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Transcription analysis of genes involved in lipid metabolism reveals the role of chromium in reducing body fat in animal models



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ABSTRACT

Chromium was proposed to be an essential trace element over 50 years ago and has been accepted as an essential element for over 30 years. The recent studies indicated that the addition of supra nutritional amounts of chromium to the diet can only be considered as having pharmacological effects. However, the precise mechanism through which chromium acts on lipid, carbohydrate, protein and nucleic acid metabolism are relatively poor studied. To uncover, at least partially, the role of chromium in lipid metabolism, in this study, we evaluated the expression status of eight important genes, involved in fat biosynthesis and lipid metabolism, in four different tissue types (liver, subcutaneous fat, visceral fat, and longissimus muscle) in domestic goat kids feeding on three different chromium levels. The quantitative real-time PCR (RT-PCR) was established for expression analyses with HSP90 gene was used as reference gene. The results showed that supplementation of goats with 1.5 mg/day chromium significantly decreases the expression of the ACC1, DGAT1, FABP4, FAS, HSL, LEP genes, but does not affect the expression of the LPL and SCD1 genes in all studied tissues. This study highlights, for the first time, the role of supra nutritional levels of chromium in lipid biosynthesis and metabolism. These findings are of especial importance for improving meat quality in domestic animals.

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1. Introduction

Understanding precise relationships between metabolic health and diet is one of the main goals of biochemistry researchers. A large number of biologically active substances, including heavy metals, may have direct, primary or secondary effects on human and animal health, thus are of particular interest to pathologists, physiologists, and toxicologists. Existing as metallic (Cr^0), trivalent (Cr^{+3}), and hexavalent (Cr^{+6}) states, chromium (Cr) is one of the most abundant elements in the earth crust and seawater [1]. Although, the hexavalent Cr has been found to be highly toxic, trivalent Cr, found in most food and nutrient supplements with very low toxicity [2]. Cr is a trace mineral involved in carbohydrate, lipid, protein and nucleic acid metabolism [1]. Over five decades ago, Cr was suggested

to be an essential element; however, the element currently can only be considered pharmacologically active [3]. Pharmacological quantities of Cr may increase insulin sensitivity in both healthy subjects and subjects with type 2 diabetes [4]. Dietary recommendation for Cr is not listed for most livestock species including goats. In addition, studies have indicated that organic sources of Cr is absorbed more efficiently, about 25–30% more than inorganic compounds which are poorly absorbed (1–3%) regardless of dose or dietary Cr status [5,6]. A variety of organic forms of Cr is now available worldwide, and different forms would be expected to have different bioavailabilities. The bioavailability of Cr sources has been determined based on their ability to alter glucose metabolism [7,8]. Recently, Emami et al. [9] found that Cr supplemental as Cr-Met (chromium methionine) decreased meat lipid peroxidation, increased plasma and tissue Cr contents, and increased glucose clearance rate response to an intravenous glucose challenge in kids.

Despite this archaism, little attention has been paid to elucidating the precise mechanisms by which Cr acts in human and animal models. The effects of Cr in fat deposition have been investigated for potential use as feed additives in animal production. Recent

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studies have suggested that trivalent Cr can alter the expression levels of some genes in laboratory animal models [10–12]. For example, we have previously shown that supplementation of domestic goat kids (*Capra hircus*) with 1.5 mg/day Cr decreases the accumulation of fats in adipose tissues such as liver, subcutaneous fat, and visceral fat through down regulation of the gene encoding Acetyl CoA Carboxylase 1 enzyme (ACC1), the main enzyme involved in biosynthesis of fatty acids in mammals [12]. Similarly, the expression of some genes involved in immunity and fatty acid metabolism has been shown to be affected by Cr [10,11,13]. To improve our understanding of the role of Cr in fatty acid metabolism, in this study, we evaluated the expression of some genes associated with biosynthesis and metabolism of fatty acids, in four tissues (liver, visceral fat, subcutaneous fat, and *longissimus lumborum* (LL) muscle) of *Mahabadi* goat kids, as animal models.

2. Materials and methods

2.1. Ethics statement

All experiments with animals were performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the Research Station of Department of Animal Science, University of Tehran, Iran. The protocols were approved by the Animal Care and Use Committee of the University of Tehran Institutional Animal Care and Use Committee and included in a Research project.

2.2. Animal models and experimental design

This study was conducted at the Research Station of Department of Animal Science, College of Agriculture and Natural Resources, University of Tehran, Iran. Twenty-four (BW = 22 ± 2 kg, 4–5 mo. of age) male goat kids, belonging to the native Iranian breed, *Mahabadi*, were selected for the experiment. All procedures of immunity and nutrition were conducted under protocols approved by this station.

The kids were weighed and allocated randomly to one of the four following dietary treatments: standard diet plus 0, 0.5, 1 and 1.5 mg Cr per day as Cr-Met (Availa®Cr 1000, Zinpro Corporation, USA). The level of Cr in this study has been selected due to previous research on goat [14–16]. The experimental diets were formulated to be isocaloric and isonitrogenous, and to meet NRC (2007) requirements (see [17]) and basal diet has contained 0.83 ppm Cr. Amount of Met in the basal diet is 0.22% and each kg of Cr-Met supplementation used in this experiment includes 1000 mg (1 mg/g) Cr and 3000 mg (3 mg/g) of Met. Dry matter intake (DMI) in all treatments was equal (about a kg; Cr-Met supplement did not cause a change in DMI; unreported data). It can be stated that all the animals intake 2.2 g (2200 mg/day) Met by basal diet plus the amount of Met available in Cr-Met supplementation.

Therefore, the Met amount of this supplementation that provided for 1–4 treatments is 0 (0 mg × 3 mg), 1.5 (0.5 mg × 3 mg), 3 (1 mg × 3 mg) and 4.5 (1.5 mg × 3 mg) mg/day, respectively. In the other word, sum of Met amount that each animals intake is 2200, 2201.5, 2203, and 2204.5 mg/day, respectively, in treatment 1–4. As can be seen, the amount of Met in the treatment 4 is higher than that of control treatment but with a negligible difference (about 0.0002 times) that can be ignored. In the ruminant nutrition researches that has used Cr-Met supplementation, the amount of Met added to the diet as this supplementation has been overlooked [18,19].

Kids were housed in individual pens (1.3 m × 1.0 m) for 100 days (10 days for adaptation and 90 days for feeding period), with constant illumination and fed diet twice daily at 0800 and 1700 h as TMR. Clean drinking water was available in plastic buckets and pens were cleaned weekly. The kids were weighed before the morning feeding meal at 21 days intervals (i.e. after 14–16 h of starvation) throughout the experimental period to determine changes in their body weight.

2.3. Slaughtering and tissue sampling

After feeding on the prepared diets for 90 days, the kids were transferred to the departmental abattoir, where they were kept for 12 h with free access to water. They were then slaughtered by decapitation and tissue samples from liver, visceral fat, subcutaneous fat, and *longissimus lumborum* muscle were taken from the corpses. The samples were immediately frozen in liquid nitrogen and transferred to the laboratory, where they were maintained at –80 °C until used.

2.4. Total RNA isolation, clean up and cDNA synthesis

Total RNA was extracted according to the method of Chomczynski and Sacchi using Trizol Reagent (Invitrogen Co., Carlsbad, CA, USA) [20]. The extracted RNA was then treated with RNase-free DNase I (TaKaRa, Shuzo, Kyoto, Japan). RNA concentrations were estimated by Nanodrop spectrophotometry at 260 nm and their purities were checked by determining the absorption ratios at 260/280 nm. The quality of extracted RNA was assessed by electrophoresis at 1% agarose-gel containing Ethidium Bromide. First-strand cDNA was synthesized from 100 ng of total RNA using an oligo (dT) primer, random hexamers and a commercially available kit (AccuPower® RocketScript™ RT PreMix) according to manufacturer's instructions.

2.5. Primer pairs and real-time PCR (RT-PCR)

Primer pairs for the eight genes including Acetyl CoA Carboxylase 1 (ACC1), Diglyceride Acyltransferase 1 (DGAT1), Fatty Acid-Binding Protein 4 (FABP4), fatty acid synthase (FAS), the Ob (LEP) gene (Ob for obese, LEP for leptin), lipoprotein lipase (LPL), hormone-sensitive lipase (HSL) and stearoyl-CoA Desaturase 1

Table 1
General properties of specific primer pairs for the nine genes used for expression analyses.

Gene name	Accession number	Sense primer sequence 5' → 3'	Anti-sense primer sequence 5' → 3'	Length (bp)	T _m (°C)
HSP-90	AF548366.1	GCCCGAGATAGAAGACGTTG	AGTCGTTGGTCAGGCTCTTG	197	59.8
LEP	EF583947.1	GAGCTGCCCTTACCACAG	GTAGAGACCCCTGTAGCCG	119	59.8
FABP	EF105407.1	CAGGAAAGTGGCTGGCATGG	GCCCAATTGAAGGACATCTCA	115	59.8
FAS	DQ223929.1	GGCAGTTTCATGGAAGCGGAG	GCTTGTGGTAGAAGGAGCG	162	59.8
LPL	GU082383.1	GCTCCAAGTCGCCTTCTCC	CCGTTAGGGTAAATGTCAACATG	128	59.8
SCD1	GU947654.1	GCTTCATCTCGCCACACTC	ATTGAGCAACAACAGCGTACCG	101	59.8
HSL	GQ927175.1	TACGTACGCTGCACAAGG	CTCAGGTCGATGGCAAAGC	195	59.8
ACAC	DQ370054.1	CGTATGGAAGTCGGCTGTG	CAGGAAGAGCGGATGGGAA	105	59.8
DGAT1	NM.001110164.1	AGTACCCCGACAACCTGAC	GGGTGAGGAACAGCATCTCC	145	59.8

Table 2

The relative expression levels of target genes in treatments.

Genes	Gene expression in treatments ^a				DF	P-value
	Control	Cr ^{0.5}	Cr ¹	Cr ^{1.5}		
ACC1	3.95 ± 0.2247 ^a	2.90 ± 0.2407 ^a	2.77 ± 0.2508 ^a	1.18 ± 0.2264 ^b	1	<0.01
DGAT1	0.332 ± 0.2178 ^a	0.213 ± 0.2137 ^{a,b}	0.132 ± 0.2286 ^b	0.122 ± 0.2187 ^b	1	0.001
FABP4	2.49 ± 0.286 ^a	1.27 ± 0.282 ^a	0.122 ± 0.228 ^a	0.418 ± 0.283 ^b	1	0.01
FAS	0.248 ± 0.203 ^a	0.136 ± 0.186 ^b	0.152 ± 0.214 ^{a,b}	0.133 ± 0.201 ^b	1	0.01
HSL	1.29 ± 0.199 ^a	1.06 ± 0.194 ^{a,b}	0.907 ± 0.270 ^{a,b}	0.610 ± 0.212 ^b	1	0.01
LEP	20.436 ± 0.217 ^a	16.681 ± 0.210 ^{a,b}	11.442 ± 0.227 ^{a,b}	10.562 ± 0.228 ^b	1	0.01
LPL	3.07 ± 0.206 ^a	3.41 ± 0.199 ^a	3.715 ± 0.223 ^a	4.639 ± 0.228 ^a	1	<0.01
SCD1	0.440 ± 0.292 ^a	0.387 ± 0.258 ^a	0.497 ± 0.277 ^a	0.655 ± 0.275 ^a	1	<0.01

^a Values were means (n = 24) of delta C_T with their standard errors. Means in rows without a common superscript letter differ (P < 0.01).

(SCD1) were designed using primer3Plus [54] and Primer3 [55] online software programs (see Table 1). The heat shock protein 90 (HSP-90) gene was selected as reference gene for normalization of expression data as its expression stability had been approved in a previous study [17]. The specificity of designed primers was evaluated using PrimerBLAST software of NCBI database [21].

RT-PCR was performed using SYBR Green I technology on iQ5 System (BioRad, USA). The reactions consisted of 1x SYBR Green PCR Master Mix (SYBR biopars, GUASNR, Iran), 300 nm of each specific forward and reverse primers, 10 ng of cDNA, and nuclease free water to a final volume of 20 µl.

The cycling conditions were as follows: cDNA was denatured at 94 °C for 3 min, followed by 35 cycles of 94 °C for 15 s and 59.8 °C for 15 s (gain set at 10 for SYBR Green). All samples were amplified in triplicate from the same RNA preparation and the mean value was considered. Two biological replications were used for each plate. The RT-PCR efficiency was assessed for each gene based on the slope of a linear regression model [22]. The bulks of each cDNA sample were used as PCR template in a range of 10-fold dilution series. The corresponding RT-PCR efficiencies were calculated based on the slope of the standard curve using the following equation: $(E = 10 - 1/\text{slope} - 1)$ [23].

2.6. Data analyses

Analysis of RT-PCR data was performed according to our previous studies [12,13,24]. Each reaction was run in triplicate and the average threshold cycle (C_T) values were used to calculate the relative expression values. In our study, the HSP-90 gene was served as reference gene to calculate the initial C_T values. Relative expression level for each gene was calculated by the differences in C_T between the reference and target genes of the same samples (delta C_T) and following the formula: $2^{-(\text{delta } C_T)}$ [53]. Statistical differences (ANOVA) in gene expression among different diets were evaluated using the GENMOD procedure (SAS 9.2). Tissue and treatment were

included in the model as the fixed effect and interaction between them was assessed. Significance was determined at P < 0.05.

3. Results

The relative expression levels of the ACC1, DGAT1, FABP4, FAS, HSL, LEP, LPL and SCD1 genes were compared by RT-PCR in four tissues and different treatments to illustrate the role of Cr in fatty acid metabolism. The specificity of all amplifications was tested through analysis of a melting curve, which was generated at the end of each reaction. All studied genes provided a single peak in the melting curve implying on the absence of primer-dimer formation during the reaction, thus confirming the specificity of the amplifications. The efficiency and linearity of RT-PCR reactions were evaluated using the 10-fold serial dilutions. The relationship between threshold cycle (C_T) and the log copy numbers of cDNA for the two studied genes were linear. Additionally, the efficiencies of our amplifications ranged between 1.79 and 2.05 which are near to the theoretical optimum level of 2 [56].

The two-way analysis of variance revealed no interaction effect (P > 0.05) between tissues and treatments in all studied genes. Among these genes, the LEP gene was expressed at the highest rates in all studied tissues. We found no significant difference in the expression of ACC1 gene in kids supplemented by 0, 0.5 and 1 mg Cr. However, the expression level of ACC1 gene was lower in 1.5 mg Cr groups of kids compared with other groups (Table 2). There were significant differences in the expression of ACC1 gene among different studied tissues, with subcutaneous fat showed the highest expression level (Table 3).

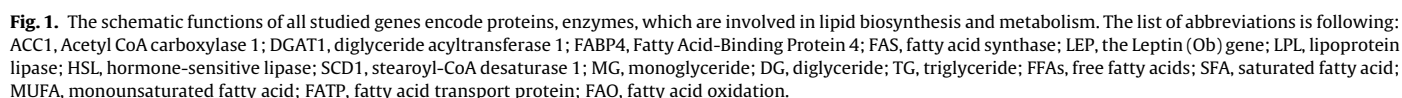
The expression of DGAT1 gene was also decreased as a result of Cr supplementation with the most reductive effects were observed in kids fed by 1 and 1.5 mg Cr (Table 2). Similar to the ACC1 gene, the expression of DGAT1 gene was statistically different among the four tissues with LL muscle showed the highest expression, while three lipogenic tissues showed a relatively low expression (Table 3).

Table 3

The relative expression levels of target genes in tissues.

Genes	Gene expression in tissues ^a				DF	P-value
	Liver	Subcutaneous fat	LL muscle	Visceral fat		
ACC1	2.528 ± 0.2238 ^a	6.240 ± 0.2174 ^b	0.947 ± 0.2985 ^c	2.540 ± 0.2142 ^a	1	<0.001
DGAT1	0.095 ± 0.2247 ^a	0.199 ± 0.2407 ^b	0.434 ± 0.2508 ^c	0.138 ± 0.2264 ^{a,b}	1	<0.01
FABP4	0.257 ± 0.261 ^a	1.41 ± 0.263 ^b	1.265 ± 0.381 ^{b,c}	3.55 ± 0.259 ^d	1	<0.01
FAS	0.054 ± 0.206 ^a	0.159 ± 0.187 ^b	0.466 ± 0.232 ^c	0.170 ± 0.184 ^{d,b}	1	<0.0001
HSL	0.233 ± 0.206 ^a	4.851 ± 0.187 ^b	0.329 ± 0.232 ^a	2.33 ± 0.184 ^c	1	<0.001
LEP	8.314 ± 0.221 ^a	24.414 ± 0.196 ^b	0.654 ± 0.268 ^c	2.33 ± 0.184 ^d	1	<0.01
LPL	13.386 ± 0.195 ^a	7.924 ± 0.215 ^a	0.182 ± 0.248 ^b	9.357 ± 0.2020 ^a	1	<0.001
SCD1	0.270 ± 0.264 ^{a,b,c}	0.743 ± 0.250 ^b	0.476 ± 0.348 ^{a,b,c}	0.581 ± 0.257 ^c	1	<0.05

^a Values were means (n = 24) of delta C_T with their standard errors. Means in rows without a common superscript letter differ (P < 0.05 or P < 0.001).



Although, a slightly descending pattern in the expression of HSL and LEP genes was observed by increase in Cr level, the expression of these genes was statistically different only in goats treated by 1.5 mg Cr (Table 2). The highest expression level of HSL gene was detected in subcutaneous fat while liver showed the lowest expression of HSL gene (Table 3). The expression of LEP gene, however, maximized in liver followed by subcutaneous fat, LL muscle, and visceral fat, respectively (Table 3).

In this study the effects of feeding different levels of dietary Cr supplementation (0, 0.5, 1, and 1.5 mg/day) on expression status of eight genes were investigated in four different tissue types of domestic goat kids (including liver, visceral fat, subcutaneous fat, and LL muscle) by using RT-PCR technique. All studied genes encode proteins, enzymes, which are involved in lipid biosynthesis and

metabolism (Fig. 1). A comprehensive knowledge on the expression status of these genes in the different tissues with different levels of Cr is useful in understanding the background genetic mechanisms involved in fat metabolism. Recently, researchers reported that reducing dietary Met from 0.86% to 0.17% alters the fat metabolism in the liver and adipose tissues in the rat [25]. It should be noted that in this study, compared to levels of Met in the basal diet (2200 mg/kg dry matter), amount of provided Met by adding Cr-Met supplement to the diet are so small and negligible. Hence, changes in the expression of genes involved in lipid metabolism are likely due to the presence of Cr not Met.

The expression of Diglyceride acyltransferase 1 (or O-acyltransferase) (DGAT1) gene was decreased by Cr and the lowest expression level was detected in kids feeding on 1.5 mg Cr per day. DGAT1 catalyzes the formation of triglycerides from diacylglycerol and Acyl-CoA. The reaction catalyzed by DGAT1 is considered the terminal and only committed step in triglyceride synthesis and to be essential for the formation of adipose tissue [2]. The DGAT1 gene is especially expressed in liver, fat tissues, and adrenal glands, which are actively involved in triglyceride biosynthesis [26].

A significant decrease in the expression of FABP4 was observed in goats fed by 1.5 mg/day Cr. The FABPs are a family of carrier proteins for fatty acids and other lipophilic substances such as eicosanoids and retinoids [2,27]. These proteins are thought to facilitate the transfer of fatty acids between extra- and intracellular membranes [28].

The expression of the fatty acid synthase (FAS) gene was significantly decreased in goats fed by all Cr levels, and the most reductive effects were observed in goats supplemented by 1.5 mg/day. In humans, the FAS gene encodes fatty acid synthase (FAS), a multi-enzyme protein that catalyzes fatty acid synthesis. FAS is a whole enzymatic system composed of two identical 272 kDa multifunctional polypeptides, in which substrates are handed from one functional domain to the next [29–32]. Its main function is to catalyze the synthesis of palmitate from acetyl-CoA and malonyl-CoA, in presence of NADPH, into long-chain saturated fatty acids.

The expressions of the hormone-sensitive lipase (HSL) and leptin (LEP) genes showed a slight descending pattern in response to increased Cr dose. However, only 1.5 mg/day Cr resulted in significant decrease in expression of these genes. HSL is an intracellular neutral lipase that is capable of hydrolyzing a variety of esters [33]. The enzyme has a long and a short form. The long form is expressed in steroidogenic tissues such as testis, where it converts cholesterol esters to free cholesterol for steroid hormone production [34]. The short form, known also as triglyceride lipase, is expressed in adipose tissue, where it hydrolyzes the first fatty acid from a triacylglycerol molecule, freeing a fatty acid and diglyceride [35,36]. The expression of short form of the HSL gene was measured in this study. HSL is activated when the body needs to mobilize energy stores, and so responds positively to catecholamines, ACTH, while inhibited by insulin. Glucagon was previously thought to activate HSL, however the removal of insulin's inhibitory effects has been recently suggested to be the source of activation.

Leptin (LEP) is one of the most important adipose-derived hormones which is encoded by Ob gene in adipocytes and play key roles in regulation of energy intake and expenditure, appetite and hunger, regulation of body temperature, and metabolism [37,38]. LEP functions by binding to the LEP receptor in the hypothalamus, where it increases energy consumption and reduces the appetite and fat accumulation via counteracting the effects of neuropeptide Y and anandamide, two potent feeding stimulants, and promoting the synthesis of α -MSH, an appetite suppressant [39].

In contrast to all abovementioned genes, despite showing a slightly ascending pattern by increase in Cr levels, the expression of lipoprotein lipase (LPL) and stearoyl-CoA desaturase-1 (SCD1)

genes was not statistically different among different treatments. LPL is a water-soluble enzyme that hydrolyzes triglycerides into two free fatty acids and one monoacylglycerol molecule in lipoproteins. The LPL gene is also involved in promoting the cellular uptake of chylomicron remnants, cholesterol-rich lipoproteins, and free fatty acids [40–42]. LPL binds to the luminal surface of endothelial cells in capillaries. It is most widely distributed in adipose, heart, and skeletal muscle tissue, as well as in lactating mammary glands [43–45].

Encoded by the SCD1 gene in humans, SCD1 is a key enzyme in fatty acid metabolism [46]. It is responsible for forming a double bond in Stearoyl-CoA. Indeed, SCD1 is an iron-containing enzyme that catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids. The principal product of the SCD1 is oleic acid, which is formed by desaturation of stearic acid. The conversion ratio of stearic acid to oleic acid has been implicated in the regulation of cell growth and differentiation through effects on cell membrane fluidity and signal transduction. In addition, the SCD1 gene affects a variety of key physiological activities, including insulin sensitivity, metabolic rate, and adiposity [57]. Divergence in the number of the SCD genes occurs among mammals with the SCD1 isoform is common among humans, mouse, cow, goat, pig, and sheep [46–52].

Altogether, results of the current study, together with our previous works [12] shows that the expression of ACC1, LEP, FABP4, HSL, DGAT1, and FAS in goats significantly decreased as a result of Cr supplementation, while the expression of LPL and SCD1 genes did not show any significant alteration despite their slight ascending pattern in response to increased Cr dose. Additionally, the expression of LPL, LEP, FABP4, HSL, and ACC1 genes was higher in lipogenic tissues compared to muscles, while the expression of SCD1, FAS, and DGAT1 genes was not different among these two tissue types. The precise functions of all studied genes in lipid biosynthesis and metabolism have been schematically illustrated in Fig. 1. The decrease in expression of the FAS and ACC1 genes can lead to decreased biosynthesis of fatty acids in adipocytes. On the other hand, decrease in expression of the DGAT1 gene may reduce accumulation of triglycerides in lipid droplets, while suppression of the HSL gene may lead to decreased production of monoglycerides and fatty acids. The expression of SCD1 gene, which contributes to biosynthesis of unsaturated fatty acids, was slightly elevated as a result of Cr supplementation, a favorable process in term of meat quality. The decrease in expression of the FABP4 gene, which facilitates the transport of fatty acids in adipocyte membrane, may result in decreased exchange of fatty acids and decreased lipid metabolism. In contrast to the abovementioned genes which are active in cytoplasm, the LEP gene is expressed in adipocyte intracellular space. Leptin counteracts the effects of neuropeptide Y in the hypothalamus, thus increases energy consumption and reduces the appetite and fat accumulation [39]. The decreased expression of LEP gene in the current study may not to be a direct effect of Cr supplementation; instead it may be related to decrease in biosynthesis and accumulation of lipids as a result of decreased expression of the FABP4, FAS, ACC1, HSL, and DGAT1 genes.

Our results, showed, for the first time, that Cr can decrease the expression of some important genes involved in biosynthesis of lipids in goat carcass. We have also previously shown that goat Cr supplementation results in improved meat quality by increasing the accumulation of energy in muscles instead of lipogenic tissues [12]. In accordance with our results, Cr has been suggested to facilitate the activity of insulin and bias the energy taken from food toward growth and muscle production. This study highlighted the key role of chromium in biosynthesis of lipids, which can be utilized in future studies to improve meat quality by hindering fat deposition in animals. Future studies may explore the precise

mechanism(s) through which chromium acts on genes involved in lipid metabolism.

Conflict of interest

The authors declared that they have no conflict of interest.

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