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# Effects of diets with different levels of fennel (*Foeniculum vulgare*) seed powder on *DLK1* gene expression in brain, adipose tissue, femur muscle and rumen of Kermani lambs

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ABSTRACT

*DLK1* (protein delta homolog 1) plays an important role in production of adipocytes, muscle development, wound healing, pancreas, liver and lung cells development and also in the development of meat quality, digestion and growth performance. Therefore, the goal of this research was to investigate the effects of different levels of fennel seed power in the diet on growth performance and on *DLK1* gene expression in brain, adipose, femur (leg) muscle and rumen muscle tissues of Kermani lambs. Dietary treatments including different levels of fennel seed powder (0, 1 and 2 % of diet DM) were fed to 3 groups of lambs (each group contained 10 animals) for three months. Some physiological parameters relating to muscle and adipose development were measured. Tissues including brain, adipose tissue, femur (leg) muscle and rumen muscle were collected for expression analysis of the *DLK1* gene. In this study, increasing the level of fennel seed powder in diets of lambs, increased the amount of *DLK1* gene expression in the femur muscle and rumen tissues. Feeding 2 % fennel in comparison to the control diet (0 %) resulted in a significant difference in the amount of *DLK1* gene expression in the femur (leg) muscle and in the rumen muscle ($P < 0.05$). Maximum gene expression rate was observed in all studied tissues of lambs fed diets with 2 % fennel in comparison to 0%. On the other hand, final weight, live daily gain, dry matter intake, warm carcass weight, weight of back muscle (loin), weight of femur (leg) muscle, weight of lean meat and eye muscle area were higher for animals fed with 2 % fennel than those fed control diet, but weight of liver was lower in animals fed with 2 % fennel than those fed control diet. In regards to the results of this investigation, it can be concluded that fennel can be used for increasing *DLK1* gene expression in some tissues, like muscle, and consequently for increasing animal growth and muscle mass, which is important in the sheep industry.

## 1. Introduction

One of the important genes that is extensively expressed during the embryonic development in mammals is *DLK1* (preadipocyte factor 1, or a protein delta homolog 1). This protein is a transmembrane epidermal growth factor (EGF)-like with an N-terminal signal sequence, six EGF-like repeats, a short juxtamembrane region of the ADAM17 cleavage site, a short C-terminal cytoplasmic tail (intracellular region) and a transmembrane domain (Bujak et al., 2015; Falix et al., 2013). *DLK1* gene is located on chromosomes 7, 12, 14 and 18 of pigs, mice, humans and sheep respectively (Oczkowicz et al., 2010). The most important functions of *DLK1* includes production of adipocytes (Smas and Sul, 1993), muscle development, wound healing (Andersen et al., 2009), liver (Tanimizu et al., 2003), lung and pancreas development (Yevtodiyenko and Schmidt, 2006) cellular proliferation and differentiation (Baladrón et al., 2005; Nueda et al., 2007). According to some studies *DLK1* gene is

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expressed in many fetal tissues and undifferentiated cells of murine and humans (Smas and Sul, 1993) whereas in adult human tissues *DLK1* is only expressed in organs including pancreas (Jensen et al., 1994) and adrenal gland (Jensen et al., 1993). Moreover, the expression of *DLK1* mRNA was also reported in ovary, testis, heart and pituitary gland (Harel et al., 2011), preadipocytes (Traustadottir et al., 2013) and neuron stem cells (Surmacz et al., 2012). *DLK1* studies in farm animals were widely performed in sheep and mostly designed to distinguish the Single Nucleotide Polymorphism (SNP) for the so called "callipyge phenotype" (muscle hypertrophy of the hindquarters). Inheritance of the callipyge (CLPG) phenotype does not follow Mendelian rules, but is a case of polar overdominance, and comes out in the offspring only when the father and mother are the origin of mutated allele and wild one respectively (Cockett et al., 1996). The level of *DLK1* gene expression is increased in CLPG phenotype. Because SNP in CLPG significantly affects sheep musculature, researchers have introduced *DLK1* as a strong candidate gene for marker-assisted selection in livestock. Unfortunately, SNP in CLPG is breed specific and observed only in callipyge flocks (Smit et al., 2003), but investigation on *DLK1* polymorphism shows an association with growth, fatness and body composition and its polar overdominant inheritance (Kim et al., 2004). Shin et al., 2009 showed that *DLK1* mRNA expression was higher in the muscles of broilers than in layers and suggested *DLK1* gene as a new selection-marker for studying the high muscle growth in chickens. The use of phytobiotics and medicinal plants as natural antimicrobial growth promoters in replacement to antibiotics in animal feeding has definitely many benefits for the progress of zootechnical efficiency parameters, suppression of specific diseases (Aćimović et al., 2016), antimicrobial and antioxidants activities (Valero and Salmeron, 2003; Miura et al., 2002), hypocholesterolemic effects (Craig, 1999), digestive enzymes enhancement (Ramakrishna et al., 2003) and improvement of liver functions (Hernandes et al., 2004). Researchers demonstrated that adding these plants to diet of poultry and other animals increased feed consumption, ratio of feed conversion and carcass yield (Abdullah and Rabia, 2009). Fennel (*Foeniculum vulgare* mill) from *Apiaceae* family, is one of the most important medical plant with very strong antioxidant, antimicrobial and hepatoprotective properties which was recognized and applied by humans since ancient times (Gharaghani et al., 2015). It was shown that using fennel in diets of broilers resulted in increased body weight, better feed conversion (El-Deek et al., 2003), improved performance and health conditions (Aćimović et al., 2016), decreased total number of bacteria, increased weight and length of small intestine and carcass yield (Saki et al., 2014), increased the number of red blood cells, hemoglobin and packet cell volume (Mohammed and Abbas, 2009), increased oxidative quality of meat (Gharaghani et al., 2013) and improved digestion and growth performance (Radwan and Khalil, 2002). There are different breeds of sheep in Iran (twenty-six breeds) with specific adaptation to a certain region (Zamani et al., 2015; Khodabakhshzadeh et al., 2016). Kermani sheep is one of the most valuable breeds of Iran's native sheep and provides substantial requirements of nomads and breeders in Kerman province (Mohammadabadi et al., 2017). This dual-purpose medium--sized fat-tailed sheep (meat and wool) with white wool has a high adaptation to the severe and unproper environmental conditions of the southeastern part of Iran with hot and dry climate, as well as poor pastures and low vegetation cover (Mohammadabadi, 2016; Vajed Ebrahimi et al., 2016). Since effect of fennel and role of *DLK1* in farm animals, especially in sheep was not studied, the aim of this research was to study the effect of different levels of fennel in the diet on growth performance and on expression of *DLK1* gene in various tissues of Kermani sheep.

## 2. Materials and methods

### 2.1. Animals, diets and experimental design

This research was done in the Animal Science Research and Training Station of Shahid Bahonar University of Kerman, Iran. Thirty male lambs of Kermani sheep with almost the same weight ($27.5 \pm 0.45$ kg) and 6-months-old were randomly allocated to three experimental groups (10 animals in each group). Lambs were placed in individual pens ($1.2 \times 1.5$ m) with bedded straw, in a sheltered, cemented-floor, open-side barn, well ventilated and had free access to feed and water. During this period, 20 days for adaptation and 90 days for data collection were considered. Before starting experimental period, all animals were sheared, anti-parasitic drugs were used and anthrotoxemia vaccine was also performed. Experimental animals were fed free and individually with three levels of fennel (0, 1 and 2 %) in the diet for three months (90 days). It should be noted that 10 animals were considered for each level. The feed was mixed completely and lambs were fed twice a day at 8:00 and 16:00, to allow about 5% orts. Water was provided ad-libitum and was replaced twice a day. All experimental diets had the same energy and crude protein.

### 2.2. Measurements

Some physiological parameters relating to muscle and adipose development, as dry matter intake, final weight, live daily gain, warm carcass weight, weight of back muscle (loin), weight of femur (leg) muscle, weight of fat –tail, weight of lean meat, eye muscle area, weight of empty rumen, weight of liver and back fat thickness were measured. The live daily gain of lambs was measured as the difference between the initial and final weights over the interval of the performance phase.

### 2.3. RNA expression analysis

After slaughter, Kermani sheep tissues including brain, subcutaneous adipose tissue, femur (leg) muscle and muscular wall of the rumen (3 repeats for each of the four tissues in each of the 3 groups of 10 animals, totally 360 samples) were collected. The samples were quickly placed in liquid nitrogen and then stored. One Step RNA Reagent Kit (Biobasic Co. Ltd., Iran) was used for extracting total RNA from each tissue and then integrity of RNA and absence of genomic DNA was assessed by agarose gel electrophoresis, checking the presence of the two bands 18S and 28S and comparing with molecular weight standards. cDNA was synthesized from RNA using standard kit (#K1631, Fermentase Co., Iran) and an oligo d(T) primer. The concentration of total RNA in each reaction was 1 microgram. RT-PCR technique was applied using primers forward 5′-CGTCTTCCTCAACAAGTGCGA-3′ and reverse 5′-TCCTCCCCGCTGTT GTAGTG-3′ (accession number NM-174037, Tm = 57 °C, product size 102 bp) for *DLK1* gene and forward 5′−CCTGGCACCCAGCACAAT -3′ and reverse 5′- GGGCCGGACTCGTCATAC -3′ (accession number NM_001101.3, Tm = 57 °C, product size 144 bp) for beta actin (*ACTB*) gene, used as reference gene. Reactions were carried out in a volume of 15 μL consisting of 2X SYBR Green PCR Master Mix (Fermentase Co., Tehran, Iran), 7.5 μL; template cDNA, 1.5 μL; 10 μM forward and reverse primers, 1 μL; ROX, 0.3 μL and ddH2O, 4.7 μL in Rotor-Gene Q MDx instrument (QIAGEN Hilden, Germany). PCR protocol was done at 94 °C for 3 min, then 35 cycles of 94 °C for 60 s, 57 °C for 60 s, and 72 °C for 60 s followed by a melt curve of 55 °C–95 °C with increments of 0.5 °C every 5 s. The sharp single peaks of the melting curves and the presence of the amplification curves of *DLK1* and *ACTB* products implies the lack of primer-dimers formation and also verify the specificity of the primers, in accordance with the lack of generation of amplification products in the negative control samples.

Pfaffl et al. (2002) method was used to analyze the data from Real Time PCR. To determine the PCR reaction efficiency, a standard curve for *DLK1* and *ACTB* was drawn with serial dilution of cDNA of a pool of samples (1, 1/10, 1/100, 1/1000); PCR efficiency of *DLK1* and *ACTB* genes were 98 % and 99 % respectively.

### 2.4. Statistical analysis

Completely randomized design using the MIXED procedure of the

SAS (2005) was used for analyzing the physiological data. The normality of the data distribution was checked by the Pair Wise Fixed Reallocation Randomisation Test© (REST, 2009). For comparing the means, the LSD test with probability level of $P < 0.05$ was used.

The softwares including SPSS 16.0 (SPSS, Inc., Chicago, IL, USA), LinRegPCR (11.0) and REST (2009) were utilized for analysis of Real-Time PCR results obtained using Pfaffl formula (Pfaffl et al., 2002).

Moreover, below statistical model was used for assessing the main effect of fennel level and the tissue effect with the interaction fennel x tissue:

$$X_{ijm} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{m(ij)}$$

Where, $X_{ijm}$ is the dependent variable score for subject *m* in treatment group *ij*, μ is the mean, $\alpha_i$ is the main effect of tissue at level *i*, $\beta_j$ is the main effect of fennel at level *j*, $\alpha\beta_{ij}$ is the interaction effect of tissue at level *i* and fennel at level *j* and $\varepsilon_{m(ij)}$ is the effect of all other extraneous variables on subject *m* in treatment group *ij*.

The STRING program was used for representing *DLK1* interactions with other genes in Ovis aries (http://string-db.org).

## 3. Results

Data on the physiological parameters related to muscle and adipose development were shown in Table 1. Dry matter intake, final weight, live daily gain, weights of warm carcass, back muscle (loin), femur (leg) muscle, lean meat and eye muscle area were higher for animals fed with 2 % fennel than those fed control diet (no fennel diet) ($P < 0.05$), whereas weight of liver was lower in animals fed with 2 % fennel than those fed control diet (no fennel diet) ($P < 0.05$). The average cycle threshold (Ct) value of the *DLK1* gene in different tissues ranged from 22 to 24. The interaction between tissue and fennel feeding level was always significant. Along with increasing the level of fennel in diets of lambs, in level 2, compared to level 0, there is greater expression of *DLK1* in femur muscle and rumen muscle (Table 2). The comparison of the expression of *DLK1* gene in the brain, adipose, femur (leg) muscle and rumen tissues at 1 % fennel level (Table 3): showed that the *DLK1* expression in femur muscle and rumen muscle was more than brain and adipose tissues ($P < 0.05$). At 2 % level of fennel feeding (Table 3), the difference on gene expression was significant between brain and other three tissues, with lower expression in brain ($P < 0.05$).

The interaction between *DLK1* and other predicted genes and the description of predicted functional partners using the STRING program is given in Fig. 1. *DLK1* had an interaction with iodothyronine deiodinase (*DIO3*) with score 0.896, thyroxine 5-deiodinase (ENSOARG00000013889) with score 0.896, retrotransposon-like 1 (*RTL1*) with score 0.859, brain-enriched guanylate kinase-associated protein (*BEGAIN*) with score 0.692, insulin-like growth factor II (*IGF2*) with score 0.677, fibroblast growth factor (acidic) intracellular binding protein (*FIBP*) with score 0.674, SRY-box 9 (*SOX9*) with score 0.649, insulin-like growth factor 2 receptor (*IGF2R*) with score 0.600, deoxynucleotidyltransferase terminal interacting protein 1 (*DNTTIP1*) with score 0.596 and CCAAT/enhancer binding protein (C/EBP) alpha (*CEBPA*) with score 0.591.

**Table 1**
The effect of fennel feeding on some physiological parameters relating to muscle and adipose development.

| Calculated parameters | Level of fennel (%) | | | SEM | P value |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | | |
| Initial weight (kg) | 27.0 | 27.9 | 27.7 | 0.49 | 0.638 |
| Final weight (kg) | $44.7^{b}$ | $45.8^{a}$ | $46.5^{a}$ | 0.23 | 0.024 |
| Live daily gain (g) | $221^{b}$ | $224^{b}$ | $235^{a}$ | 3.06 | 0.035 |
| Dry matter intake (kg/day) | $1.367^{b}$ | $1.406^{a}$ | $1.433^{a}$ | 0.015 | 0.01 |
| Warm carcass weight (kg) | $20.74^{b}$ | $21.20^{ab}$ | $21.99^{a}$ | 0.36 | 0.039 |
| Weight of liver (kg) | $0.73^{a}$ | $0.62^{b}$ | $0.64^{b}$ | 0.02 | 0.013 |
| Weight of back muscle (loin) (kg) | $3.67^{b}$ | $3.84^{ab}$ | $4.07^{a}$ | 0.11 | 0.043 |
| Weight of femur (leg) muscle (kg) | $5.53^{b}$ | $5.86^{ab}$ | $5.96^{a}$ | 0.12 | 0.039 |
| Weight of fat –tail (kg) | 2.23 | 2.31 | 2.48 | 0.14 | 0.426 |
| Weight of lean meat (kg) | $14.28^{b}$ | $14.70^{ab}$ | $15.43^{a}$ | 0.34 | 0.029 |
| Back fat thickness (cm) | $4.50^{a}$ | $3.10^{b}$ | $4.00^{a}$ | 0.28 | 0.018 |
| Eye muscle area ($cm^2$) | $17.08^{b}$ | $16.96^{b}$ | $20.44^{a}$ | 0.77 | 0.034 |
| Weight of empty rumen (kg) | $0.89^{a}$ | $0.78^{b}$ | $0.81^{ab}$ | 0.03 | 0.040 |

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

**Table 2**
The effect of fennel feeding on expression of *DLK1* gene in brain, adipose, femur (leg) muscle and rumen tissues of Kermani sheep.

| Tissue | Relative expression of *DLK1* | | | SEM | P value |
|---|---|---|---|---|---|
| | 0 % fennel | 1% fennel | 2% fennel | | |
| Brain | 1 | 2.0 | 2.9 | 0.16 | 0.515 |
| Adipose tissue | 1 | 1.2 | 2.9 | 0.18 | 0.427 |
| Femur muscle | 1 | 1.6 | 3.6* | 0.12 | 0.026 |
| Rumen muscle | 1 | 1.7 | 3.8* | 0.11 | 0.019 |

Treatments marked with * have a significant difference ($P < 0.05$) in comparison of control diet (0% fennel) for any tissue.

**Table 3**
Comparison of effect of fennel feeding at two levels on *DLK1* gene expression in the brain, adipose, femur (leg) muscle and rumen tissues of Kermani sheep. Mean comparison (mean of three replications) was performed using LSD test ($P < 0.05$).

| Level of fennel | Relative expression of *DLK1* | | | | SEM | P value |
|---|---|---|---|---|---|---|
| | Brain | Adipose tissue | Femur muscle | Rumen muscle | | |
| 0% | 1 | 1 | 1 | 1 | 0 | – |
| 1% | $1.70^{b}$ | $1.65^{b}$ | $2.07^{a}$ | $2.03^{a}$ | 0.22 | 0.038 |
| 2% | $2.75^{b}$ | $3.4^{a}$ | $3.70^{a}$ | $3.65^{a}$ | 0.35 | 0.029 |

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

## 4. Discussion

The average cycle threshold (Ct) value of the *DLK1* gene in different tissues ranged from 22 to 24. These results indicated that the transcript

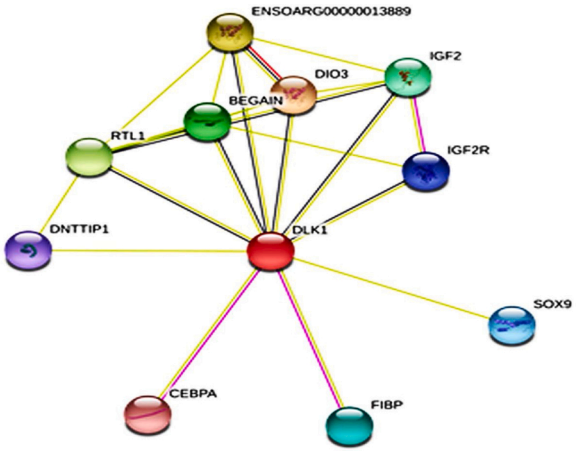


**Fig. 1.** *DLK1* interaction with other predicted genes and description of predicted functional partners using the STRING program in *Ovis aries*. Iodothyronine deiodinase (*DIO3*) score 0.896, thyroxine 5-deiodinase (*ENSOARG*00000013889) score 0.896, retrotransposon-like 1 (*RTL*1) score 0.859, brain-enriched guanylate kinase-associated protein (*BEGAIN*) score 0.692, insulin-like growth factor II (*IGF2*) score 0.677, fibroblast growth factor (acidic) intracellular binding protein (*FIBP*) score 0.674, SRY-box 9 (*SOX*9) score 0.649, insulin-like growth factor 2 receptor (*IGF2R*) score 0.600, deoxynucleotidyltransferase terminal interacting protein 1 (*DNTTIP*1) score 0.596 and CCAAT/enhancer binding protein (C/EBP) alpha (*CEBPA*) score 0.591.

abundance of *DLK1* in different tissues is high (Radoni et al., 2004). However, Ct value also depends on factors including the efficiency of Real-Time PCR, the amount of cDNA and the instrument settings. Along with increasing the level of fennel in diets of lambs, the amount of *DLK1* gene expression in the brain and adipose tissues numerically increased (Table 2). Some studies (Yin et al., 2006; Rocha et al., 2007; Oczkowicz et al., 2010) reported the expression of the *DLK1* gene in brain tissue and Yevtodiyenko and Schmidt (2006) and Deiuliis et al. (2006) observed *DLK1* gene expression in the adipose tissue that confirms our results. Karami et al. (2010) added herbal antioxidants to diets of goats and showed that these additives remarkably reduced back fat. Moon et al. (2002) demonstrated that *DLK1* plays an important role in many aspects of energy metabolism, specially as an inhibitor of adipogenesis. In another study, Charalambous et al. (2014) concluded that *DLK1* function modifies the metabolic way toward oxidation of peripheral lipid and prevention of lipid storage. In our study, adding fennel in the diet of animal at 1 % level compared to 0% level numerically increased the expression of *DLK1* gene in the adipose tissue and reduce the back fat thickness, suggesting the role of *DLK1* as an inhibitor of adipogenesis. Unfortunately, this effect is not confirmed for 2 % level, where a numerically greater gene expression is observed, but a lower thickness of the back fat is not observed.

In the current study, weight of liver in sheep fed with 1 and 2 % fennel diets was lower than those fed the control diet, which can be explained by the estrogenic effect of essential olis of fennel, particularly the phenolic components anethole and estragole. Hosseinzadeh and Farhoomand (2014) fed broilers with different levels of tarragon (rich in estragol and anethole). They concluded that the liver weight decreased in response to adding 0.5 % tarragon's powder in diet of broilers. In another study, Amad et al. (2013) reported that using phytogenic feed additive with combination of anethole and thymol in broiler chicks diets enhances the digestive and bile enzyme secretions resulting in higher lipid digestibility. In another study (Visavadiya and Narasimhacharya, 2011), it was shown that feeding diets containing *Glycyrrhiza glabra* and *Withania somnifera* to rats with high cholesterol level can increase HMG-COA reductase activity and secretion of bile acids but decrease liver weight. Charalambous et al. (2014) showed that overexpression of *DLK1* reduces fat storage, pituitary insulin-like growth factor 1 (IGF1) resistance and feedback regulation of growth hormone. These changes result in higher blood GH toward whole body in the reduction of hepatic metabolism and reduction in hepatic steatosis and liver weight. Hence, *DLK1* gene mediates important physiological adaptations and metabolic disease resistance. In our study, liver weight decreased in response to adding fennel in the diet of lambs and other researchers (Charalambous et al., 2014) have shown that *DLK1* decreases hepatosteatosis and alters the whole metabolism of the organism. These results imply that using fennel in the diet of animals can be useful to preserve animals from steatosis when the animals are exposed to metabolic stress conditions. Weight of empty rumen of sheep fed 1% fennel was lower than those fed 0% fennel, which agrees with the results from Kamra et al. (2006) and Patra et al. (2010).

The expression of *DLK1* gene in the femur muscle and in the rumen muscle was higher in 2 % fennel diet compared to 0 % fennel diet (Table 2). The expression of *DLK1* in muscle tissue was reported in some studies (Davis et al., 2005; Yevtodiyenko and Schmidt, 2006; Fleming-Waddell et al., 2009; Oczkowicz et al., 2010; Falix et al., 2013; Su et al., 2014) which is in line with our results. Yevtodiyenko and Schmidt (2006) reported higher *DLK1* expression in skeletal muscle of sheep during embryogenesis but it is down-regulated postnatally. They concluded that this gene seems to have an important role as a growth-promoting factor in this tissue. According to Davis et al. (2005), over-expression of *DLK1* gene in skeletal muscle of transgenic mice had a significant increase in muscle mass and muscle fiber size compared to normal mice which could implies the role of *DLK1* in muscle hypertrophy in Callipyge sheep. Su et al. (2014) demonstrated a positive and significant correlation between *DLK1* and *IGF1* gene expression concerning muscle fiber diameter and muscle fiber shear stress, but negative correlation with muscle fiber density, which could confirm the positive effects of fennel at 1% and 2% levels on final weight, and at 2 % level on live daily gain of animals as observed in our study (Table 1). Also the increase of weight of back muscle (loin), femur muscle (leg) and lean meat in 2% fennel diet compared to control diet could be related to an increase of the expression of *DLK1* gene in muscle tissues. The effect of fennel on muscle tissue might be due to estrogenic effects of essential oils of fennel, such as anethole constituents. Mauvais-Jarvis et al. (2013) stated that estrogens can have positive effects on the production of protein kinase B and in turn higher entry of glucose to muscle. Notelovitz (2002) reported that steroid hormones have receptors on all bone cells which enhance bone and lean tissue mass. Saeedi et al. (2016) also reported that estrogens in fennel has positive effects on body weight of Holstein dairy calves.

Our results showed that diets including fennel affected dry matter intake of animals, so that animals fed 1 % and 2 % of fennel consumed more dry matter than those fed control diet (no fennel). This might be due to the positive effects of fenchone and anethole of fennel on diet palatability. Cabuk et al. (2003) reported that anethole and estragole have appetite stimulating effects. Saeedi et al. (2016) found that supplementation of the starter diets with 0.4 and 0.8 % (dry matter basis) fennel increased dry matter intake in Holstein dairy calves. In contrast to our results, Zolfaghari Moheb et al. (2015) showed that feeding fennel by-product (including the leaves, stems and some seeds) had no effect on dry matter intake of growing lambs. Asemi Esfahani et al. (2016) also reported that adding 0.25 and 0.5 % of anise seed powder to the suckling calf diet did not affect dry matter intake.

In our study, there was a significant increase of dry matter intake between animals fed with control diet and animal fed with fennel (Table 1), and there was a significant difference in the *DLK1* expression among 0 % and 2 % fennel levels ($P < 0.05$) in the femur (leg) muscle and rumen muscle (Table 2). Thus, it can be concluded that although fennel can partially increase muscle mass through increased feed intake (dry matter intake), it can also do some of this increase through increased *DLK1* gene expression. The weights of warm carcasses, lean meat, back muscle (loin), femur (leg) muscle, and eye muscle area were higher for animals fed with 2 % fennel than those fed control diet which might be due to greater final body weight of studied sheep. In contrast to our results, Karami et al. (2010) added herbal antioxidants to diet of goats and showed that these additives remarkably reduced eye muscle area. On the other hand, Waddell et al. (2010) proposed a model in which *DLK1* gene expressed by aborning or regenerating myofibers non-cell autonomously increases the differentiation of their neighbor satellite cells and leads to muscle hypertrophy.

Considering the significant effect of fennel on increasing the *DLK1* gene expression and the role of this gene in muscle size increase, especially at an early age, it can be concluded that fennel (especially at level of 2 %) can be used in lamb diets as a suitable and beneficial natural growth promoter in the sheep production industry and also can be proposed that the fennel feeding mimics the callipyge phenotype. Andersen et al. (2013) studied the role of *DLK1* in skeletal muscle development and showed that *DLK1* fails to alter the adipogenic commitment of muscle-derived progenitors in vitro, as well as intramuscular fat deposition during in vivo regeneration. Our results showed that fennel feeding had increasing effect on *DLK1* gene expression in a muscle tissue and the weight of lean meat was higher for animals fed with 2% fennel, in comparison to 0 % level. Thus, based on what Andersen et al. (2013) found, it can be speculated that, in animals fed with 2 % fennel, intramuscular adipose tissue doesn't grow, and probably animals have tougher meat. Although no studies regarding the role of *DLK1* gene expression in rumen have been reported, the similarity of the expression pattern of this gene in the muscular wall of the rumen and muscle tissue implies that fennel can be used in lamb diets to improve the structure of rumen muscle.

Comparison of effect of fennel feeding at 1 % level on *DLK1* gene

expression in the brain, adipose, femur (leg) muscle and rumen tissues (Table 3) showed that the increase in *DLK1* gene expression in femur muscle and rumen tissues is significantly ($P < 0.05$) more than brain and adipose tissues. At 2 % level of fennel feeding, the brain had the lowest expression (Table 3). These results propose the possible role of *DLK1* in regulating the activity of muscle development and muscle growth promotion in femur muscle and in the muscular wall of the rumen. The highest expression of *DLK1* gene was observed in femur muscle at both level (1% and 2%) of fennel feeding. Davis et al. (2005) reported that *DLK1* gene causes significant increase in muscle mass and muscle fiber size. Concerning our study, we could speculate that fennel can have a positive effect on expanding of muscle mass through increasing *DLK1* gene expression.

As seen in Fig. 1, based on description and function of different genes, *DLK1* has an interaction with retrotransposon-like 1 (*RTL1*) gene. Concerning *DLK1* and *RTL1* interaction, Fleming-Waddell et al. (2009) studied the effect of *DLK1* and *RTL1* on muscle gene expression in callipyge lambs and identified a number of genes that are regulated by *DLK1* and *RTL1* expression and exert control on postnatal skeletal muscle growth. They demonstrated that the genes identified in this model are primary candidates for naturally regulating postnatal muscle growth in all meat animal species, and may serve as targets to ameliorate muscle atrophy conditions including myopathic diseases and age-related sarcopenia. Callipyge lambs have normal muscle development at birth, and just prior to birth no differences in *DLK1* and *RTL1* expression were detected compared to normal phenotype (Perkins et al., 2006). One possibility to explain this condition is that mechanical tension and nerve stimulation that occurs when lambs begin to walk and run is the initiating stimulus for differential *DLK1* and *RTL*1 expression and the onset of muscle hypertrophy. Changes in gene expression during muscle hypertrophy can be categorized as the primary causative genes, *DLK1* and *RTL1*, which are known due to the inheritance model, the secondary effector genes that have a direct transcriptional response to *DLK1* and *RTL1* activities, and the tertiary responses associated with hypertrophy, such as protein accretion, myofiber type and metabolic changes. The mechanisms by which elevated *DLK1* and *RTL1* protein levels initially induce changes in gene expression are not likely to be detected by RNA analysis but the changes in transcripts abundance of the secondary and tertiary genes can be collectively identified by gene expression profiling. This information suggests broader roles of *DLK1* protein in other tissues. Yu et al. (2018) examined 23 transcripts and showed that among them, five genes, deoxynucleotidyltransferase terminal interacting protein 1 (*DNTTIP1*), Parkinson Protein 7 (*PARK7*), cAMP specific phophodiesterase 4D (*PDE4D*), Solute carrier family 22 member 3 (*SLC22A*3), and protein-lysine methyltransferase 21E (*METTL21E*), were up-regulated specifically in hypertrophied muscles, resembling *DLK1* expression patterns in seven muscles, and concluded that these genes can be considered as the secondary targets in response to *DLK1* signaling. They also reported that only *DNTTIP1* and *PDE4D* were up-regulated in *DLK1*-treated myoblasts and myotubes, suggesting a direct signaling effect of *DLK1* on the transcriptional expression of these two genes. Taken together, these combined results indicated that *DNTTIP*1 and *PDE4D* are potential secondary effector genes responding to *DLK1* signaling. The up-regulation of Myosin Heavy Chain 4 *MYH*4 in *DLK1*-treated myotubes was consistent with analyses of hypertrophied muscle from callipyge sheep, indicating *DLK1* signaling have an effect on fast-twitch myofiber formation. *DNTTIP1* positively regulated *MYH4* and negatively influenced Myosin Heavy Chain 7 (*MYH7*) luciferase activity, implying a direct effect of the transcription factor on muscle fiber switch in callipyge muscles. The study of Yu et al. (2018) provided additional supports that *RTL*1 alone was insufficient to induce muscle hypertrophy and concluded that *DLK1* was likely the primary effector of the hypertrophy phenotype. Their results also suggested that *DNTTIP1* and *PDE4D* were secondary effector genes, responding to *DLK1* signaling and resulting in muscle fiber switch and muscular hypertrophy in callipyge lamb, and that *DNTTP1* may respond to *DLK1* signaling and modulate myosin heavy chain gene expression.

Concerning to *DLK1* and CCAAT/enhancer binding protein (C/EBP) alpha (*CEBPA*) interaction, Armengol et al. (2012) found that the transcription factor *CEBPA* activates *DLK1* in brown adipocytes in mice by binding to its promoter and that knockdown of *CEBPA* resulted in reduced expression of *DLK1*. Given that *DLK1* functions as a repressor of adipogenesis (Rosen and MacDougald, 2006), it can be concluded that *CEBPA* is a transcriptional regulator of *DLK1* and has a key role in brown adipocyte differentiation in mice. Wang et al. (2012) reported that the expression of *CEBPA* in subcutaneous adipose tissue was the highest among 14 other Qinchuan cattle tissues. The function of *CEBPA* in sheep tissues is not clear, however, the differential expression of this gene in fetuses whose mothers were fed different energy sources suggests an important role in fetal programming of sheep subcutaneous adipose tissue. In regards to the results of the current study and the results of other researchers, it seems that *DLK1* gene interacts with different genes via various mechanisms and as a pleiotropic gene can have different major and minor roles in various tissues. On the other hand, the results of the present study have shown that fennel increases the *DLK1* expression in different tissues.

## 5. Conclusions

Based on the results of the present study, it can be concluded that fennel could be used in lamb diets to improve the structure of muscle (expanding of muscle mass and muscle fiber size) through positive effects on expression *DLK1* gene. As fennel has increased the expression level of *DLK1* gene in some tissues, like femur muscle, it could be considered for increasing animal growth and muscle mass, which is important in the sheep industry. It can be concluded that fennel can be used for different purposes in livestock, but for each effect in every tissue, further research needs to be performed considering different genetic, epigenetic, and physiological conditions in order to reach final conclusion. Furthermore, the interesting results of the current study in response to using different levels of fennel in diets of lambs opens the new direction to more extensive research in this area.

## Ethics statement

The project was found to be in accordance to the ethical principles and the national norms and standard for conducting animal research in Iran and animals were maintained according to the guidelines set by the Iranian Council of Animal Care (Guide to the Care and Use of Experimental Animals, 1. IUT, Iran).

## Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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