Shahid Bahonar University of Kerman
Agricultural Biotechnology Journal
Iranian Biotechnology Society
Print ISSN: 2228-6705 Online ISSN: 2228-6500

Measurement of the relative expression pattern of the UCP2 gene in different tissues of the Raini Cashmere goat

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Abstract

Objective

The Raini cashmere goat is one of the most momentous Iranian goat breeds. These animals are bred for production of not only cashmere but also meat. Factors such as lipid peroxidation, oxidative stress, and changes in membrane properties are among the factors that cause the death of germ cells at different stages of development and ultimately have a negative effect on production and reproduction. To investigate this process, the UCP2 gene, a protein from the inner membrane of the mitochondrial organelle that pertains to uncoupling proteins family that performs a dominant function in reducing the membrane potential of the mitochondrial organelle and the loss of metabolic energy by preventing the accumulation of oxidative stress, was studied. Moreover, this gene is implicated in the physiology of several tissues, and the dominant isoform of its unconjugated proteins performs a dominant function in preventing inflammation and

inhibiting cell death. The goal of the present study was therefore to consider the relative expression level of the UCP2 gene in several tissues of the Raini cashmere goat applying the Real-Time PCR technique.

Materials and Methods

For this purpose, samples of humeral muscle, back muscle, femur muscle, spleen, heart, testis, liver and back fat tissues (three replicates of each tissue) were collected from four Raini cashmere goats of approximately the same weight at the time of slaughter. Total RNA of these tissues was extracted and their cDNA amplified. Real-Time PCR reaction using the non-specific SYBR Green dye method was performed to examine the relative gene expression. In this study, the GAPDH gene was used as an internal control. Prism software was applied to analyze the raw data, analyze the data got from Real-Time PCR and calculate the extent of changes in gene expression.

Results

The results of current investigation showed that the UCP2 gene was expressed in all tissues examined, with the highest expression level in the humeral muscle tissue (9.5-fold) and back muscle (4.5-fold), while the lowest expression level was observed in the back fat tissue (1.3-fold) and liver (1.5-fold) ($P<0.01$).

Conclusions

Considering that the UCP2 gene was expressed in all tissues examined and showed different expression levels in the different tissues, it could be inferred that this gene performs a function in different mechanisms and activities. Therefore, by conducting further experiments and understanding the relevant mechanisms, the cause of the change in expression can be found and action can be taken to improve livestock performance by changing the expression of this gene.

Keywords: Livestock, Real-Time PCR, tissue, UCP2 gene

Paper Type: Research Paper.

Citation: Mohammadabadi M, Babenko O, Borshch OO, Kalashnyk O, Ievstafiieva Y, Buchkovska V (2024) Measurement of the relative expression pattern of the UCP2 gene in different tissues of the Raini Cashmere goat. *Agricultural Biotechnology Journal* 16 (3), 317-332.

Agricultural Biotechnology Journal 16 (3), 317-332. DOI: 10.22103/jab.2024.24337.1627

Received: July 22, 2024. Received in revised form: September 25, 2024.

Accepted: September 26, 2024. Published online: September 30, 2024.

Publisher: Faculty of Agriculture and Technology Institute of Plant Production, Shahid Bahonar University of Kerman-Iranian Biotechnology Society.

Introduction

Researchers have reported that the goat was the first ruminant to be domesticated by humans (Mohammadabadi and Asadollahpour 2021). It has also been reported that domestication of goat began 9,000 to 11,000 years ago (Noori et al. 2017; Mohammadabadi et al. 2022). Some reports suggest that goat remains found in Europe or on the banks of the Nile are among the earliest traces, but further reports have shown that domestication of goats first began among Aryan tribes in Iran. From this, it can be conducted that domestication of goats first began in the Middle East in the areas of Kurdistan (Mohammadabadi and Tohidinejad 2017). It has been shown that about 30 million cashmere goats are bred worldwide and approximately 5 million in Iran (Noori et al., 2017). The Raini cashmere goat is one of the most momentous of these breeds (Mohammadabadi and Asadollahpour 2021). In the province of Kerman, the number of Raini cashmere goats is estimated around three million (Mohammadabadi et al. 2022). The breeding priorities of this breed in Iran are the production of cashmere, meat, and milk (Mohammadabadi and Tohidinejad 2017). Breeding this animal has many advantages, including high twinning rate, low-fat meat production, adequate milk production, low feed consumption, minimal capital investment, low space and equipment requirements, easy digestibility of milk, job creation and contribution to the economy of the breeder's family and villagers (Mohammadabadi et al. 2022). In addition, DNA replication, transcription, translation and gene regulation have been shown to be important molecular mechanisms (Jafari Ahmadabadi et al. 2023). Not all genes in a cell are expressed simultaneously, but a number of them are expressed at a given time and produce and deliver the product required by the cell (Barazandeh et al. 2016). The switching on and off, i.e.; the expression or non-expression of genes, is determined by the cell environment (Mohammadabadi et al. 2023). This means that the tissues implement their own specific expression pattern (Shokri et al. 2023).

Based on the results of the studies in evolutionary biology, it has been determined that there is a close link between metabolism, growth, and reproduction. In other words, in order for living organisms to achieve the optimal level of metabolism for reproduction, they must reach full maturity. Growth, metabolism, and reproduction are interlocked because of the without an intermediary connection of signaling pathways and co-regulatory networks (Safaei et al., 2024). The fertility in livestock is crucial, because the production of offspring is the top priority for livestock and very important. Therefore, any fertility defect leads to reduced profits and causes numerous challenges. Several factors such as nutrition, health management, production management, environmental conditions, and genetics influence the livestock fertility. The right combination of these factors and maintaining with productivity in agriculture and animal husbandry can help to increase the fertility of animal and consequently increase the production of

animal products. Therefore, reduced fertility or fertility disorders are important challenges in livestock farming, especially in sheep farming, which should be given special attention in research. Uncoupling proteins (UCPs) are part of the mitochondrial transport protein family and play an essential role in reducing mitochondrial membrane potential and metabolic energy loss such as heat, maintaining respiration, speeding up glucose utilization, insulin secretion, and preventing the accumulation of reactive oxygen species (ROS) (Pierelli et al., 2017). In mammals, the UCP family includes minimum six members: UCP1, UCP2, UCP3, UCP4, UCP5, BMCP1 or brain mitochondrial carrier protein-1, and UCP6. These proteins perform a fundamental role in regulating thermogenesis, regulating energy expenditure, and protecting versus oxidative stress. Although UCP proteins contain the same molecular structure, but different expression levels in various tissues, containing white adipose tissue, brown adipose tissue, thymocytes, skeletal muscle, testis, and liver (An et al., 2018). UCP2 is a member of the mitochondrial anion carrier family and shares 60% sequence similarity with UCP1. The gene encoding UCP2 is located on chromosome 1 in rats, chromosome 7 in mouse, chromosome 11 in humans, and chromosome 15 in sheep (Ferreira et al., 2020). The UCP1-UCP3 genes assign six coding exons and two extra untranslated exon regions. UCP2 is very conserved among species, with 95% amino acid sequence identity among human and mouse. The UCP2 promoter includes putative response elements for the peroxisome proliferator-activated receptor (PPARα, PPARγ, and PPARδ), sterol-responsive element-binding protein (SREBP), and cyclic AMP response element-binding proteins (CREB) (Bugge et al., 2010). Several regulatory factors influence UCP2 at both transcriptional and post-transcriptional levels. Purine nucleotides are negative regulator of UCP2, while volatile unsaturated fatty acids (FFA), glucose, retinoic acid, lipopolysaccharides, and ROS can activate UCP2. Notably, FFA has been shown to forcefully increase UCP2 expression when it acts on either the PPAR or SREBP element (Echtay et al., 2002). Expression level of UCP2 in adipose tissue is high. Significant levels are also observed in skeletal muscle, heart, lung, spleen, thymus, immune cells, testis and vascular cells, while lower levels are found in brain, kidney and liver. All UCP family members are integral membrane proteins placed in the inner mitochondrial membrane. They are characterized by three repeating domains, each consisting of two α-helical regions within the lipid bilayer (Berardi et al., 2011). The carboxy- and amino-terminal regions are located in the mitochondrial intermembrane space, while the α-helical regions are connected by long hydrophilic loops on the matrix side. Given the role that UCP2 performs in mitochondria, it is therefore clear that reduced UCP2 expression may be involved in the development of mitochondrial dysfunction, cell death and ROS reposition and thus in the pathogenesis of multiple diseases (Pierelli et al. 2017). Moreover, as a gene involved in testicular physiology, UCP2 and

the dominant isoform of its unconjugated proteins perform an important role in preventing inflammation and inhibiting cell death in this tissue. Therefore, the aim of the current research was to study the expression of the UCP2 gene in the humeral muscle, back muscle, femur muscle, spleen, heart, testis, liver and back adipose tissue of the Raini cashmere goat using the Real-Time PCR technique.

Materials and Methods

Twenty-four tissue samples including humeral muscle, back muscle, femur muscle, spleen, heart, testis, liver and back adipose tissues (three replicates of each tissue) were prepared from four Raini cashmere goats at slaughter at the abattoir. Immediately after slaughter, small pieces of the required organs were separated and placed in 1.5 mL microtubes. Several microtubes were then wrapped in aluminum foil and immediately transferred to a liquid nitrogen container. After rapid freezing, the samples were stored in a -80°C freezer. RNA extraction was conducted in an RNase-free environment. Prior to RNA extraction, all equipments were made RNase-free with DEPC water (Cynagen, MR8244). Total RNA extraction from the tissue was performed based on the instructions of the One Step RNA Reagent Extraction Kit (Biobasic, Canada). The quality and quantity of extracted RNA was evaluated by agarose gel electrophoresis and UV spectrophotometry. The extracted RNA was transferred to a freezer at -80°C until the next steps of the experiment. For cDNA synthesis, the Fermentase cDNA synthesis kit was used (RerertAid^{TM} H Minus First Strand cDNA Synthesis Kit #K1631). For this purpose, 1 µg of total RNA was used. Since different amounts of RNA may be generated during extraction from each tissue, 1 µg of RNA was used to equalize the RNA used for cDNA synthesis, . For RT-PCR reactions, the RNA sample must be free of DNA contamination for this purpose, the RNA was treated with DNaseI. The reverse transcription reaction product was stored at -20°C. The primers (Table 1) were manufactured by Bioneer (South Korea) with the support of Tekapozyst Company, Iran (Restelli et al. 2015).

To perform the reaction, 4.7 µL of double-distilled water, 7.5 µL of SYBRPermixTaq II, and 0.3 µL of ROX were combined with 0.5 µL of forward primer, 0.5 µL of reverse primer, and 1.5 µL of template cDNA in a 2.0 mL microtube. The microtubes were spun to collect all the materials in one spot and then placed in a Gene 3000 rotor with the following conditions. For UCP2 and GAPDH genes, initial denaturation at 95°C for 5 min, secondary denaturation at 95°C for 20 sec, annealing at 60°C for 1 min, 40 cycles of steps 2-4, and extension at 72°C for 30 sec were performed. The data obtained from Real-Time PCR were analyzed using the method described by Pfaffl et al. (2002).

Table 1. Oligonucleotide primer pairs used for UCP2 gene expression in various tissues of Raini Cashmere goat

Gene name	Primer sequence	Fragment Length (bp)	Accession number	Reference
UCP2 Gene	5'-GCATAGGCATCCAGGAATCA-3' 5'- AGGACGCTTCTGTCTCC-3′	112	AF127029	Restelli et al. 2015
GAPDH Gene	5'-GGCGTGAACCACGAGAAGTATAA-3' 5'-CCCTCCACGATGCCAAAGT-3'	119	NM_001034034	Restelli et al. 2015

In this method, a standard curve for the leptin and beta-actin genes was first created to examine the percentage of PCR reaction efficiency. Different cDNA concentrations (1, 0.1, 0.01, and 0.001) for PCR were used to construct the standard graph, and the PCR reaction efficiency for the UCP2 and GAPDH genes was estimated to be 98% and 99%, respectively. PCR reactions were then performed on the samples, and the results were calculated to examine the relative expression amplification rate using the formula of Pfaffl et al. (2002).

$$\text{ratio} = \frac{(\text{E}_{\text{target}})^{\Delta\text{CT}_{\text{target(control-sample)}}}}{(\text{E}_{\text{ref}})^{\Delta\text{CT}_{\text{ref(control-sampl)}}}}$$

In this formula, Etarget and Eref are the PCR efficiency of the gene under study and the reference gene or internal control, respectively. ΔCt is difference between the Ct (threshold cycle) value of the UCP2 gene and the Ct value of the GAPDH gene. Rotor-Gene Q Series Software was used for statistical analysis of the data obtained from Real-Time PCR. GraphPad Prism 8 (https://www.graphpad.com/updates/prism-802-release-notes) and Excel were also used for data analysis.

Results and discussion

The results of the absorbance ratios of the extracted samples at the A260/A280 wavelength were between 1.8-1.9, which demonstrated the suitable and advisable quality of the extracted RNAs, and the presence of two 18S and 28S bands in the RNA indicated the integrity of the RNA and the absence of an extra band indicated its cleanness (Figure 1).

Agricultural Biotechnology Journal; Print ISSN: 2228-6705, Electronic ISSN: 2228-6500

To realize the optimal binding temperature of the target gene (UCP2) and control (GAPDH) primers, a temperature gradient PCR was performed and the most suitable temperature for binding of the specific primers (60 °C) was selected.

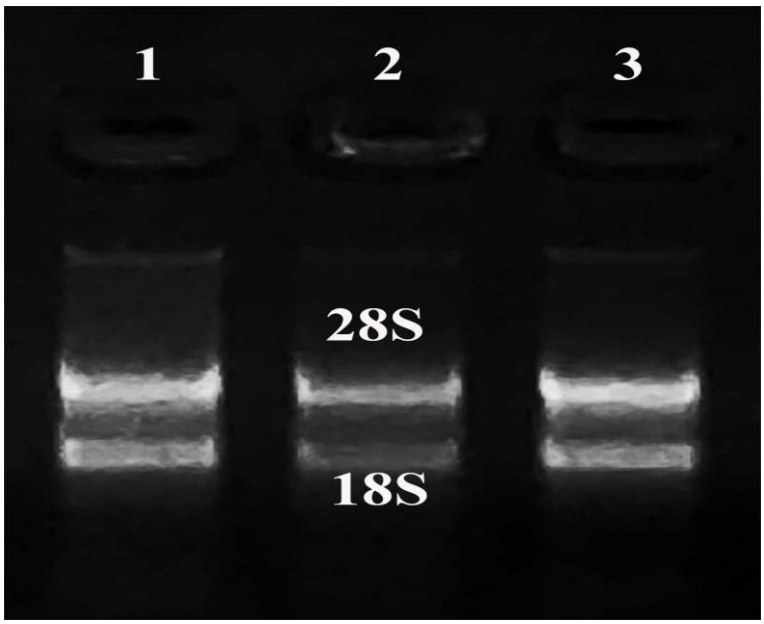
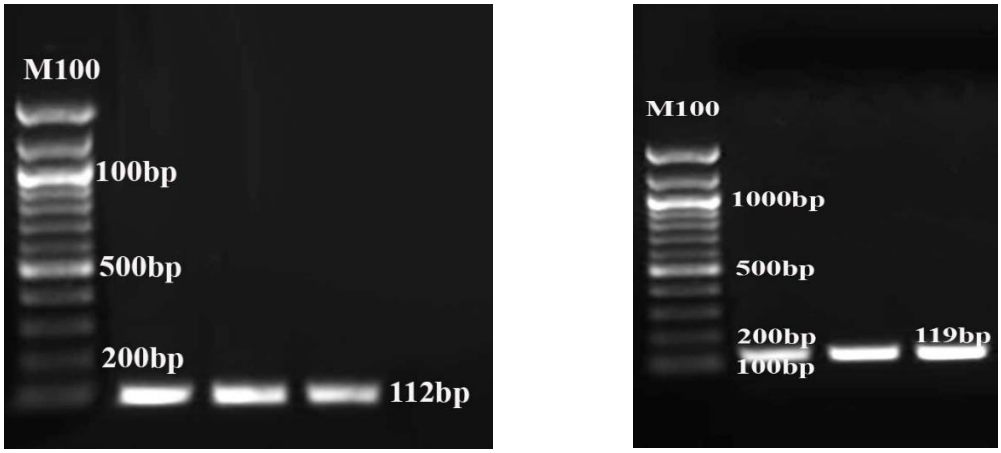


Figure 1. Some extracted RNA samples from some tissues of Raini Cashmere goat qualified on agarose gel

The results of the Real-Time PCR curves and the PCR products by agarose gel electrophoresis (2%) showed that the UCP2 gene was successfully amplified in different tissues. A single band at 112 bp for the UCP2 gene (Figure 2A) and a band at 119 bp for the GAPDH gene (Figure 2B) in all samples confirmed the accuracy of the experiment and the successful amplification of the target fragments.

In the time of the reaction, the Real-Time PCR device displays changes in fluorescent light intensity for each cycle as an amplification curve. For the amplification curve of all samples, a threshold value was defined in the exponential phase, which indicates the cycle in which the intensity of the fluorescent light emitted by the amplification reaction reaches the threshold value. The melting curve of the UCP2 and GAPDH genes shows the specificity and Tm (melting temperature) of the product (the temperature at which half of the product is released from the double-stranded state) of the Real-Time PCR reaction of these two genes.



A B

Figure 2. Results of electrophoresis on agarose gel for some studied samples applying UCP2 primers (A) and GAPDH primers (B) in Raini Cashmere goat. M is the size marker

In the present study, UCP2 gene expression in eight different organs of the Raini Cashmere goat was analyzed by Real-Time PCR. The results of the current research demonstrated that this gene was expressed in all tissues examined (humeral muscle, back muscle, femur muscle, spleen, heart, testis, liver and back fat), with the highest expression level ($P<0.01$) observed in the humeral muscle tissue (9.5-fold) and back muscle (4.5-fold) and the lowest expression level in the back fat tissue (1.3-fold) and liver (1.5-fold) (Figure 3).

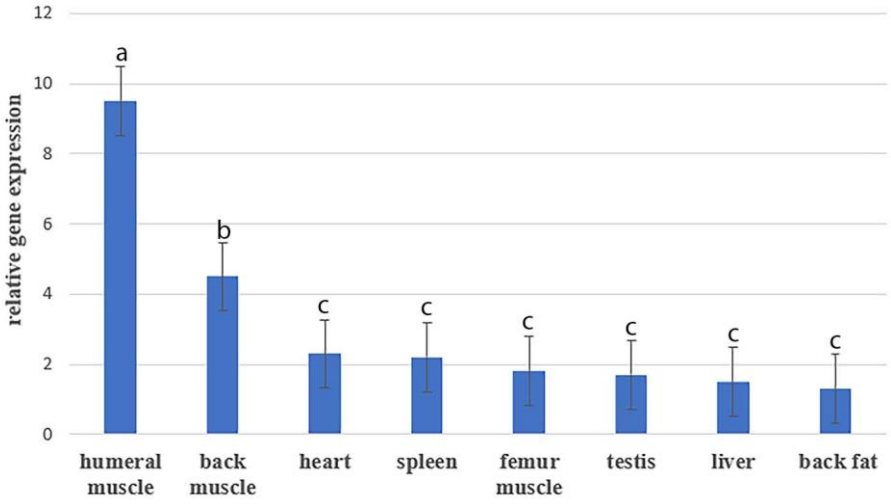


Figure 3. Relative gene expression level of UCP2 gene in humeral muscle, back muscle, femur muscle, spleen, heart, testis, liver and back fat tissues of Raini Cashmere goat

The results demonstrated that the UCP2 gene was expressed in different tissues. In most mammals, low testicular temperature is necessary for sperm production. Factors such as varicocele or cryptorchidism or other pathological conditions that cause testicular hyperthermia reduce male fertility because they cause apoptosis of spermatocytes and spermatids at certain periods (Xu et al., 2000). Researchers believe that the pathway by which hyperthermia induces apoptosis is the intrinsic mitochondrial pathway rather than the extrinsic death receptor pathway (Green & Kroemer, 2004). One class of these pro-apoptotic molecules are radical oxygen species (ROS), which are mainly made by the electron transport chain of mitochondria. Induction of apoptosis by excessive ROS acts by causing irreversible damage to proteins, membrane lipids, and DNA. Actually, hyperthermia has been related to increase ROS production through mitochondrial electron transport, which in turn induces cellular apoptosis (Adam-Vizi & Chinopoulos, 2006).

Researchers have shown that superoxide regulates UCP2, a gene that intercede proton flow and decreases ATP production (Krauss et al., 2002). Since superoxide production in a positive way associates with changes in mitochondrial membrane potential and is highly sensitive, the physiological function of UCP2 may be to decline mitochondrial ROS production and secure cells from ROS-induced apoptosis. Therefore, it can be concluded that when the expression level of UCP2 is too high, the apoptosis rate of cardiomyocytes, macrophages, and endothelial cells and oxidative damage are reduced. When UCP2 is genetically silenced or pharmacologically inhibited, more ROS are made in macrophages, hepatocytes, endothelial cells, and pancreatic beta cells. UCP2 reduces oxidative stress and prevents neuronal and cardiac cell death under ischemic conditions (McLeod et al., 2005).

In a study (Fleury et al., 1997), it was shown that UCP2 is expressed in the testis. UCP2 mRNA was also detected in mouse testis using Affymetrix DNA arrays (Li et al., 2006). In a study (Jiang et al. 2014), to investigate the expression of genes involved in lipid metabolism, the sheep genome and its transcripts from 40 different tissues were analyzed. The results indicated that the UCP2 gene is significantly expressed in sheep testis tissue, which was consistent with the results reported previously in Kermani sheep. In another study (Zhang et al., 2007), UCP2 was shown to secure germ cells from apoptosis, and its existence in elongating spermatids can describe why these cells are more stable to spontaneous apoptosis under natural conditions. They observed that UCP2 could be detected in almost all testicular cell types when the testes were briefly exposed to heat (43°C for 5 minutes), and its overall abundance increased about six-fold. Designated likewise a considerable grow in the levels of UCP2 protein and the cytoprotective role of UCP2 in other tissues, it is probable that UCP2 performs a significant function in inhibiting hyperthermia-induced testicular cell apoptosis. Apoptosis-inducing hyperthermia increases

testicular ROS levels. Their findings forcefully suggest that UCP2 can secure germ cells from ROS-induced apoptosis, however other elements cannot be ruled out. They also concluded that UCP2 could be show an efficient weapon for testicular germ cells to battle the apoptotic effect of hyperthermia induced by ROS task.

UCP2 expression in skeletal muscle can be influenced by a variety of factors. For example, in mice, exercise has been demonstrated to increase UCP2 expression in skeletal muscle, leading to improved glucose metabolism and insulin sensitivity (Cortright et al. 1999). Conversely, a high-fat diet has been shown to decrease UCP2 expression in skeletal muscle in mice, potentially contributing to the development of obesity and insulin resistance (Felipe et al. 2003). In mice, studies have shown that UCP2 expression in skeletal muscle is managed by thyroid hormones. Specifically, thyroid hormone treatment has been demonstrated to increase UCP2 expression in skeletal muscle, leading to increased energy expenditure and improved metabolic health (Masaki et al. 1997). Furthermore, UCP2 expression is reduced in response to high-fat diets and fasting in rodents (Joseph et al. 2002). Overall, studies in animal models suggest that UCP2 expression in skeletal muscle is managed by multiple factors, including exercise, diet, and hormonal regulation.

The heart is a vital organ of the goat that pumps blood through the body and supplies the tissue with oxygen and nutrients. Due to its anatomical and physiological similarities to the human heart, the goat heart can also serve as an animal model for the study of cardiovascular disease in humans. Former investigations have demonstrated that UCP2 protects against atherosclerosis in animal models of atherosclerosis by inhibiting ROS production in endothelial cells or by inhibiting monocyte reposition in the arterial wall (Lee et al. 2005). Therefore, strategies to grow UCP2 expression in vascular endothelial cells can help prevent the growth and improvement of atherosclerosis in patients with metabolic syndrome (Mattiasson and Sullivan 2006).

The expression of UCP2 is low in healthy, normal hepatocytes, but can be expanded in consequence of oxidative stress, steatosis, or the systemic response to bacterial infection (Ruzicka et al. 2005). The task of UCP2 in the liver is unclear, but as in other tissues, UCP2 comes into view to play a role in antioxidant defense (Collins et al. 2005) and possibly in metabolic control. In an experimental mouse model, obstructive jaundice was associated with increased hepatic UCP2 expression (five-fold), rapid weight loss, and intact insulin action on skeletal muscle glucose metabolism (Isaksson et al. 2002).

UCP2 may be of certain significance for the management of lipid metabolism in adipose tissue and skeletal muscle. Moreover, its capacity to prevent insulin secretion may also increase the application of fat for storage. UCP2 expression in lung and adipose tissues is also strongly influenced by diet. In contrast to UCP1, UCP2 is upregulated in white adipose tissue in response

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to fat feeding. In one study, it was seen that the expression of UCP2 and UCP3 in adipose tissue was lower in low-birth-weight animals than in high birth weight animals (Ricquier and Bouillaud 2000).

Conclusions: The highest increase in expression was observed in the humeral muscle and back muscle tissue, which were significantly different from each other and from other tissues. The decrease in gene expression in the other tissues was not significant. In general, it can be concluded that the expression of the UCP2 gene in various tissues is complex and tissue-specific. Although the role(s) of UCP2 in the normal physiology of tissues is not fully recognized, it is clear that this mitochondrial protein is existent and effective in various tissues. Considering that the UCP2 gene is expressed in all the tissues studied and has different expression levels in different tissues, it can be finalized that this gene performs a task in several mechanisms and activities. Therefore, by conducting additional experiments and understanding the relevant mechanisms, the cause of the change in expression can be found and action can be taken to improve the performance of livestock by changing the expression of this gene.

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