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Expression of calpastatin gene in Kermani sheep using real-time PCR

Zohre Hajalizadeh[1], Omid Dayani[1], Amin Khezri[1], Reza Tahmasbi[1], Mohammadreza Mohammadabadi[1], Tetiana Solodka[2], Oleksandr Kalashnyk[3], Volodymyr Afanasenko[4] and Olena Babenko[5]*

[1]Department of Animal Science, College of Agriculture, Shahid Bahonar University of Kerman, Kerman, Iran.
[2]National University of Water Management and Nature Resources Use, Ukraine.
[3]Sumy National Agrarian University, Sumy, Ukraine.
[4]National University of Life and Environmental Sciences of Ukraine, Ukraine.
[5]Bila Tserkva National Agrarian University, Ukraine.

*Corresponding author,
Tel: +989133987534
E-mail address:
mrm@uk.ac.ir

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ORCID
Zohre Hajalizadeh
0000-0002-8052-8133
Omid Dayani
0000-0002-7067-8242
Amin Khezri
0000-0002-5371-9831
Reza Tahmasbi
0000-0002-5211-657X
Mohammadreza Moahammadabadi
0000-0002-1268-3043
Tetiana Solodka
0000-0002-0666-6626
Oleksandr Kalashnyk
0000-0003-2354-3473
Volodymyr Afanasenko
0000-0002-2782-5403
Olena Babenko
0000-0002-8404-3272

Abstract The aim of this study was to investigate the calpastatin gene expression in different tissues of Kermani sheep using the real-time PCR. Tissue samples from the brain, humeral muscle, femoral muscle, liver, adipose tissue, rumen and testis were taken from 30 Kermani sheep. Total RNA was extracted using RNXTM plus solution. To determine the quantity (concentration) and quality of the extracted RNA, two methods of RNA; electrophoresis on 1% agarose gel and a Nano drop device were used. A Thermoscientific kit (Iran) was used for cDNA synthesis. After performing normal PCR reactions and obtaining the desired binding conditions and temperature for the genes, real-time PCR was performed to study the relative gene expression. The *Beta-actin* gene was used as a housekeeping gene. The Pfaffl method was used to analyze the data. The quality of the extracted RNAs was good. The presence of two 18S and 28S bands in the rRNA indicated that the RNA was healthy and the absence of an additional band was an indication of its purity. For the calpastatin gene, the 189bp fragment, and for *Beta-actin*, the 206bp fragment was observed in all tissues. The real-time PCR findings showed that calpastatin gene was expressed in all tissues (brain, humeral muscle, liver, adipose, femoral tissue, rumen and testis) with the highest level of expression in the humeral and femoral muscles and the lowest level in adipose tissues. This study lays a foundation for further calpastatin research in sheep. It is suggested that this study be conducted on a greater number of animals, and different breeds, sexes, ages and physiological stages to reach a more comprehensive conclusion.

Keywords: calpastatin, gene expression, Kermani sheep, tissue

Introduction

Goat and lamb meat is one of the most important sources of protein for humans (Savell et al., 1991). Consumers consider meat tenderness as one of the most important characteristics of meat quality (Morgan et al., 1991). If the causes of changes in meat tenderness are identified, the desired goals can be achieved by modifying the mechanism (s) influencing the tenderness process (Wulf et al., 1996; Roudbari et al., 2020).

Although the texture of meat, especially its tenderness, is highly variable in different animals, it has similar structures and the same chemical properties in all vertebrates (Mohammadabadi et al., 2021). It has been shown that among all skeletal muscle proteolytic systems, only the calpain system is involved in meat tenderness (Koohmaaraie, 1996). In general, the calpain system consists of two calcium-dependent proteases called–m-calpain and

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µ-calpain, and a polypeptide called calpastatin, which inhibits the activity of these two proteases (Leal-Gutiérrez et al., 2018). In addition, three members of the calpain family, namely–m-calpain and µ-calpain and calpain 3, have been well studied for their involvement in myofibrillar protein breakdown (Leal-Gutiérrez et al., 2018). µ -Calpain has been shown to be responsible for post-mortem muscle proteolysis in mice in which µ-calpain had been inactivated (Leal-Gutiérrez et al., 2018). Calpastatin is a specific inhibitor of calcium-dependent proteases, -m calpain and µ-calpain (Juszczuk-Kubiak et al., 2008) and has been shown to be involved in cell survival and cell proliferation *in vivo* (Van Ba et al., 2015). In addition, overexpression of calpastatin significantly inhibits calpain activity in muscle, heart, and nerve cell tissues (Liang et al., 2010). The calpastatin gene is located on chromosome 7 in goats (Antonius et al., 2020), cows (Enriquez-Valencia et al., 2017), and yaks (Yang et al., 2012), and chromosomes 5 in sheep (Palmer et al., 1998) and humans, and contains 4 exons and 3 introns (Inazawa et al., 1990). Calpastatin can prevent unlimited cell growth by suppressing the calpain activity (Farr, 2011). Involvement of calpains in apoptosis is still a matter of debate, being limited to certain cell types and specific stimuli (Kidd et al., 2000). Some studies, using overexpression of calpastatin, reported that calpains are clearly involved in apoptosis in response to specific apoptotic signals (Ruizvela et al., 2001). Calpains have been reported to act as both negative and positive regulators of apoptosis (Nakagawa and Yuan, 2000). As reported by some studies, the possible involvement of calpains in apoptosis is through their ability to break down their own caspases (McGinnis et al., 1999). Conversely, Chua et al. (2000) reported that calpains could potentially break down and inactivate caspases 7, 8, and 9. There are conflicting reports on the involvement of the calpain-calpastatin system in apoptosis (Van Ba et al., 2015). Interfering RNA (RNAi), mediated by small interfering RNA (siRNA), has been widely used in functional studies of genes (Yang et al., 2012). Small ruminants play important socio-economic roles in the livelihood of a significant portion of the human population in warm regions (Pour Hamidi et al., 2017). Therefore, combination experiments with emphasis on genetic management and development are crucial for improving animal production (Ebrahimi et al., 2015; Mohammadabadi et al., 2017). The economic and biological efficiency of sheep breeding industries generally improves with increases in productivity and reproductive performance of ewes. Twenty-six sheep breeds and ecotypes are found in Iran, which include more than 50 million heads, each of which is adapted to a specific part of the country (Ghotbaldini et al., 2019). Kermani sheep is one of the most important Iranian sheep breeds. The breed is appropriately adapted to the harsh and unfavorable habitat in south-eastern areas of Iran, principally the areas with dry and hot weather as well as low vegetation and poor pastures (Masoudzadeh et al., 2020b). Kermani sheep is a fat-tailed dual-purpose (meat and wool) breed of medium size and white wool that satisfies most needs of the ranchers and nomads. Therefore, paying attention to the breeding of this animal from a genetic and phenotypic point of view will be very useful (Masoudzadeh et al., 2020a).

The expression of eukaryotic genes is temporarily and multidimensionally controlled (Mohammadabadi, 2021). Only a relatively small set of the entire genome is expressed in each tissue, and the expression of genes depends on the stage of development; therefore, gene expression in eukaryotes is specific to each tissue (Tohidi Nezhad et al., 2015). Also, the amount of gene products that are made in a specific tissue, as well as in any other tissue that makes that product, regulates the expression of that gene (Mohammadabadi, 2019a). Of interest in domestic animals is the study of genes and proteins related to economic traits and their study at the cellular or chromosomal level (Mohammadabadi and Asadollahpour Nanaei, 2021). One of these important genes is the key gene of calpastatin, which plays an important role in various tissues. On the other hand, various studies have shown that calpastatin is involved in growth and meat quality; therefore, the aim of the present study was to determine the expression of the calpastatin gene in different tissues of Kermani sheep using real-time PCR.

Materials and methods

This research was performed in the research farm of the Faculty of Agriculture, Shahid Bahonar University of Kerman, using tissues from 30 healthy male Kermani lambs with an average body weight of 27.5 ± 0.45 kg and an age of about 6 months. The animals were slaughtered on the same day. To investigate the expression of calpastatin gene, small pieces of tissues were dissected by a sterile surgical blade and placed in 1.5 mL RNase-free microtubes and quickly frozen in nitrogen. The frozen samples were stored at -80 °C. The primers were designed using the GeneRunner software (Table 1), and the *Beta*-actin gene was used as the internal control. The primers were synthesized by Microgen company (Korea) via Iranian Takapozist Company (Iran).

Total RNA was extracted using RNXTM plus solution (Sinaclone Company, Iran). To determine the quantity (concentration) and quality of the extracted RNA, two methods of RNA; electrophoresis on 1% agarose gel and a Nano drop device were used. Agarose gel electrophoresis was performed using 1% agarose and 0.5× TBE buffer for 20 minutes. In addition, Gel Red was used to stain the gel and in order to observe the samples, the gel was placed in a UV Gel document (model SR1X3) and photographed. A Thermoscientific kit (Iran) was used for cDNA synthesis.

After performing normal PCR reactions, and obtaining the desired binding conditions and temperature for the genes, the real-time PCR was performed using a Biorad

device. Real-time PCR reaction, using the Syber Green method, was used to evaluate the relative expression of genes. The Syber Green kit included Taq DNA polymerase, $MgCl_2$, dNTPs, PCR buffer, Syber Green and -ROX. The real-time PCR reactions were performed at a final volume of 25 µL. The reaction mixture consisted of the ingredients listed in Table 2. The real-time PCR reactions were performed to amplify each gene according to the reaction thermal profile (Table 3).

Table 1. Oligonucleotide sequences of the primer pairs used for gene expression

Locus	Primers sequence	Fragment length (bp)	Annealing temperature (°C)
Beta-actin	F: 5'GGA CAT CCG CAA AGA CCT GA 3' R: 5'ACA TCT GCT GGA AGG TGG ACA 3'	206	57
Calpastatin	F: 5' ACA GGC TCA GTG GTC GTG 3' R: 5' GTT TGA CTG GCA GAT GAAGG 3'	198	57

Table 2. The materials and amounts used in each real-time PCR reaction

Material	Amount
Syber green Master-Mix	12.5µL
Fed primer (10 pmol/µ)	1µL
Rev primer (10pmol/µ)	1µL
cDNA (20 ng/µ)	2µL
Water (ddw)	Up to 25µL

A standard diagram for calpastatin and beta-actin genes was drawn for defining the quantity of PCR output with distinct concentrations (one, 1/10, 1/100, 1/1000) of cDNA. For calpastatin and beta-actin genes the PCR reaction yields were 98 and 99%, respectively. Data resulting from the real-time PCR were analyzed using the Pfaffl formula (Pfaffl et al., 2002) of the REST software.

Table 3. Real-time PCR thermal program

Steps	Temperature (°C)	Time (minutes)
Primary denaturation	94	3
Degradation of each cycle	94	1
Annealing	57	1
Synthesis	72	1

Results

The quality of the extracted RNAs was good. The presence of two 18S and 28S bands in the rRNA indicated that the RNA was healthy, and the absence of an additional band indicated its purity (Figure 1).

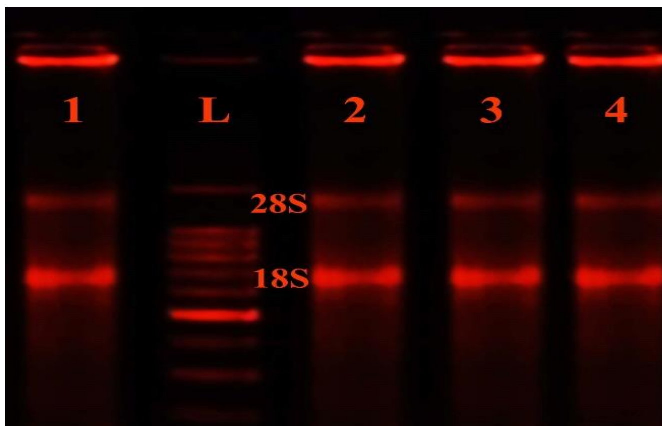

Figure 1. Quality of the extracted RNA from four tissues of Kermani sheep on agarose gel. Samples are 1, 2, 3 and 4 and Ladder is L

The sharp single peaks were observed in the amplification and melting curves of calpastatin (Figure 2 and 3) and beta-actin gene (Figure 4 and 5) PCR products. The PCR amplification curve of calpastatin gene samples in the liver tissue (Figure 2) from cycle 22 to 25, began to amplify and entered the exponential phase. In the next step, PCR products entered the linear phase, and finally samples of the liver tissue from cycle 32 entered the plateau phase. The same trend was observed for samples studied for the beta-actin gene (Figure 4).

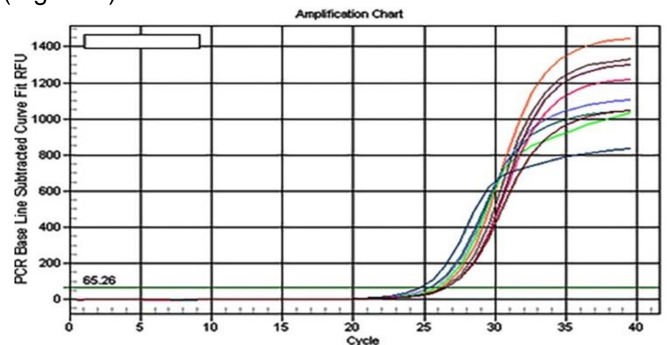

Figure 2. The amplification curve of calpastatin gene in the liver tissue

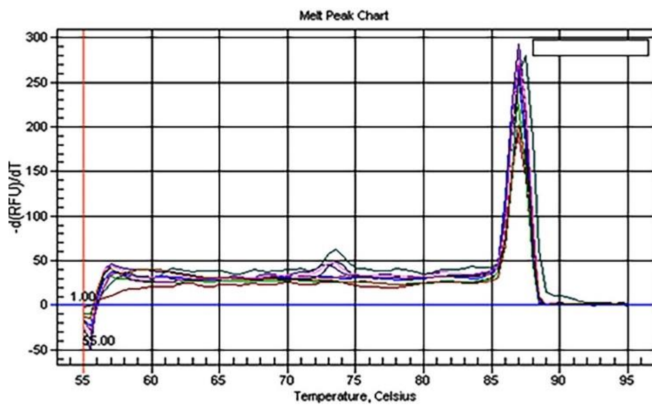

Figure 3. The melting curve of calpastatin gene in the liver tissue

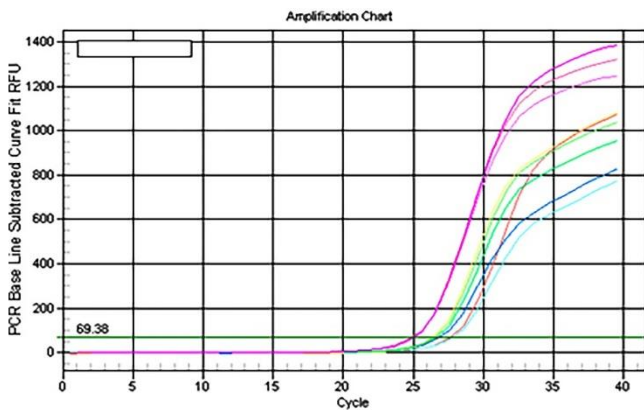

Figure 4. The amplification curve of *beta-actin* gene in the liver tissue

The samples related to calpastatin and beta-actin genes produced a peak at 87 ° C (Figures 3 and 5), which indicated the production of a specific product in this reaction. The fragment size of PCR products for the calpastatin and beta-actin gene was 198 bp and 206 bp, respectively (Figure 6). Calpastatin gene was expressed in all tissues studied (brain, humeral muscle, liver, adipose, femur, rumen and testis) with the highest level of expression in the humeral and femoral muscles, and the lowest level of expression in adipose tissue (Figure 7).

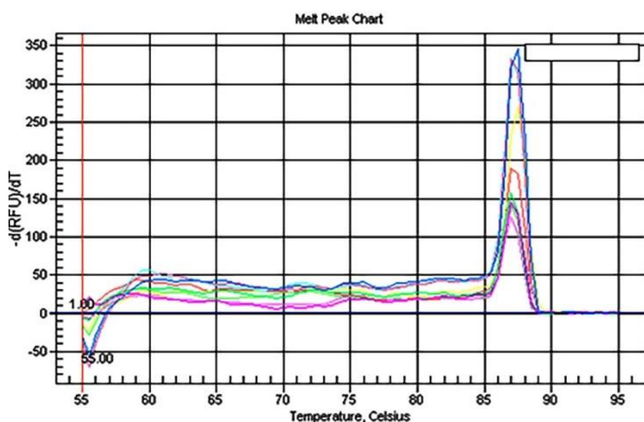

Figure 5. The melting curve of *beta-actin* gene in the liver tissue

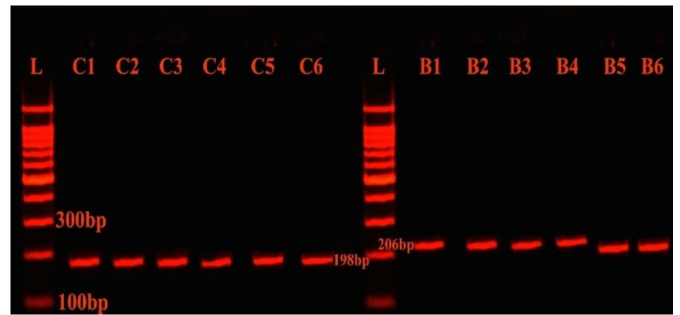

Figure 6. Electrophoresis of studied samples using calpastatin and beta-actin primers in Kermani sheep on agarose gel. C1 to C6 are calpastatin fragments (198 bp), B1 to B6 are beta-actin fragments (206 bp), and L is 100bp size marker

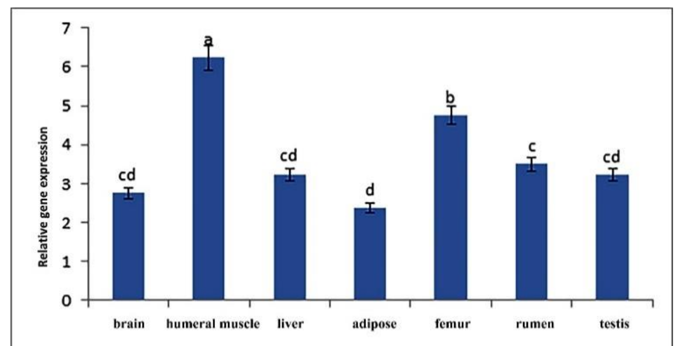

Figure 7. Relative expression of calpastatin gene in brain, humeral muscle, liver, adipose tissue, femoral muscle, rumen and testicular tissues of Kermani lambs. [a,b] Means with common alphabet do not differ (P <0.05)

Discussion

The expression pattern of calpastatin gene in seven tissues of Kermani sheep is consistent with the results of other researchers (Wang et al., 2015; Zheng et al., 2011; Mohammadabadi, 2019b). Mohammadabadi (2019b) studied calpastatin gene expression in Raini Cashmere goats using real-time PCR and showed that calpastatin gene was expressed in several tissues (muscle, adipose, kidney, spleen, liver, lung and heart) with the highest level of expression in the heart, spleen and liver and the lowest level in adipose tissue. Zheng et al. (2011) also examined the expression of calpastatin gene in different tissues of domestic yak and observed the highest level of expression in the heart, liver and spleen and the lowest level in adipose tissue. They reported that the expression of the calpastatin gene in the muscle of young *Longissimus* muscle of young yaks was lower than in adult ones. This may indicate that the *Longissimus* muscle growth in young yaks is less inhibited than in adult animals, which is in line with the faster growth rate of young animals. Because the calpastatin gene is a negative regulator of skeletal muscle growth (Barnoy et al., 1997), it was suggested that calpastatin is not a key factor in the calf size, at least in the *Longissimus* muscle. In the present study, it was

observed that the expression level of calpastatin gene was higher in the humeral and femoral muscles (Figure 7), which may be related to controlling the natural structure and function of the muscle by preventing uncontrolled calcium ion-dependent protease activity (Zheng et al., 2011). Calpastatin is an inhibitor of calcium ion-dependent protease in cells, can prevent the breakdown of muscle proteins and has a negative effect on meat tenderness (Koohmaraie, 1996; Zheng et al., 2011). Interestingly, the expression level of μ-calpain mRNA was in contrast to the expression level of calpastatin mRNA. Expression of μ-calpain mRNA was significantly increased with increasing the digestive energy levels, and significantly decreased with increasing crude protein levels. These results may be related to the fact that calpastatin is a calpain-specific endogenous inhibitor. Studies have shown that diets with low dietary levels tend to increase the amount of intramuscular fat and decrease the shear strength of Warner-Bertzler (WBSF) in pork, and increase the level of μ-calpain mRNA in skeletal muscles (Tang et al., 2010). The obtained results suggested that a diet with relatively reduced energy and protein can increase the tenderness of meat and intramuscular fat. Leal-Gutiérrez et al. (2018) showed that the main effect of μ-calpain and calpastatin on Warner-Bertzler shear force (WBSF) in cattle was through muscle proteolysis. To investigate the effects of cooking damage on tenderness, independent of muscle proteolysis (Bouhrara et al., 2011, 2012), the cooking damage was included as a dependent variable in correlation analysis. They found a very significant relationship between calpastatin 5 marker and water holding capacity in raw meat. However, calpastatin 5 marker did not significantly influence the cooking damage. The ARSUSMARC116 was associated with Warner-Bertzler shear force (WBSF) in their studied population and this association was significant when the SNP was analyzed individually or as a tag SNP for the calpastatin LD-block 3. When considering total calpastatin LD-block 3, SNIP-tagged diplotypes were able to segment populations for the higher-specific Warner-Bertzler shear force (WBSF).

In summary, calpastatin gene was expressed in the brain, humeral and femoral muscles, liver, adipose tissue, rumen and testis of Kermani lambs, with the highest level of expression in the humeral and femoral muscles, and the lowest level of expression in adipose tissues. This study also provided a basis for further research on calpastatin in sheep. It is suggested that this study be performed on a larger number of animals of various livestock, using both males and females of different ages and physiological states to reach a more comprehensive conclusion.

Conflict of interest statement

The authors declare that they do not have any conflict of interest.

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