Article

Transcriptomic and Metabolomic Insight into the Roles of α-Lipoic Acid in the Antioxidant Mechanisms of Sheep

Zhaohui Yao, Gaiying Li, Xinlu Yang, Liyang Zhang, Chuanyou Su, Tong Fu, Linfeng Wang, Hongxia Lian, Tengyun Gao and Kaizhen Liu *

Henan International Joint Laboratory of Nutrition Regulation and Ecological Raising of Domestic Animal, College of Animal Science and Technology, Henan Agricultural University, Zhengzhou 450046, China; 13295045831@163.com (Z.Y.); ligaiying@henau.edu.cn (G.L.); yangxinlu@163.com (X.Y.); zhangliyang@henau.edu.cn (L.Z.); suchuanyou@henau.edu.cn (C.S.); futong@henau.edu.cn (T.F.); wanglinfeng@henau.edu.cn (L.W.); lianhongxia@henau.edu.cn (H.L.); gaotengyun@henau.edu.cn (T.G.)
* Correspondence: liukaizhen@henau.edu.cn; Tel.: +86-15321607930

Abstract: Alpha lipoic acid (α-LA) is a powerful antioxidant, which can reduce oxidative damage and inflammation in the host. In previous research, we found that 600 mg/kg α-LA supplemented in the diet could improve the activity of antioxidant enzymes and regulate the secretion of inflammatory factors in rumen of sheep. However, the mechanism of α-LA driving the antioxidant capacity in rumen of sheep remains unknown. The aim of this current research was to investigate the role of α-LA in antioxidant and inflammatory processes in the rumen of sheep. Transcriptomic and metabolomic analyses were performed to assess the variation of genes and metabolites of rumen epithelial tissue in sheep induced in the LA (600 mg/kg α-LA) group compared with the CTL (0 mg/kg α-LA) group. The results showed that some discovered core genes in the rumen epithelial tissue were negatively correlated with antioxidant activity. *G6PD* and *HK2*, the downregulated and upregulated core genes in the comparison of LA/CTL, were enriched in the pentose phosphate pathway (PPP) and the pathways of mannose and oligosaccharide metabolism, respectively. The PPP is a metabolic pathway within cells, primarily functioning to produce nicotinamide adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate. The differential metabolites were enriched in the pathway of 2-oxocarboxylic acid metabolism, which improves the antioxidant capacity of the rumen epithelium by increasing enzymatic activities of SOD. In conclusion, the present study demonstrated that α-LA improved antioxidant activity by regulating PPP and 2-oxocarboxylic acid metabolism. This study will provide a theoretical basis for the application of α-LA in the raising of sheep.

Keywords: α-lipoic acid; rumen epithelial tissue; transcriptomics; metabolomics; antioxidant

1. Introduction

The rumen is a complex microbial ecosystem that evolved approximately 35 to 40 million years ago [1]. Additionally, the rumen epithelium tissue is critical to nutrient translation and host health, and also has key functions in the antioxidant and immune processes of the host [2]. Alpha-lipoic acid (α-LA), a vitamin-like disulfide compound, is a key coenzyme in mitochondria, which is widely found in nature in plants, animals, humans, and microorganisms. It can be supplemented in animals through the consumption of dark green leafy vegetables and meat [3,4]. α-LA exerts a powerful antioxidant action in vivo through scavenging of free radicals and regeneration of other antioxidants, such as ubiquinone, glutathione, and vitamin C [5,6]. In animals, α-LA participates in the oxidative decarboxylation reaction of pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase complex, and catalyzes acyl group production and transfer in the host [7,8]. Thus, α-LA plays a key role in animal growth and metabolism, and anti-inflammatory and antioxidant functions [9,10]. A significant reduction in pro-inflammatory cytokines and significant increase in anti-inflammatory cytokines were observed in the livers of α-LA

treated rats [11]. Organ damage is tightly related to hypertension-induced oxidative stress, and its role in reducing oxidative stress and kidney and heart damage had been proved in spontaneously hypertensive rats [12].

A number of researchers have reported that α-LA exerts positive effects on metabolism and oxidative stress in ruminants. The supplementation of 600 mg/kg α-LA in the diet of lambs enhanced the antioxidant capacity of the muscle, effectively inhibited muscle oxidation, and maintained the stability of meat color [13]. α-LA supplementation in early lactating high-yielding dairy cows promoted hepatic metabolism [14]. In addition, α-LA acts as an effective cryoprotectant for buffalo semen because of its potent antioxidant properties [15]. In ruminants, the effects of α-LA on the host have focused on changes in antioxidant and immune markers in serum; yet research on rumen tissues and their antioxidant mechanisms is limited. In previous research, results showed that the addition of α-LA in the diet promoted growth in sheep, along with rumen epithelial tissue antioxidant capacity and immune function [16]. However, the mechanism by which α-LA regulates rumen epithelial tissue is unknown. The aim of this current research was to investigate the role of α-LA in the antioxidant processes of rumen in sheep. We conducted transcriptomic and metabolomic analyses of the genes and metabolites of a CTL (0 mg/kg α-LA) group and an LA (600 mg/kg α-LA) group. By exploring the effects of α-LA on differential metabolites and expressed genes in the rumen epithelium tissue of sheep, we aimed to further elucidate the mechanism of the antioxidant activity changes in the rumen epithelium after the addition of α-LA to sheep diets.

2. Materials and Methods

2.1. Animal Experiment and Sample Collection

The experimental procedure was performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Henan Agriculture University (Permit Number 12-1328).

A total of 100 healthy Duhu F1 hybrid (Dupo♀× Hu sheep♂) sheep with an average age of 2.5 months and similar body weight (27.49 ± 2.10 kg) were randomly divided into five groups, and they were fed a diet with 0 (CTL), 300 (LA300), 450 (LA450), 600 (LA600), and 750 (LA750) mg/kg α-LA, respectively. α-LA was incorporated into the daily ration and mixed uniformly. The composition and nutritional content of the diet is shown in Table S1 [16]. The sheep pen was disinfected, and the sheep were dewormed. The pre-experiment period was 7 d and the experiment period was 60 d. The purity of α-LA was 99.08%, purchased from Xi'an Tian Guangyuan Biotechnology Co., Ltd. (Xi'an, China). On the morning of the last experimental day, sheep were transported to the slaughterhouse for the experiment. The muscular layer was carefully dissected away to obtain the rumen epithelium, and a defined quantity of epithelial tissue was sequentially excised from various regions of the rumen. We then minced the rumen tissue samples to achieve homogenization. Rumen epithelium tissues of each sheep were collected in 2 mL freezing tubes. Subsequently, samples were quickly immersed in liquid nitrogen, and finally transferred to −80 °C refrigerator for preservation.

The oxidative immunity indexes of rumen epithelial tissues were measured, and the results showed that the LA600 group exhibited greater antioxidant activity compared to the control group [16]. Along with previous results, the gene expression and metabolites of the rumen epithelial tissue in CTL and LA (LA600) groups were explored by transcriptome sequencing and metabolomic analysis. The sheep in the CTL group and the LA group were deemed as the control group and the treatment group, respectively, with the aim of exploring the antioxidant mechanism of the rumen epithelial tissue through correlation analysis of histological techniques and antioxidant indexes.

2.2. Transcriptome Sequencing of Rumen Epithelial Tissue

Total RNA of the rumen epithelial tissue was extracted using the Trizol reagent method (DP762-T1C), and purified using the RNeasy micro kit (QIAGEN, shanghai, China). RNA

concentration and integrity were detected using the Qubit (Qubit RNA HS, Thermo Fisher Scientific, Waltham, MA, USA). The results of Tophat2 (v 2.1.0) comparison were assembled by Cufflinks (v 2.1.1) and compared with known gene sequences using Cuffcompare. After the results were compared using tools such as Picard-tools (v 1.96) or samtools (v 0.1.18) for sorting, de-PCRing duplicates, etc., SNP and InDel detection were performed using GATK, respectively. Gene expression levels of each sample were analyzed using HTSeq (v 0.5.4p3) software, and genes with a log fold change > 1.5 were selected for differential expression analysis using DESeq (1.10.1). The criterion for differential gene screening was $p < 0.05$. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment was used to analyze the relevant metabolic pathways in which the differential metabolites were involved.

2.3. Gene Expression Quantified by RT-PCR

In verifying the stability of transcriptome results, RT-PCR was used to quantify the significantly up- and downregulated genes (Table 1), while RNA was used on the same samples with transcriptome sequencing. The primers of *SLC15A1*, *LPL*, *MGAT4C*, *FRZB*, *LIPH*, *CA14*, *HSD17B3*, *GRIA1*, and *DDO* were synthesized by Nanjing Prime Biotechnology Co., Ltd. (Nanjing, China), and the details of the primers for each target gene are shown in Table 1. The upstream primers were labeled with -S or -F, and the downstream primers were labeled with -A or -R.

Table 1. Primers of genes selected for RT-PCR.

Genes	Primer Sequence (5′-3′)	Amplicon Size (bp)	Annealing Temperature (°C)
SLC15A1	S: ATCGTGTCGCTGTCCATCGT A: ATCTGTTCCTTTGCTTTTCCTGG	235	60
LPL	S: GGCTACGAGATCAACAAGGTCA A: GGAAACTTCAGGCAGGGTAAAA	213	60
MGAT4C	S: GATTGGCTATTGACTCATTTCCG A: GACATCTCCTGTTGAAGGTGGTTT	279	60
FRZB	S: GTGAAAGAAATAAAGACCAAGTGCC A: GGTGTAAAGGTTCACAGTCTCCC	111	60
LIPH	S: GTGGCAACAGTCTTGAAGGAATT A: GGTCCAGACCTGTAATTCTCCCC	157	60
CA14	S: TTACAACGGCTCGCTTACCACT A: AAAGACCATCCGCTGATTGAGA	193	60
HSD17B3	S: AAGGGAATCATCATCCAGGTGT A: AACTCATCAGCAGTCTTGGTTATCA	98	60
GRIA1	S: GCTCAAGAAGAATGCCAACCAG A: CCATTCCAAGCCTTTGTGTCAG	165	60
DDO	S: AGATGCTGGTGTTATTCTGGTGTC A: AGGTCTTCTATTCGTCGGGTGAG	252	60

Note: SLC15A1 = solute carrier family 15 member 1; LPL = lipoprotein lipase; MGAT4C = mannosyl (alpha-1,3-)-glycoproteinbeta-1,4-N-acetylglucosaminyltransferase, isozyme C (Putative); FRZB = frizzled related protein; LIPH = lipase H; CA14 = carbonic anhydrase 14; HSD17B3 = hydroxysteroid 17-beta dehydrogenase 3; GRIA1 = glutamate ionotropic receptor AMPA type subunit 1; DDO = D-aspartate oxidase.

2.4. Metabolite Identification and Analysis of Rumen Epithelial Tissue

Taking 50 mg of rumen epithelial tissue samples in a 1.5 mL centrifuge tube, we added mL μL of extract containing internal standard (methanol: acetonitrile: water volume ratio = 2:2:1, internal standard concentration 20 mg/L), then vortex mixed them for 30 s. Adding the steel beads, we ground the samples with a homogenizer at 45 Hz for 10 min, left them at −20 °C for 1 h, and then centrifuged them for 15 min in a 4 °C centrifuge at 12,000 rpm. We then added 500 μL of supernatant to a 1.5 mL centrifuge tube, and the extract was dried in a vacuum concentrator. Adding 160 μL of the extract (acetonitrile: water, v/v = 1:1) into the centrifuge tube, we vortexed the mixture for 30 s, sonicated it for 10 min in an ice-water bath, and centrifuged it at 12,000 rpm for 15 min in a 4 °C centrifuge, and then took 120 μL of the supernatant for the assay.

The metabolite analysis was performed by ultra-performance liquid chromatography (Waters Company, Milford, MA, USA) coupled with an G2-XS QTOF high-resolution mass spectrometer.

2.5. Statistical Analysis

One-way analysis of variance (ANOVA) in SPSS software (version 27, SPSS Inc., Chicago, IL, USA) was applied to compare the differences in the indicators of DEGs (differentially expressed genes) between the two groups. A result of $p < 0.05$ was judged to be statistically significant. The weighted gene co-expression network analysis (WGCNA) algorithm (Langfelder and Horvath, 2008) was employed through the R package BiocManager to identify modules of genes and metabolites related to immunity and antioxidants. Gene networks were constructed using Cytoscape (version 3.7.1, Bethesda, MD, USA) for visualization and further analysis of the relationships between genes. Hub genes in the network, which represent highly connected genes, were identified utilizing the MCODE plugin in Cytoscape (version 3.0, http://apps.cytoscape.org/apps/mcode, accessed on 24 October 2024) with specific programming parameters set (degree cutoff: 2; K-core: 2; maximum depth: 100).

The Metabo-Analyst 6.0 platform (https://www.metaboanalyst.ca/, accessed on 11 November 2024) was used to identify enriched biological pathways and functions associated with the metabolites.

3. Results

3.1. Effect of α-LA on Rumen Epithelial Tissue Genes

The expression of 29,448 genes was detected in the rumen epithelial tissue of each sheep. In comparison between the LA group and the control CTL group, 133 differentially expressed genes (DEGs) were observed ($p < 0.05$, 63 upregulated and 70 downregulated, Figure 1A). To investigate the comprehensive gene expression affected by factors including rumen epithelial rumen epithelial antioxidant activity and α-LA effects, WGCNA was conducted to identify gene modules. Consequently, 63 coexpressed gene modules were identified (named in color, MEmediumpurple2-MEnavajowhite4, Figure 1B,C). The MEsalmon1 module was selected for further analysis because it had the strongest correlation with SOD. The results of antioxidant indexes were shown in previous research [16].

The MEsalmon1 module comprises a total of 303 genes, and a total of 30 core genes were identified through the utilization of the MCODE plug-in within the Cytoscape software (version 3.10.1). There were 22 genes with functional annotations as described above. They were visualized for network analysis (Figure 2A), then they were subjected to KEGG enrichment analysis, which enriched a total of 21 pathways, and the six most significantly enriched pathways were selected for display (Figure 2C). The most enriched pathways were neomycin, kanamycin, and gentamicin biosynthesis, and the pentose phosphate pathway. The screened core genes were mainly active in the glucose metabolism pathway. We selected the significantly up- and downregulated genes for RT-PCR validation, and the validation results were consistent with the transcriptome (Figure 2B).

3.2. Effect of α-LA Addition on Metabolites of Rumen Epithelial Tissue

Metabolomic analysis was conducted to identify metabolites present in the rumen epithelium following α-LA supplementation. A total of 3818 metabolites were identified using liquid chromatography–mass spectrometry (LC-MS). A total of 135 differential metabolites were identified ($p < 0.05$); 76 metabolites that were upregulated and 59 metabolites that were downregulated. (Figure 3A). Principal component analysis (PCA) revealed notable disparities in metabolite composition between the CTL and LA groups (Figure 3B). In addition, WGCNA was performed to cluster metabolites and identify modules associated with antioxidant capacity, immune indices, and the α-LA. Consequently, 18 modules designated from MEblue to MEpurple were categorized (Figure 3C). There were 68 metabolites in the metabolite module of MEred, which were significantly negatively correlated with SOD.

As the core metabolites, LysoPA, 3-dehydroquinate, 9-methylthiononanaldoxime and 1-heptadecanoylglycerophosphoethanolamine were significantly enriched in 2-oxocarboxylic acid metabolism.
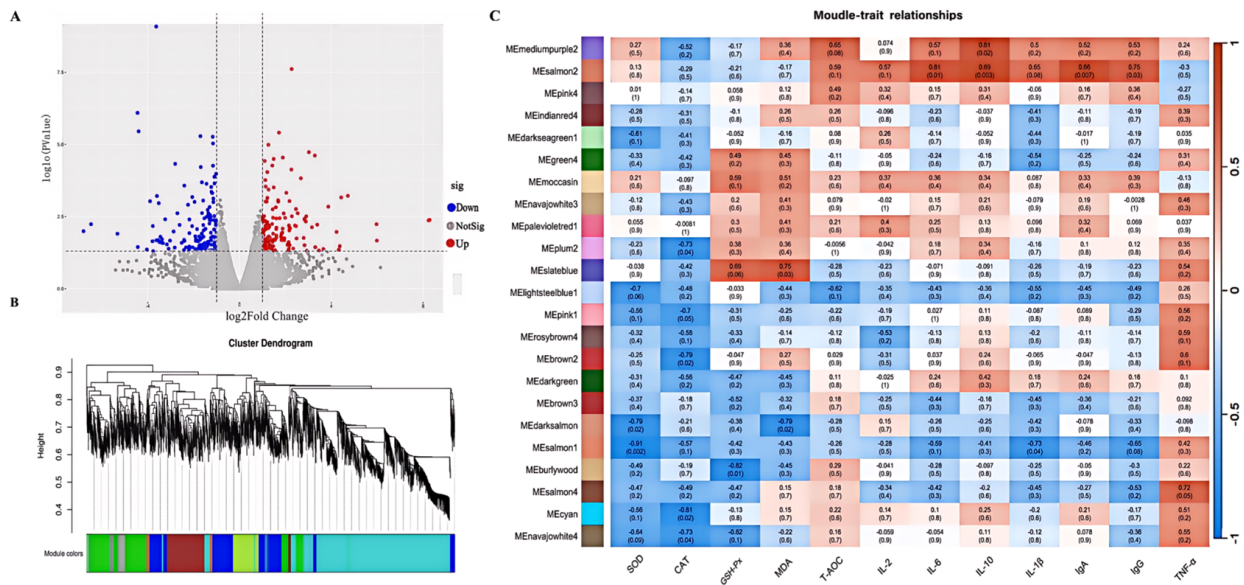

Figure 1. The gene coexpression network and its association with phenotypes of sheep. Volcano plots of transcriptomic differential genes: (**A**) volcano plots of the DEGs in the two comparison groups. The X axis indicates the difference multiple values after log2 conversion, and the Y axis indicates the significant value after log10 conversion. Red dots represent downregulated DEG, blue dots represent upregulated DEG, and gray dots represent non-DEG. (**B**) The dendrogram generated from the gene coexpression network of samples. (**C**) The relationship between antioxidant indexes and the 63 modules of genes calculated by WGCNA: in the presented data visualization, blue hues indicate negative correlations, while red hues signify positive correlations. The numerical values displayed within each square correspond to the Pearson correlation coefficient r and the associated *p*-values.
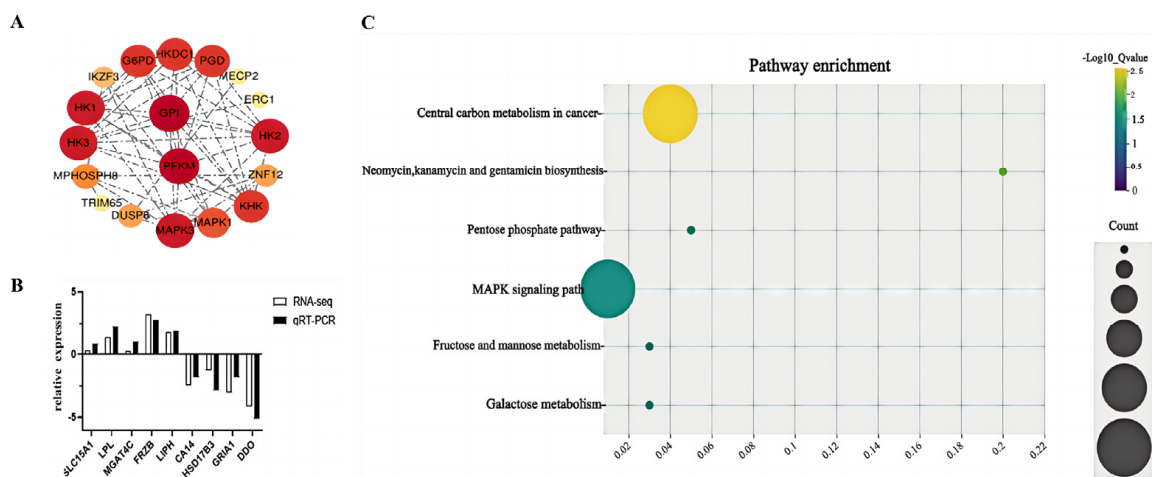

Figure 2. Network analysis and functional annotation of hub genes in the Mesalmon1 Module; validation of transcriptomic stability using PCR. (**A**) Network analysis of the hub genes. (**B**) The relative expressions of these 8 genes were quantified by RT-PCR. (**C**) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the hub genes. The circle shape represents the hub gene.
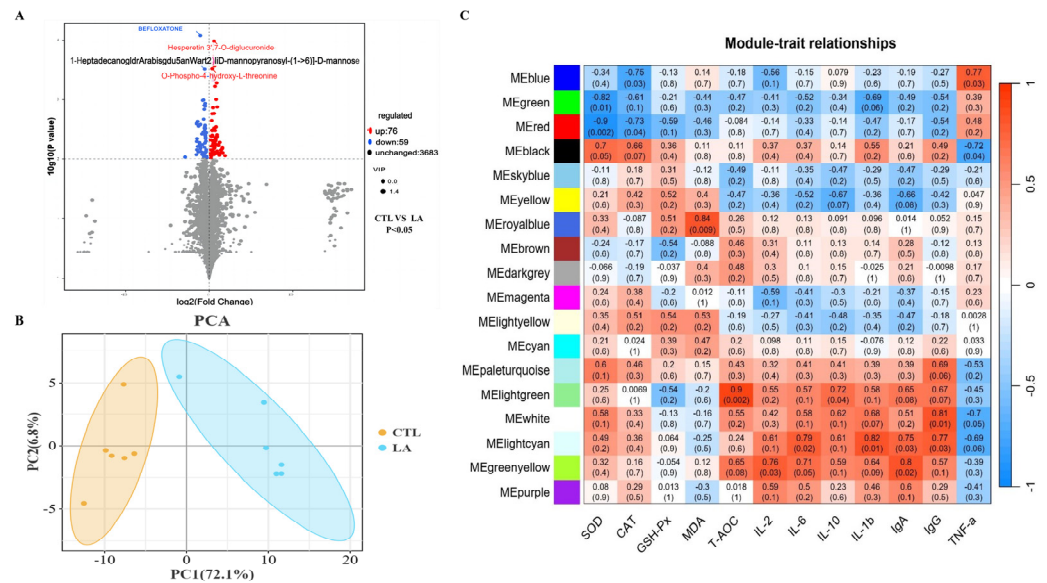
Figure 3. Screening of core metabolites related to α-LA addition. (**A**) Volcano plots of the differences between metabolites in the two comparison groups. The X axis indicates the different multiple values after log2 conversion, and the Y axis indicates the significant value after log10 conversion. Red dots represent differentially upregulated metabolites, blue dots represent differentially downregulated metabolites, and gray dots represent non-differentially expressed metabolites. (**B**) The principal component analysis (PCA) of metabolite composition in different groups. Each point represents a unique sample, and different colors represent different groups. (**C**) The relationship between antioxidant indexes and the 18 modules (MEblue– Mepurple) of metabolites calculated by WGCNA.

4. Discussion

α-LA as an additive plays an important role in ruminants. The addition of meso-alpha-LA to the diet improved DMI, energy digestibility, antioxidant capacity, and immunity in summer sheep studies [16,17]. It was found that supplementation of 32 mg/kg body weight of α-LA upregulated and maintained the expression of the heat shock protein 90 (HSP90) gene in heat-stressed male buffaloes, which acted as an antioxidant and ameliorated the deleterious effects of heat stress on male buffaloes [18]. In addition, research has demonstrated that the incorporation of α-LA into the diet can augment the antioxidant capacity, enhance growth performance, and improve the meat quality of Hainan black goats [19]. Most studies on α-LA in ruminants have focused on its antioxidant effects on the organism. However, the mechanisms underlying its action remain unexplored. This study firstly investigates the mechanism by which α-LA exerts its antioxidant effects on rumen epithelial cells using multi-omics technology. We found that α-LA enhances the antioxidant capacity of the rumen epithelium by regulating factors associated with the PPP, such as G6PD and NADPH.

The oxidative phase of the PPP provided precursors for the biosynthesis of nucleotides, lipids, and amino acids. The dehydrogenases of this pathway produce NADPH for anabolic purposes, but are also involved in several defense processes against oxidative stress [20]. G6PD is the first rate-limiting enzyme in the PPP, whose main function is to regenerate NADPH to maintain cellular redox homeostasis [21–24]. It has been found that skin cells rapidly produce NADPH through the oxidizing branch of PPP to support the regeneration of reduced glutathione when exposed to UV light or hydrogen peroxide, and then scavenging reactive oxygen species (ROS) and reducing cellular damage, and NADPH inhibits G6PD activity [25]. When PPP is activated, overexpression of *G6PD* inhibits NADPH production and, consequently, antioxidant capacity. *G6PD* expression was negatively correlated with NADPH production [26,27]. In the present study, enhancement of the PPP pathway further promoted NADPH synthesis; simultaneously *G6PD* was inhibited, which is consistent with these findings.

Hexokinase (HK) is a key enzyme that catalyzes the phosphorylation of glucose to generate glucose-6-phosphate (G6P) in cells, playing a key role in the uptake and utilization of glucose in cellular metabolism [28]. *HK* catalyzes the phosphorylation of glucose, initiating glucose metabolism and producing energy (ATP). In cardiomyocytes, overexpression of *HK2* can protect cardiomyocytes from oxidative damage by enhancing the flux of the pentose phosphate pathway (PPP) and reducing reactive oxygen species (ROS) [29]. Therefore, we speculate that the overexpression of *HK2* in this study may have enhanced the antioxidant performance of rumen epithelial cells by enhancing the flux of the PPP.

SOD is a ubiquitous and potent antioxidant enzyme that mainly scavenges ROS such as superoxide radicals, which cause oxidative damage to essential proteins and lipids of organisms and their DNA [30]. Our study found that α-LA increased SOD activity in rumen epithelial tissues and the expression of *G6PD* was inhibited as verified, which is consistent with the results of previous research [16,31]. Oxidant treatment leads to inactivation of *G6PD*, which inhibits anxiolysis and increases the pentose phosphate pathway flow to increase NADPH production and improve cellular antioxidant capacity [32]. α-Ketoglutarate is an intermediate in the 2-oxocarboxylic acid metabolic pathway. α-Ketoglutarate is able to increase the enzymatic activity of SOD, CAT, and GSH-Px [33]. Therefore, we infer that α-LA exerts its antioxidant effects by regulating the 2-oxocarboxylic acid metabolic pathway.

5. Conclusions

This study showed the expression of *G6PD* was suppressed and *HK2* was promoted, which further promoted glycolysis, increasing PPP flux. In addition, PPP in transcription and 2-oxocarboxylic acid metabolism were significantly enriched by α-LA. Therefore, the present study demonstrated that α-LA improved antioxidant activity by regulating PPP and 2-oxocarboxylic acid metabolism. This study will provide a theoretical basis for the application of α-LA in the raising of sheep.

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