-like growth factor 1 (*IGF1*) gene expression in the liver tissue of growing lambs.

Methods: Three groups (including 0, 10, and 20 g/kg dry matter of fennel) of animals, with 10 animals in each group, were studied. The Pfaffl method was applied to assess the real-time polymerase chain reaction (qPCR) output.

Results: Mixing fennel into the feed of lambs increased the weights of their testis and gallbladder and decreased the weight of their liver in comparison to feed without fennel. It was also found that by increasing the fennel level in the feed, the expression level of *IGF1* in the liver increased significantly ($p < 0.05$).

Conclusions: Fennel had a useful effect on the expression of *IGF1* in the liver tissue of sheep and may be applied to their diet to attain better liver function.

Introduction

The liver is one of the organs that plays a key role in insulin-mediated regulation of metabolism as well as in glucose, whole-body, and lipid homeostasis.¹ The functioning of the autocrine, parac-

rine, and endocrine systems and nutrition are factors that control the secretion of insulin-like growth factor 1 (*IGF1*) by the liver, although many other tissues produce this hormone.^{2,3} The main synthesizer of *IGF1* and cytoprotective hormone is the liver.⁴ It is a regulator of proliferation, survival, cell senescence, and so on.^{5,6} The *IGF1* gene is well-conserved among 25 different mammalian species representing 15 different orders and ranging over ~180 million years of evolutionary diversification.⁷ Pre- and postnatal growth in mammals is controlled by *IGF1*.^{8,9} It also acts as the main mediator of growth hormone functions and is involved in controlling tissue repair, intermediary metabolism, and disease pathogenesis throughout life.¹⁰ Grochowska *et al.* have reported that the molecular structure of *IGF1* is similar to that of insulin.¹¹ The length of the *IGF1* gene is 59.3 kb, is located on chromosome 3 of sheep, consists of five exons, and encodes a protein of 172 amino acids.³ In addition, Jeanplong *et al.* have identified *IGF1* as a key mitogen.¹² Moreover, Grochowska *et al.* have demonstrated that *IGF1* participates in the somatotrophic axis along with growth

Keywords: Liver; Diet; Real-time PCR; Sheep; Fennel.

Abbreviations: BPs, binding proteins; DM, dry matter; GH, growth hormone; GHRH, growth-hormone-releasing hormone; *IGF1*, insulin-like growth factor 1; IGF2, insulin-like growth factor II; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.

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hormone (GH), growth-hormone-releasing hormone (GHRH), insulin-like growth factor II (IGF2), binding proteins (BPs), and their associated receptors (IGF1R, IGF2R, GHRHR, and GHR).¹¹

Small ruminants, especially indigenous breeds, provide a significant portion of the dairy products, red meat, wool, and fiber needed by human populations living in hot, dry regions,^{13,14} and they have a significant impact on the economies of these regions.^{15,16} Therefore, the use of new methods for studying these animals and managing them to improve their genetic function and production seems very necessary.^{17,18} This increase in efficiency can be done via different aspects of production and reproduction. In Iran, the purpose of sheep farming is to produce meat, and other products are of secondary importance.¹⁷ Currently, more than 50 million sheep, including 27 breeds, are bred in Iran.^{19,20} Kermani sheep is one of the most important sheep breeds in Iran and is considered as a meat-wool breed.^{17,21} Due to the climatic conditions of Kerman Province, this animal has been able to adapt to hot and dry conditions as well as poor pastures and is a valuable breed that meets many needs of the nomadic people and ranchers of this province.^{22,23}

Phytobiotics and medicinal plants are widely used in animal feed.^{24,25} These additives have many benefits, including increasing liver function,²⁶ preventing certain diseases,²³ improving antioxidant and antimicrobial activities, and enhancing the efficiency of the carcass, the ratio of feed conversion, and feed intake.^{27–31} Fennel (*Foeniculum vulgare* Mill.), which belongs to the Apiaceae family, is one of these important and widely used medicinal plants. Fennel can create many of the above-mentioned effects in animals.^{23,32–37}

Kita *et al.* have reported that fasting and food-restriction reduce *IGF1* gene expression and that feeding recovers *IGF1* gene expression to the level of fed animals.^{38,39} In addition, Leili *et al.* have demonstrated that these changes are in line with the variations in the levels of *IGF1* in plasma.⁴⁰ Moreover, Kita *et al.* have shown that even though the restoration of the *IGF1* concentration in plasma via feeding after fasting requires approximately 48 h, the expression of *IGF1* is improved at 24 h after feeding fasted kids.³⁸ Furthermore, Izuddin *et al.* have reported that using postbiotics in the diet of newly weaned sheep increases their feed intake, weight gain, consumption and digestibility of nutrients, concentration of butyrate and ruminal ammonia-N, blood total protein, glucose, urea nitrogen, population of fiber-degrading bacteria, and mRNA expression of ruminal monocarboxylate transporter 1 and hepatic *IGF1*.⁴¹

The results of past studies show that fennel can probably affect the gene expression of *IGF1* and thereby improve some functional factors of livestock. However, to date, the role of food additives, especially fennel, on the expression of *IGF1* in sheep, especially Kermani sheep, has not been studied. Thus, the aim of this study was to investigate the effect of fennel (*F. vulgare*) consumption on the gene expression of *IGF1* in the liver tissue of growing lambs for the first time.

Materials and methods

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.

Animals

The experimental animals used in this study were selected from

the Animal Science Research and Training Station of Shahid Bahonar University of Kerman, Iran, and comprised 30 male lambs with an approximately similar weight (27.5 ± 0.45 kg). The lambs were immunized using a standardized procedure. All lambs were kept in a single pen (1.2×1.5 m) at the Animal Science Research and Training Station of Shahid Bahonar University of Kerman, Iran, for 110 days, including a 20-day transition period and a 90-day experimental period. The lambs had free access to food and water.

Feeding

The animals were fed twice a day. The diets used all had the same energy and protein (Table 1). Three groups of animals, with 10 animals in each group, were studied. The first group was the control group and was not fed fennel (0 g/kg dry matter (DM)), the diet of the second group contained 1% fennel (10 g/kg DM), and the diet of the third group contained 2% fennel (20 g/kg DM). The chemical composition of the three diets is shown in Table 1. To calculate the components of the diets, such as dry matter, nitrogen, ether extract, and ash, standard AOAC methods were used.⁴² The Van Soest method⁴³ was applied to determine the ash-free neutral detergent fiber and ash-free acid detergent fiber contents.

Blood sampling

On the day before slaughter at 3 h after the morning feeding, blood samples were collected. The collected samples were centrifuged at 6,000 rpm for 10 min and then frozen at -20°C for further analyses. An automatic analyzer (Technicon RA 1000; Bayer Co., Whippany, NJ, USA) using an Immunotech A, Beckman Coulter/REF 2121 (Parsazmun Laboratory, Tehran, Iran) testing kit was applied to determine the concentrations of serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT). The concentration of testosterone was calculated using an enzyme-linked immunosorbent assay kit (Stat Fax) along with a testosterone set (Patangostar-e-Eisar Co., Iran). This kit is based on a competitive enzyme immunoassay. Testosterone in the samples competes with the testosterone bound to horseradish peroxidase for binding to the coated antibody on the wells. After the incubation time, the wells were drained and washed. Then enzyme substrate was added to each well, and the activity of the enzyme was inversely proportional to the concentration of testosterone in the samples. Testosterone standards with specific concentrations were tested together with unknown samples, and the concentrations of unknown samples were obtained based on the standard curve of light absorption against the testosterone concentration. At the end of the investigation, all studied lambs were immediately slaughtered. Then, the weights of the liver, testis, and gallbladder were recorded.

Tissue sampling

Tissue sampling (three tissue samples from each animal in the study, for a total of 90 samples) from liver tissue was done. The samples were quickly placed in liquid nitrogen and then stored at -80°C .

RNA expression analysis

A standard One Step RNA Reagent kit (Biobasic Co. Ltd., Markham, Ontario, Canada) was applied for total RNA extraction. Extracted RNA was treated with RNase-free DNase I to remove any contaminating genomic DNA. Then, the quality of the extracted RNA was evaluated using agarose gel electrophoresis. Observation of only the 28S and 18S bands on the agarose gel affirmed the

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		

optimal quality of the purified RNA. An oligo d(T) primer along with a standard kit (#K1631, Fermentase Co., Iran) was used to synthesize the cDNA from the extracted total RNA.

For the *IGF1* target gene (NCBI accession number, NM_001009774.3; melting temperature (T_m), 57 °C; and product size, 265 bp), the following primers were used: forward 5'-ATTACAGCTGCCTGCCCTT-3' and reverse 5'-CACATCTGCTTACACCTTACCCG-3'. For the glyceraldehyde-3-phosphate dehydrogenase reference gene (NCBI accession number, NM_001190390.1; T_m , 57 °C; and product size, 76 bp), the following primers were used: forward 5'-ACCACTTTGGCATCGTGGAG-3' and reverse 5'-GGGCCATCCACAGTCTTCTG-3'. The total volume of each real-time polymerase chain reaction (qPCR) contained 15 µL and qPCR was done in Rotor-Gene Q MDX device (QIAGEN Hilden, Germany). Each qPCR reaction tube included template cDNA (1.5 µL), 2× SYBR Green PCR Master Mix (Fermentase Co., Tehran, Iran) (7.5 µL), dd H₂O (4.7 µL), 10 µM forward and reverse primers (1 µL), and carboxy-X-rhodamine as a fluorescent dye (0.3 µL). The following program was used to perform the qPCR reactions: 95 °C for 5 min, followed by a cycle of 95 °C for 20 s, 57 °C for 30 s, and 72 °C for 30 s, for 38 cycles. Melting curve analysis after completion of the amplification cycles was applied to affirm that the desired amplification had been done. To define the annealing temperature for the studied genes (target and reference), the gradient protocol was performed. The Pfaffl method⁴⁴ was employed to evaluate the qPCR data as follows: ratio = $\frac{(E_{\text{target}})^{\Delta CT_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta CT_{\text{ref}}(\text{control-sample})}}$

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta CT_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta CT_{\text{ref}}(\text{control-sample})}}$$

where, E_{target} is the PCR yield of the studied target gene, E_{ref} is the PCR yield of the internal control (reference) gene, and $\Delta ct = ct_{\text{GADPH}} - ct_{\text{IGF1}}$.

Statistical analysis

The mixed procedure with a completely randomized design was applied for data analysis using SAS software. To test the normality of the distribution of data, the pairwise fixed reallocation randomization test⁴⁴ was used. In addition, the least significant differences test was used to compare the means ($p < 0.05$).

Results

The weights of the liver, testis, and gallbladder in the studied lambs according to the amount of fennel in their feed were assessed (Table 2). The results showed that adding fennel to the diet increased the weights of the testis and gallbladder and decreased the weight of the liver in comparison to diets without fennel ($p < 0.05$).

In addition, the levels of blood liver enzymes (SGOT and SGPT) and testosterone in the studied lambs according to the amount of fennel in their feed were assessed (Table 3). The results demonstrated that adding fennel to the diet increased the concentration of blood testosterone and decreased the concentrations of SGOT and SGPT in comparison to diets without fennel ($p < 0.05$).

The cycle threshold values and gel electrophoresis of the synthesized cDNA showed that the transcript abundance level of *IGF1* in the liver was high. The results showed that *IGF1* was expressed in the liver of all studied lambs at a high level. The findings of the current investigation demonstrated that mixing the feed with fennel at different levels can significantly ($p < 0.05$) increase the expression of *IGF1* in sheep liver tissue (Table 4).

Discussion

In this research, the effects of different levels of fennel (0, 10, and 20 g/kg DM) in the diets of Kermani lambs on their liver, gallbladder, and testis weights, blood liver enzyme (SGOT and SGPT)

Table 2. Weights of the liver, testis, and gallbladder with different levels of fennel in the feed of studied lambs

Parameter	Level of fennel (g/kg DM)			SEM	p-value
	0	10	20		
Weight of testis (kg)	0.290 ^b	0.350 ^a	0.360 ^a	0.020	0.040
Weight of gallbladder (kg)	0.027 ^b	0.031 ^{ab}	0.041 ^a	0.004	0.049
Weight of liver (kg)	0.740 ^a	0.640 ^b	0.630 ^b	0.030	0.014

^{a,b,c}Values within a row with different superscripts differ significantly at $p < 0.05$. DM, dry matter; SEM, Standard Error of Mean.

Table 3. Levels of blood liver enzymes and testosterone with different levels of fennel in the feed of studied lambs

Parameter	Level of fennel (g/kg DM)			SEM	p-value
	0	10	20		
Serum glutamic oxaloacetic transaminase (U/L)	112.600 ^a	79.250 ^b	78.890 ^b	2.760	0.002
Serum glutamic pyruvic transaminase (U/L)	30.510 ^a	25.010 ^b	24.920 ^b	1.110	0.039
Testosterone (ng/dL)	1.700 ^b	3.500 ^a	4.400 ^a	0.230	0.025

^{a,b}Values within a row with different superscripts differ significantly at $p < 0.05$. DM, dry matter; SEM, Standard Error of Mean.

concentrations, testosterone concentration, and *IGF1* expression in the liver were studied. The results showed that adding fennel to their diet increased their testis and gallbladder weights, decreased their liver weight, increased their blood testosterone concentration, and decreased their SGOT and SGPT concentrations in comparison to diets without fennel ($p < 0.05$).

The lower weight of the liver in animals fed with fennel compared to control animals is probably due to the presence of compounds such as estragole and anethole in this additive. Meanwhile, the larger gallbladder weight in the fennel-fed animals could be due to the presence of oleic acid in fennel. In addition, the active compounds in fennel, such as various polyphenolic compounds, as well as the antioxidant activity of fennel may be the reasons for the low concentrations of SGPT and SGOT in the animals fed with fennel compared to the control animals.

The qPCR results are in line with different investigations that have demonstrated *IGF1* expression in the livers of sheep,⁴⁵ mice,^{46,47} goats,⁴⁸ pigs,⁴⁹ and rats.⁵⁰ Furthermore, Lara-Diaz *et al.* have revealed that *IGF1* deficiency causes significant changes in gene expression patterns.⁴⁷

Many studies have shown that *IGF1* is a growth stimulant, protects the liver, and plays an important role in liver metabolism, tissue repair, and the pathogenesis of liver disease.^{2,8,9,51} For example, Adamek and Kasprzak have shown that in liver diseases such as liver steatosis, fibrosis, and cirrhosis, the serum *IGF1* levels and tissue expression are reduced.¹ However, an increase in the *IGF1* level can improve liver function and fibrosis. In addition, it has been demonstrated that enhancing *IGF1* in cirrhotic patients can improve liver protection.^{52–55} For instance, Sobrevals *et al.* transferred the *IGF1* gene to animal models of liver cirrhosis and showed that it increases the expression of antifibrogenic molecules, decreases the expression of profibrogenic factors, and

relieves pain.⁵⁶ Therefore, increasing the level of *IGF1* by adding fennel to the diet can help to fight liver diseases more easily.

The results of many studies have shown that *IGF1* increases the survival and longevity of cells, suggesting that *IGF1* may be used to suppress premature cell aging and improve liver disease.^{57–59} Additionally, Adamek and Kasprzak have revealed that to prevent premature aging of liver cells, the p53/progerin pathway could be downregulated by *IGF1* overexpression or prolonged treatment with exogenous *IGF1*.¹

Furthermore, Shahsavari *et al.* have demonstrated that *IGF1* interacts with various genes, including insulin-like growth factor-binding protein 4 (*IGFBP4*), tyrosine-protein kinase receptor (*IGFR1*), insulin-like growth factor binding protein 5 (*IGFBP5*), insulin-like growth factor binding protein 3 (*IGFBP3*), insulin-like growth factor-binding protein 1 (*IGFBP1*), tyrosine-protein kinase receptor (*INSR*), hepatocyte growth factor (*HGF*), vascular endothelial growth factor A (*VEGFA*), tyrosine-protein kinase receptor or insulin receptor-related receptor (*INSRR*), and insulin-like growth factor-binding protein 2 (*IGFBP2*).²⁵ Interestingly, among the above-mentioned genes, *IGFBP4*, *IGFR1*, *IGFBP5*, and *IGFBP3* possess the closest interactions with *IGF1*.

Since the use of fennel in this study increased the expression of *IGF1* in the liver and *IGF1* has been shown to increase liver function, it can be hypothesized that the addition of fennel to the sheep diet can be used to improve liver function. However, further studies are needed to reach a definitive conclusion.

Conclusions

Based on the results of the current study, fennel has a useful effect on the expression of *IGF1* in the liver tissue and may be applied to the sheep diet to increase liver function. Although it can be hypoth-

Table 4. The influence of fennel consumption on the relative *IGF1* gene expression in the liver of studied lambs

Tissue	Fennel consumption (g/kg DM)			SEM	p-value
	0	10	20		
Liver	1 ^c	2.6 ^b	3.3 ^a	0.04	0.001

^{a,b,c}Values within a row with different superscripts differ significantly at $p < 0.05$. DM, dry matter; SEM, Standard Error of Mean.

esized that fennel may be applied for various aims in sheep breeding, future investigations are needed so that various and more complex epigenetic and physiological conditions can be considered.

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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

Study concept and design (MRM, OB), acquisition of the data (MS, VK), assay performance and data analysis (VF, OK, OB, MRM), drafting of the manuscript (MRM, AK), critical revision of the manuscript (MRM), supervision (MRM).

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.

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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