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Stimulatory effects of nano-selenium and conjugated linoleic acid on antioxidant activity, trace minerals, and gene expression response of growing male Moghani lambs

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Abstract

Sheep keepers need suitable strategies to improve animal immunity and the quality of their products. This study was aimed to evaluate the effect of nano-selenium (nano-Se) and conjugated linoleic acid (CLA) on an antioxidant statue, trace minerals, and mRNA expression of glutathione peroxidase 1 (GPX1) and selenoprotein W1 (SEPW1) genes in the liver and peroxisome proliferator-activated receptor-gamma (PPARγ) and stearoyl COA desaturase 1 (SCD1) genes in fat- tail of male Moghani lambs. Thirty male Moghani lambs, three months old and average weight 30.00 ± 0.25 kg, were assigned to a completely randomized design in a 2×3 factorial arrangement with dietary supplementation of nano-Se (0, 1.00 and 2.00 mg kg^{-1} dry matter) and CLA (0.00 and 15.00 g kg^{-1} dry matter). The lambs were slaughtered at the end of the experiment, on day 90 of the experiment. Results showed that dietary inclusion of nano-Se significantly improved antioxidant enzymes glutathione peroxidase and superoxide dismutase in blood, however, did not show any differences in trace mineral treatments. The analysis of qPCR showed that nano-Se inclusion at the highest level (2.00 g kg^{-1} dry matter) enhanced gene expression of GPX1 (0.64 vs 0.34) and SEPW1 (0.72 vs 0.35) in the liver. Dietary inclusion of CLA increased the expression of PPARγ (0.63 vs 0.38) and decreased SCD1 (0.63 vs 0.33) genes in fat- tail. It could be concluded that selenium inclusion in the growing lamb's diet could improve antioxidant status, however, no synergistic interaction was observed along with CLA on the mentioned parameters.

Introduction

In recent years, new technologies have caused developing minerals with more impact on performance, health, growth, safety, and reproduction of animals than ordinary minerals.[1] Interest in nano-selenium has been increasing recently because of their outstanding bioactivities. Nano-selenium (nano-Se) showed significant anti-cancer activities and can significantly inhibit cancer cell growth at μM range.[2] Additionally, nano-Se have exhibited immunomodulation activities in several livestock species at the level of μg g^{-1}, such as broilers and sheep.[3,4] Selenium participates in some physiological properties and molecules including selenoproteins and glutathione peroxidases and biological processes such as antioxidant defense, endocrine function, and immune reactions.[5,6] Several investigations have suggested utilizing nano-selenium as an animal feed supplement for boosting the immune ability of livestock.[7]

Conjugated linoleic acids (CLAs) are known as positional and geometrical isomers corresponding to linoleic acid with conjugated double bonds. It is known to have useful impacts on animal models such as reducing fat in milk and body.[8] However, investigations reported that dietary inclusion of selenium increased polyunsaturated fatty acids particularly CLA in bull meat, meat, and liver of sheep, cow's and goat's milk.[9-12] Investigations showed that CLA increased oxidation of fatty acid in mouse liver.[13] On the other hand, dietary inclusion of selenium disturbed the plasma levels of zinc, copper and other trace minerals in young ruminants, however, the impacts of CLA on trace elements is unclear.[14]

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Selenium plays role in the antioxidant system by participating in the selenoproteins structure. Seleno-enzymes are the selenoproteins products produced by cellular metabolism and peroxide radicals.[15] Glutathione peroxidase 1 (GPX1) contains the amino acid seleno-cysteine, which is utilized for specific functions in the body such as reducing intracellular hydrogen peroxide and reducing reactive oxygen species (ROS) generated by all the cells in the standard flow of oxidative respiration. Selenoprotein W1 (SEPW1), is another selenoprotein, which is similar to GPX1 and plays a main role in protecting cells from oxidative stress in the cellular defense system.[16] Reports indicated a direct relationship between SEPW1 expression and selenium content of the spleen of rats and sheep.[17] Also, it has been shown that selenium could participate in lipid metabolism by modulating mRNA expression of associated genes.[18]

Peroxisome proliferator-activated receptor gamma (PPARγ) produces bands with non-esterified fatty acids and long-chain unsaturated fatty acids and converts fat signals into transcriptional changes. This affects the metabolic procedures including synthesis, storage, transfer, and oxidation of fats.[19] It has been found that the profile corresponding to dietary fatty acids, particularly unsaturated fatty acids, controls PPARγ gene expression. It affects the diversity of the adipocyte cells and the energy-related genes metabolism.[20] Stearoyl COA desaturase 1 (SCD1) enzyme regulates liver lipogenesis and lipid oxidation.[21] SCD1 was reported as a key enzyme involving in the preparation and contexture of saturated and unsaturated fatty acid in heifers and beef cattle.[22] Some nutritional and hormonal parameters like glucose, saturated fatty acids, fructose, and insulin increased the concentration of SCD1, while other factors such as unsaturated fatty acids and leptin decreased its levels.

To date, all of the in vivo works with CLA has been done with a commercial-free fatty acid preparation containing a mixture of cis-9, trans-11, trans-10, cis-12 and cis-11, trans-13 isomers, although CLA content of food predominantly (80.00-90.00%) contained cis-9, trans-11-isomer in the form of triacylglycerols. Therefore, in this study, we used protected CLA (PCLA) containing a special complex of cis-9, trans-11 and trans-10, cis-12 CLA isomers, and saturated fat of vegetable origin and silicic acid. The PCLA contained 788 g lipid and 212 g ash kg^{-1} dry matter (DM). Of the lipid component (as free fatty acid equivalent) 11.80% was the cis-9, trans-11 isomer of CLA, 12.10% was the trans-10, cis- 12 isomer of CLA.

Though, no data is available in the studies on the impacts of nano-Se and CLA on immune, trace minerals, and gene expression response in Moghani lambs. It was hypothesized that nano-Se and CLA might alleviate adverse impacts corresponding to antioxidant status and some trace minerals in lambs. Thus, this study was aimed to evaluate the effects of the feeding of nano-Se and CLA on immune, trace minerals, and gene expression response of growing male Moghani lambs.

Materials and Methods

Thirty male Moghani lambs, 3-months-old and bodyweight of 30.00 ± 0.25 kg, were assigned in a completely randomized design with a 2×3 factorial arrangement with dietary inclusion of CLA (0.00 and 15.00 g kg^{-1} DM) and nano-Se (0.00, 1.00 and 2.00 mg kg^{-1} DM) for 90 days. Animals were classified into six groups of five animals each. Experimental treatments were 1) Control, 2) 1.00 mg nano-Se kg^{-1} DM, 3) 2.00 mg nano-Se kg^{-1} DM, 4) 15.00 g CLA kg^{-1} DM, 5) 15.00 g CLA + 1.00 mg nano-Se kg^{-1} DM, 6) 15.00 g CLA + 2.00 mg nano-Se kg^{-1} DM. The selenium (liquid) and CLA (powder) were supplied by Golbar Navid Bahar Company (Tehran, Iran). Animals were preserved in individual pens. Similar diets were formulated and justified for lipid and selenium concentrations. The concentrate comprised barley (68.50%), wheat bran (24.20%), soybean meal (1.40%), salt (0.70%), sodium bicarbonate (1.40%), dicalcium phosphate (1.40%), and minerals and vitamins (2.40%). It contained crude protein (CP), 14.29%, Neutral detergent fiber (NDF), 31.96%, and 2.20 Mcal kg^{-1} of DM. The lambs were fed on basal diets containing 0.30 mg per kg Se on DM basis. Total selenium content of the basal diet was estimated using hydride generation atomic absorption spectrometry (AA6800; Shimatzu, Tokyo, Japan). Feed samples were collected weekly in the morning feeding and frozen for subsequent analyses of DM, ash, ether extract (EE), and crude protein (CP) contents according to AOAC.[23] Neutral detergent fiber (NDF) was determined according to Van Soest *et al* using heat-stable α-amylase (Sigma, St. Louis, MO) in a fiber analyzer (Ankom-200, New York, USA). Acid detergent fiber (ADF) was analyzed according to Van Soest *et al*.[24] Determination of calcium and phosphorus were carried out based on Talapatra *et al* method.[25] Lambs were weighed every 10 days within the experiment for determination of the growth efficiency. The protocol for animal research was accepted by the Ethics Committee of the University of Mohaghegh Ardabili.

On day 90 of the trial, blood specimens samples were collected in heparinized tubes to assess the plasma concentrations of trace minerals including selenium, zinc, copper, and iron.[26] This was performed through working standards for inductively coupled plasma optical emission spectrometry (Spectroblue, Kleve, Germany) analysis prepared from proper standard solutions having 1000 ppm from per tested element obtained from Perkin Elmer (Waltham, USA).

At the same time, another blood sample (8.00 mL) were collected and centrifuged at 1,700 *g* for 10 min to achieve the serum then the specimens were frozen at – 20.00 ˚C. The serum concentration of cortisol was

determined by solid-phase radioimmunoassay by a Coat-A-Count® radioimmunoassay kit (Siemens Medical Solutions Diagnostics, Los Angeles, USA).[27] Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were measured in hemolysates with a commercially accessible kit (Randox Laboratories, Crumlin, UK).[28] Blood kit was used as recommended by manufacturer's instructions.

At the end of the trial (90 days), lambs were slaughtered. Liver and fat- tail tissue specimens were obtained and frozen directly in liquid nitrogen and kept at – 80.00 ˚C to subsequent analyses. Two-hundred mg of liver and fat- tail tissues were immediately collected in RNA-later stabilization reagent (Thermo Scientific, Waltham, USA) and processed to keep at – 80.00 ˚C, according to the instructions of the producer.

Total RNA was isolated from liver and fat- tail samples by the High Pure RNA tissue kit (SinaClon, Tehran, Iran) as recommended by producer instructions. Assessment of qualitative and quantitative parameters corresponding to the separated RNA were carried out using spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, USA). Only specimens with RNA concentration above 100 ng and absorbance ratios of A260/280 and A260/230 of about 1.90 were applied for additional analyses. Transcription First Strand cDNA Synthesis Kit (Thermo Scientific) was used to reverse transcribe the RNA into cDNA. Denaturing of 1.00 µg of RNA was performed for 5 min at 95.00 ˚C in the existence of 50.00 µM Oligo (dT). The reverse-transcription mixture (20.00 µL) comprised RNA (13.00 µL), reverse transcriptase buffer (4.00 µL), 10.00 mM dNTP (2.00 µL), protector RNase inhibitor (40.00 U μL^{-1}; 0.50 µL), and reverse transcriptase (20.00 U μL^{-1}; 0.50 µL). Incubation of the resulted solution was performed for 60 min at 50.00 ˚C, next for 10 min at 70.00 ˚C, and lastly kept at – 20.00 ˚C. The web-interface Primer 3Plus was used to design primers of gene expression based on GenBank Ovis Aries sequences and amplicon sequence according to exon-exon boundaries (Table 1). Relative mRNA abundances corresponding to genes were tested. Selected targets included two selenoprotein genes (GPX1 and SEPW1) and two genes associated with lipid metabolism (PPARγ and SCD1).

Quantitative studies are commonly realized in biomedical research to compare RNA expression in different experimental or clinical conditions. These quantifications are performed through their comparison to the expression of the housekeeping gene transcripts like glyceraldehyde-3-phosphate dehydrogenase (G3PDH), albumin, beta actins (ACTB), tubulins and cyclophilin and hypoxanthine phosphoribosyltransferase (HRPT) are also used as internal standards. In this study, we used ACTB as housekeeping gen, and its stability was tested using GeNorm software (version 3.4; PrimerDesign, Southampton, USA).[29] The RT-PCR was performed in a total of 10.00 µL PCR mixture containing SYBR Green PCR Master Mix in 96-well plates of C1000™ Thermal Cycler (Applied Biosystems, Foster City, USA). The PCR conditions were as follow: One cycle of 1 min at 95.00 ˚C, 45 PCR cycles of 10 sec at 95.00 ˚C, 10 sec at the annealing temperature of the primers 15 sec at 72.00 ˚C, plate reading, followed by an extension of 10 min at 72.00 ˚C, plate reading every other 0.20 ˚C from 65.00 ˚C to 94.00 ˚C for drawing melting curves, then the reactions were ended with an extension of 5 min at 72.00 ˚C. Each reaction was run in triplicate and the mean value was applied to compute the relative amount of the target gene. The relative gene expression was normalized against a factor which was according to the geometric average of the expression level corresponding to the housekeeping gene based on the recommendation of Vandesompele *et al*.[30] Relative mRNA abundances of the target genes in the tissue samples were determined using the Δ cycle threshold (ΔCt) method as outlined in the protocol of Applied Biosystems. In brief, a *ΔCt* value was the *Ct* difference between the target genes and the reference gene (ACTB):[31]

$$\Delta Ct = Ct^{target} - Ct^{reference}$$

For statistical analysis, the effects of CLA and nano-Se and their interactions were evaluated by analysis of variance using the command PROC GLM of SAS (version 9.12; SAS Institute, Cary, USA) software according to the following model:

$$Yi = \mu + Ti + Sj + TSij + eijk$$

Table 1. Characteristics of the used primers.

Target gene	Accession number	Primer	Sequence (5′–3′)	PCR fragment length (bp)
GPX1	XM-004018462.1	Fwd	CCTGGTCGTACTCGGCTTC	154
		Rev	CCTTCTCGCCATTCACCTC	
SEPW1	XM-015100623.1	Fwd	CTATGGCGCTTGAGGCTACA	152
		Rev	TGGAGTGAACCAGCTTTCCC	
PPARγ	XM-004007050.1	Fwd	ATGGCTTCATAACCCGTGAG	206
		Rev	AATCCCCTCCTGCATTTTCT	
SCD1	NM-001009254.1	Fwd	GTGCCGTGGTATCTATGGGG	150
		Rev	GGGGTTGATGGTCTTGTCGT	
ACTB	NM-001009784.1	Fwd	CTCTTCCAGCCTTCCTTCCT	178
		Rev	GCAGAAAGAGATCACTGCCC	

GPX1: Glutathione peroxidase 1, SEPW1: Selnoprotein W1, PPARγ: Peroxisome proliferator-activated receptor-gamma, SCD1:Stearoyl COA desaturase 1, ACTB: Beta-actin.

where, *Yi* = dependent variable; μ = the overall mean; *Ti* = effect of CLA; *Sj* = effect of selenium; *TSij* = interaction between CLA and selenium and *eijk* = residual error. The effects of treatment according to the diet were determined with averages calculated by command LSMEANS and Duncan test was used to determine the significant differences among the treatment groups at significant level of $p < 0.05$.

Results

The results indicated that dietary inclusion of nano-Se significantly improved ($p < 0.05$) antioxidant enzymes in the blood (Table 2).

The effects of selenium and CLA on trace mineral concentration are presented in Table 3. Results indicated that the dietary inclusion of selenium only increased the plasma concentration of selenium with no differences in the rest of the parameters. CLA did not have any effects on trace elements concentration.

This study tried to comprise the highest levels of selenium on Moghani male lambs (2.00 mg kg^{-1} DM) in nano form. Nano-Se improved the expression corresponding to GPX1 (0.64 vs 0.34) and SEPW 1 (0.72 vs 0.35) in liver ($p < 0.05$), however, it had no significant effect on the expression of PPARγ (0.63 vs 0.38) and SCD1 (0.63 vs 0.33), ($p < 0.05$), (Table 4). Conversely, CLA did not have an important impact on the expression corresponding to GPX1 and SEPW 1 in liver ($p < 0.05$), however, its effects on PPARγ and SCD1 were increasingly significant ($p < 0.01$).

Discussion

The key role for antioxidants available in the diet is to prevent oxidative damage to the cells and their physiological functions. The interactions between antioxidant nutrition and immune system functions are logical fields for study. For example, investigations have reported that immune activity is markedly altered with dietary selenium.[32] It was proposed that the impaired immune activity as caused by selenium deficiency might be related to altered blood antioxidants concentration. Under ROS-induced oxidative stress, antioxidant defense systems counting SOD and GPX are vital to scavenge the ROS.[33] The SOD enzyme plays role in dismutation of the superoxide anion to hydrogen peroxide, SOD acts as the first line of defense converting O_2^- into H_2O_2, which is then applied as a substrate using GPX enzymes. The greatest vital biological activity of Se is associated with its antioxidant activity as it generates selenocysteine, which is part corresponding to the active center of the GPX.[16] The outcome of this research indicated higher GPX and SOD function in the blood of Moghani lambs in all Se-supplemented groups compared to the controlled ones. As same as our outcomes, Saffari *et al*. observed that the maximum GPX and SOD activity were in carp fed on 2.00 mg kg^{-1} nano-selenium.[34] Stimulatory impacts of selenium on antioxidant parameters like GPX and SOD were also shown in turkey, chickens, rats, mice, lamb, and calves.[35-37]

Our results indicated that dietary inclusion of selenium only increased the plasma level of selenium but not other

Table 2. Effects of treatments on superoxide dismutase and glutathione peroxidase levels in Moghani lambs.

CLA (g kg^{-1} DM)	0			15			*p*-value			SEM
Nano-Se (g kg^{-1} DM)	0	1	2	0	1	2	Se	CLA	Se × CLA	
SOD (U g^{-1} of Hb)	56.92^{b}	61.92^{a}	63.21^{a}	55.12^{b}	61.21^{a}	62.21^{a}	*	NS	NS	1.54
GPx (U g^{-1} of Hb)	39.24^{b}	42.81^{a}	44.39^{a}	34.29^{b}	44.12^{a}	44.11^{a}	*	NS	NS	1.60

SOD: Superoxide dismutase; GPx: glutathione peroxidase.

^{ab} Different letters in each row indicate a significant difference. * $p \leq 0.05$. NS: Not significant ($p > 0.05$).

Table 3. Effects of dietary nano-Se and CLA on trace mineral concentration (μg L^{-1}) in Moghani lambs.

CLA (g kg^{-1} DM)	0			15			*p*-value			SEM
Nano-Se (g kg^{-1} DM)	0	1	2	0	1	2	Se	CLA	Se × CLA	
Selenium	45.00^{c}	52.10^{b}	61.20^{a}	44.20^{c}	51.10^{b}	61.10^{a}	*	NS	NS	1.63
Cupper	531	528	527	529	531	533	NS	NS	NS	40.99
Zinc	1079	1094	1011	1051	1066	1086	NS	NS	NS	47.40
Iron	615	621	627	610	631	621	NS	NS	NS	14.10

^{abc} Different letters in each row indicate significant difference. * $p \leq 0.05$. NS: Not significant ($p > 0.05$).

Table 4. Effects of treatments on the expression rate (%) of the desired genes.

CLA (g kg^{-1} DM)	0			15			*p*-value			SEM
Nano-Se (g kg^{-1} DM)	0	1	2	0	1	2	Se	CLA	Se × CLA	
Liver										
GPX1	0.30^{b}	0.30^{b}	0.48^{a}	0.38^{b}	0.37^{b}	0.80^{a}	*	NS	NS	0.112
SEPW1	0.30^{b}	0.36^{b}	0.56^{a}	0.40^{b}	0.42^{b}	0.87^{a}	*	NS	NS	0.128
Fat- tail										
PPARγ	0.29^{b}	0.36^{b}	0.48^{b}	0.53^{a}	0.55^{a}	0.80^{a}	NS	**	NS	0.104
SCD1	0.21^{b}	0.30^{b}	0.48^{b}	0.58^{a}	0.65^{a}	0.66^{a}	NS	**	NS	0.093

^{abc} Different letters in each row indicate significant difference. * $p \leq 0.05$. ** $p < 0.01$. NS: Not significant ($p > 0.05$).

parameters. The CLA did not have any impact on trace elements concentration. Moeini *et al*. have reported that serum Se levels in heifers and their calves did not change, however, administration of selenium in calves increased the serum Se concentration.[38] Conversely, Kojouri and Shirazi have reported that the selenium supplementation increased serum copper concentration in lambs fed on selenium and changed zinc-copper ratios after four weeks. In turn, zinc concentration was decreased, whereas administration of selenium enhanced iron level.[18]

Many investigations have found that dietary inclusion of selenium enhanced the expression of GPX1 and SEPW1, which was in agreement with our findings. Conversely, other investigations indicated that the types and concentrations of selenium did not enhance the concentrations of selenoproteins enzymes and gene expression of GPX1 and SEPW1.[31,39] On the other hand, Liu *et al*. found that selenium supplementation at high concentrations (3.00 mg kg^{-1}) decreased the amount of mRNA of SEPW1 in the liver of pigs compared to other levels (0.30 mg Se kg^{-1} diet).[40] The enhanced expression of GPX1 can progress animal health by inhibiting oxidation. Antioxidants like GPX1 and SEPW1are synthesized within the cells and play a role as a primary defense system against free radicals.[35] The GPX1 and SEPW can reduce free radical formation. Extensive oxidation increases the formation of unstable compounds known as free radicals, which would damage the biological components in the body and cause peroxidizing lipid, protein carboxylation, and DNA strand breakages, ultimately causing various clinical consequences.[41,42] Earlier investigations showed that some antioxidants like vitamins and selenium were a secondary defense system.[43] Our results confirmed the function of selenium in these metabolic mechanisms suggesting that selenium was not only an important antioxidant but also a regulator of gene expression.

Dietary supplementation with CLA increased the gene expression of PPARγ and reduced the expression of SCD1. PPAR is an active group of nuclear protein receptors regulating the genes expression that are involved in energy metabolism, cell differentiation, cell death, and metabolism of lipids, carbohydrates, and proteins. PPARγ is the key regulator of fat cells differentiation and production and has a vital role in controlling the overall body metabolism and is mostly expressed in fat tissues.[44] CLA is known as an agonist component for PPARγ. PPARγ isoforms are a part of the regulatory impacts of dietary fatty acids on gene expression and interfere with the fat storage that is found at a high level in adipose tissue. According to the outcomes of this research, CLA enhanced the expression of PPARγ in fat tissue. This probably indicated a direct relationship between the inclusion of CLA in the diet and the gene expression level of PPARγ.

The SCD1 has the main role in adding a double bond in Delta 9 position in an extensive range of fatty acids leading to produce CLA in ruminants' meat and milk.[45] SCD1 was one of the major candidate genes to attract researchers to alter the ratio of saturated to unsaturated fatty acids and increase the level of conjugated linoleic acids in milk.[46] The function of SCD1 is reduced with the existence of unsaturated fatty acids in the diet and increased with their absence because of the existence of CLA in the diet ($p < 0.001$). This is an excellent explanation of how manipulating the diet could progress the animal product quality via altering the gene expression of SCD1.

Based on our results, no synergistic interaction effect was observed between selenium and CLA. This could be described using the existence of selenium in the diet reducing oxidation of unsaturated fatty acids and affecting gene expression.[47] Also, high concentrations of selenium in the diet can damage the liver and consequently affect the pathways corresponding to fatty acids and selenium.[48] Wang *et al.* found that a high concentration of selenium could disrupt liver function and, consequently, impaired the pathway of fats. Therefore, they reported that a high concentration of selenium might increase oxidative stress.[49]

In conclusion, nano-Se and CLA had different important impacts on the gene expression corresponding to GPX1, SEPW1, PPARβ1, and SCD1. It indicated that diets and its nutrient contents like selenium and CLA could have an important role in changing the gene expression which influences the quality of the products derived from ruminants. However, synergistic interaction impact was not observed which needs further investigation. The existence of nano-selenium in lamb's diet showed useful impacts on immune blood parameters, however, no important impacts on iron, copper, and zinc level were observed in the blood.

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Conflict of interest

The authors declare no conflict of interest.

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