Small Ruminant Research 154 (2017) 23–28

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Effects of liquorice extract on the activity and gene expression level of antioxidant enzymes in *longissimus dorsi* muscle of Tan lamb

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ARTICLE INFO

Keywords:
Liquorice extract
Flavonoid
Antioxidant enzyme
Free radical
Lamb

ABSTRACT

The objective of this study was to investigate the role of liquorice extract (LE) as a dietary antioxidant supplement in the regulation of muscle antioxidant enzyme in lambs. Fifty Tan male lambs were randomly allocated among five groups with LE supplementation at levels of 0, 1000, 2000, 3000 and 4000 mg/kg feed, respectively. After 120 days of feeding, the lambs were slaughtered and the *longissimus dorsi* muscle was sampled for measuring the antioxidant enzymes activities and genes expression levels. In addition, superoxide anion ($O_2\cdot^-$), hydrogen peroxide (H_2O_2) and thiobarbituric acid reactive substance (TBARS) levels were also tested in our study. The results showed that supplementation with LE increased ($P < 0.05$) glutathione peroxidase (GPx) and catalase (CAT) activities, but LE had no effect on Cu/Zn superoxide dismutase (Cu/Zn SOD) and Mn superoxide dismutase (Mn SOD) activities. For gene expression, no significant differences were observed in mRNA levels of Cu/Zn SOD, Mn SOD, GPx and CAT genes among the five groups. In addition, LE supplementation decreased ($P < 0.05$) $O_2\cdot^-$, H_2O_2 and TBARS levels in muscle. The results demonstrated that LE as a supplement could improve GPx and CAT activities, then prevent muscle against lipid oxidation.

1. Introduction

Oxidative damage is the main factor to induce meat quality deterioration and reduce shelf life of meat products. Reactive oxygen species (ROS), a molecular culprit, such as superoxide radical ($O_2\cdot^-$) and hydrogen peroxide (H_2O_2) attacks biologically essential molecules, such as lipid and protein, by donating hydrogen atom or electron to induce oxidative damage (Shin et al., 2010). Lipid oxidation produces conjugated dienes, hydroperoxides and aldehydes (Raharjo and Sofos, 1993). In particular, malondialdehyde (MDA), an aldehydic product, is widely used as a marker of oxidative damage. For living animals, oxidation products as a causative agent can lead to diseases, such as reproductive disorders in cow (Kankofer, 2002), ascites syndrome in chicken (Xiang et al., 2002) and sperm motility decline in sheep (Gundogan et al., 2010). Furthermore, when oxidation products accumulate in meat, it will adversely impact its color, flavor, odor, texture and nutritive value (Devatkal et al., 2010). In order to improve the oxidative stability of meat products, plant antioxidants have been received a wide attention, because they can scavenge ROS and overcome the side effects of synthetic antioxidants to the body. Among these, natural polyphenolic compounds, especially flavonoids that occur naturally in fruit, vegetable, seed, flower and even bark, have been largely studied in cultured cells (Ramos, 2008), animal models (Turgut et al., 2016) and meat processing (Kumar et al., 2015) for their strong antioxidant capacity.

Liquorice (*Leguminosae*), one of the most popular medicinal plant, is widely distributed in the middle-east, Mediterranean area and North China (Siracusa et al., 2011). It contains high levels of glycyrrhizin, flavonoids and polysaccharides (Zhang and Ye, 2009). Previous studies paid close attention to glycyrrhizin for its antiphlogosis and ability to relieve asthma (Ram et al., 2006), but liquorice flavonoids were often ignored and even most flavonoids were discarded during the process of extracting glycyrrhizin. Liquorice flavonoids depending on hydroxyl groups can scavenge free radials and chelate metal ions, which help to improve oxidation resistance. Belinky et al. (1997) reported that glabridin, a flavonoid isolated from *Glycyrrhiza glabra*, reduced the susceptibility of low density lipoprotein (LDL) to oxidation induced by copper ion and 2,2′-azobis dihydrochloride (AAPH) free radical. Laura et al. (2011) also demonstrated the scavenging free radicals capacity of flavonoids extracted from *Glycyrrhiza glabra*.

Currently, the antioxidant actions of flavonoids have been generally related to its free radical scavenging capacity, but emerging findings seem to indicate that flavonoid may play a role in increasing endogenous antioxidant defense potential. Several studies have shown

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http://dx.doi.org/10.1016/j.smallrumres.2017.06.012
Received 14 October 2016; Received in revised form 12 June 2017; Accepted 14 June 2017
Available online 15 June 2017

that epigallocatechin gallate (Na and Surh, 2008), cocoa flavonoid (Martín et al., 2010) and quercetin (Molina et al., 2003) induce a varied set of antioxidant enzymes in diverse organs or cultured cells. Moyo et al. (2012) reported that supplementation with flavonoids extracted from *Moringa oleifera* leaves increased antioxidant enzyme activity in goat liver. Furthermore, some authors have found that a number of cellular kinases, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K), are activated in response to treatment with flavonoids, which may stimulate the transcription of antioxidant enzyme gene (Williams et al., 2004). For liquorice flavonoids, its role as free radical scavenger has been demonstrated by some studies (Laura et al., 2011), but there is little data on the antioxidant enzyme impact of liquorice flavonoids when supplemented to animals.

Our previous study revealed that liquorice extract (LE, containing 16.37% total flavonoids) could improve meat color and we speculated that it was possibly caused by the antioxidant activity of LE (Zhang et al., 2013). Further, we found that LE could scavenge free radicals to improve antioxidant activity of meat (Zhang et al., 2015). However, we are quite curious if there are other ways to improve antioxidant capacity. Therefore, the objective of the study was to investigate the role of liquorice extract (LE, containing 16.37% total flavonoids) as dietary supplement in the regulation of antioxidant enzymes activities and genes expression levels in lamb. In addition, antioxidant enzyme substrates ($O_2\cdot^-$ and H_2O_2) and thiobarbituric acid reactive substance (TBARS) levels in meat were tested in our study.

2. Materials and methods

2.1. Animal breed and welfare

The animal experiment was conducted with Tan lamb, a local breed that is celebrated for its meat in Ningxia, China, where the meat is authorized by the government as a geographically symbolic product (AGI2008-07-00061). Tan lamb, with white and shiny coat color, belongs to Mongolia sheep group. In our experiment, all procedures were undertaken following the guidelines of the China Agricultural University Animal Care and Use Committee on animal ethics.

2.2. Experimental design and diets

Fifty male Tan lamb at the age of 4 months ± 10 days, with average initial body weight of 17.56 ± 0.39 kg and the same genetic background, from the Nuanquan farm, Ningxia, China, were randomly assigned into five equal groups of 10 animals each for 120 days of feeding trial before slaughter. The lambs were housed individually in pens (1.1 m long × 1.0 m wide), were allowed visual contacts each other and had water supplied *ad libitum*.

Liquorice extract (LE) from *Glycyrrhiza uralensis* (the licorice plant) containing 16.37% total flavonoids, was supplied by Shaanxi Sciphar Biotechnology Co. Ltd., China. Total flavonoids content was determined according to Moreno et al. (2000). Briefly, 1 g of LE was homogenized (FSH-2, Ronghua Instrument Manufacture Co. Ltd., Jintan, Jiangsu, China) with 5 ml of 80% ethanol and then the homogenate was centrifuged (3K15, Sigma Laborzentrifugen GmbH, Osterode, Niedersachsen, Germany) at 1400 × *g* for 10 min. An aliquot of 0.5 ml supernatant was added to test tubes containing 0.1 ml of 10% aluminum nitrate, 0.1 ml of 1 M aqueous potassium acetate and 4.3 ml of 80% ethanol. After 40 min incubation at room temperature, the absorbance was determined at 415 nm (752, Shanghai Spectral Instrument Co. Ltd., Shanghai, China). Total flavonoid content was calculated using quercetin (Analytical Reagent, Sigma Chemical Co., St. Louis, MO, USA) as standard. The control group (LE0) was fed a basal diet without LE supplementation, while the four experimental groups were fed the basal diet with LE supplementation at levels of 1000 (LE1000), 2000 (LE2000), 3000 (LE3000) and 4000 (LE4000) mg/kg feed (on dry matter basis).

Table 1
Ingredients and chemical composition of the basal diet.

Ingredients (on dry matter basis)	Concentration
Corn silage (%)	50.00
Corn (%)	27.81
Soybean meal (%)	13.00
Wheat bran (%)	4.93
Shelled sunflower meal (%)	2.11
Sodium chloride (%)	0.68
Calcium carbonate (%)	0.23
Calcium hydrophosphate (%)	0.11
Premix (%)[a]	1.13
Chemical composition (on dry matter basis)	
Metabolizable energy (MJ/kg)[b]	8.95
Crude protein (%)	12.30
Ether extract (%)	5.08
Neutral detergent fiber (%)	48.77
Acid detergent fiber (%)	33.89
Calcium (%)	0.51
Phosphorus (%)	0.36

[a] Per kilogram of premix: 100, 000 IU vitamin A, 20, 000 IU vitamin D_3, 60 IU vitamin E, 1 g Fe, 1 g Mn, 0.78 g Zn, 0.27 g Cu, 0.012 g Se, 0.01 g I.
[b] Analyzed values except metabolizable energy.

The basal ration (Table 1) was formulated to meet the nutritional requirements of growing lamb for 100 g weight gain per day and the amount of ration was adjusted according to their bodyweight, as described by NRC (2007). The concentration of metabolizable energy was calculated from the ingredient values based on the feeding standard of meat-producing sheep and goats (NY/T816-2004). Concentrations of crude protein, ether extract, calcium and phosphorus in basal diet were determined as described in AOAC (1990), while neutral detergent fiber and acid detergent fiber levels were measured according to Van Soest et al. (1991). During the feeding period, lambs were fed twice daily (08:00 h and 18:00 h). In all five groups, < 0.5% nutritional differences, such as energy and protein, due to LE supplementation were ignored.

2.3. Slaughter procedure and muscle sampling

After the feeding trial, lambs were fasted for 12 h with free access to water and weighed immediately before slaughtering by the standard Halal procedures according to Santos et al. (2007). All lambs were electrically stunned and slaughtered by exsanguination. Muscle cuts of approximately 30 g were dissected out from the *longissimus dorsi* muscle between 11th thoracic to 6th lumbar vertebrae from each carcass. 10 g of muscle sample was stored at −80 °C until determination of $O_2\cdot^-$, H_2O_2 and thiobarbituric acid reactive substance (TBARS) content. Another 20 g muscle sample was stored at −80 °C until determination of antioxidant enzymes activities and gene expression levels, including Cu/Zn superoxide dismutase (Cu/Zn SOD), Mn superoxide dismutase (Mn SOD), glutathione peroxidase (GPx) and catalase (CAT).

2.4. $O_2\cdot^-$ and H_2O_2 concentration analytical procedure

$O_2\cdot^-$ concentration was determined by hydroxylamine oxidation method, as described by Wang et al. (2012). Briefly, 1 g of meat sample was homogenized (FSH-2, Ronghua Instrument Manufacture Co. Ltd., Jintan, Jiangsu, China) with 10 ml of ice-cold (< 4 °C) medium containing 10 μM phosphopyridoxal, 1 mM Na_2EDTA and 5 mM dithiothreitol (DTT) in 0.25 M phosphate buffer at pH 8, and the homogenate was centrifuged (3K15, Sigma Laborzentrifugen GmbH, Osterode, Niedersachsen, Germany) at 10,000 × *g* for 25 min. Subsequently, the supernatant was collected and 0.5 ml was incubated with 1 ml of 1 mM NH_4Cl and 0.5 ml of 0.05 M phosphate buffer (pH 7.8) in a test tube. After 60 min at 25 °C, the test tube was added with 1 ml of 17 mM sulfanilic acid and 1 ml of 7 mM α-naphthylamine and subsequently continued to incubate at 25 °C for 20 min. Then, the absorbance at