Dietary supplementation of thiamine down-regulates the expression of mitophagy and endoplasmic reticulum stress-related genes in the rumen epithelium of goats during high-concentrate diet feeding

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ABSTRACT
Several studies have demonstrated that high-concentrate (HC) diet with thiamine supplementation can alleviate rumen epithelium inflammation and protecting the barrier function in goats. This study aimed to investigate the effects of dietary supply of thiamine on mitophagy and endoplasmic reticulum stress (ERS) during high-concentrate diet feeding. Twenty-four Boer goats (35.62 ± 2.4 kg) were used in this study, goats were subsequently assigned to 3 treatment groups (8 goats in each group) as follows: a low-concentrate diet (CON; concentrate: forage 30:70), a high-concentrate diet (HC; concentrate: forage 70:30) and high-concentrate diet with 200 mg thiamine/kg DMI (HCT; concentrate: forage 70:30) for 12 weeks. Compared with the HC group, the goats of HCT group had a markedly higher final weight, net weight gain, and average daily gain (ADG). The blood physiological and biochemical results showed that the HCT group expressed a lower the content of lymphocytes and leukocytes but a higher total protein and monocytes compared with that of HC group. The rumen epithelial mitochondrial membrane potential change (ΔΨm), relative mitochondrial DNA (mtDNA) content, adenosine triphosphate (ATP) level, together with the activities of the respiratory complexes I, III, and IV were markedly higher in the HCT group relative to HC ones. Relative to the HC group, the unc-51 like autophagy activating kinase 1 (ULK1), microtubule-associated protein 1 light chain 3 (LC3), autophagy-related 5 (ATG5), autophagy-related 7 (ATG7), Beclin1, PTEN induced putative kinase 1 (PINK1), PERK (PKR-Endoplasmic Reticulum Kinase), activating transcription factor 4 (ATF4), activating transcription factor 6 (ATF6), X-box binding protein 1 (XBP1), heat shock 70 kDa protein 5 (HSPA5), DNA damage-inducible transcript 3 (DDIT3) and DNA damage-inducible transcript 4 (DDIT4) mRNA levels were decreased, but the breast cancer 1 (BRCA1), ataxia telangiectasia mutated (ATM), heat shock factor binding protein 1 (HSPBP1) were increased in rumen epithelium of the HCT group. The results of transmission electron micrographs showed that the cell structure of the HCT group was higher integrity than that in the HC group, and the damage degree of mitochondria as well as endoplasmic reticulum being lower than the HC group. These results demonstrated that dietary thiamine could enhance rumen epithelial integrity by suppressing the responses of ERS and mitophagy during long-term HC diet feeding.

HIGHLIGHTS
• This study is the first to demonstrate that the reduction of inflammatory response is related to the down-regulation of autophagy and endoplasmic reticulum stress in goats.
• Thiamine has good anti-inflammatory and anti-oxidation properties, which can be applied in intensive industry to reduce the negative effects caused by long-term high-concentrate diet.

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Introduction
In the intensive industry, it is generally accepted that HC diets promote growth in most ruminants (Krause and Oetzel 2006; Giger-Reverdin et al. 2014). However, it has been reported that when HC diets were added during ruminants fattening, the rate of fermentation of non-structural carbohydrates exceeds ruminal efflux, in which beyond the ability of the buffers rumen fluid...
to maintain the pH under normal physiological conditions, resulting in subacute ruminal acidosis (SARA) (Nocek 1997; Danscher et al. 2015). SARA can markedly affect the metabolic function characterised by episodes of low pH in rumen below 5.6 for at least 3 h per day. The accumulation of ruminal free lipopolysaccharide (LPS) generated by lysis of gram-negative bacteria is associated with long-term feeding of HC diet (Plaizier et al. 2008; Khafipour et al. 2009). Prior data has revealed the negative effect of low pH (Gaebel et al. 1989), hyperosmolarity (Lodemann and Martens 2006), and LPS (Zhang, Li, et al. 2019) on ruminal epithelial barrier function. More importantly, the endotoxin can transfer into the systemic circulation and trigger inflammation, which increases the threats of liver abscesses, laminitis, and other metabolic diseases, which results in conspicuous economic losses in the ruminant field (Gozho et al. 2007; Plaizier et al. 2012).

Endoplasmic reticulum (ER), as a multi-functional organelle, plays an important role in the synthesis of functional proteins and the secretion of transmembrane proteins (Iurlaro and Muñoz-Pinedo 2016). Multiple stimuli such as endotoxin, calcium imbalance, oxidative stress and inflammatory cytokines lead to the accumulation of unfolded proteins that cause endoplasmic reticulum stress (ERS) (Yang et al. 2017). Under stress, the unfolded protein response (UPR) is activated. Numerous studies have demonstrated a fact that ERS is associated with multifarious physiological and pathological responses, including autophagy, inflammation, mitochondrial diseases (Cubillos-Ruíz et al. 2017; Lenzen 2017; Liu et al. 2017). Mitochondria are dynamic organelles that regulate their function, distribution, and structure in response to metabolic state of the cell. Optimal mitochondrial state provides not only the energy requirement (Mehta et al. 2017) but the fatty acid synthesis (Kastaniotis et al. 2017) and iron-sulfur protein biogenesis (Stehling et al. 2014). Recent studies have provided strong evidence that excessive mitophagy harms cellular homeostasis (Zhang, Peng, et al. 2020). It seems universally acknowledged that cellular homeostasis is frequently damaged by invading microbes and misfolded proteins. The cells have evolved a sophisticated mechanism that eliminates impaired organelles and cytoplasmic components (Gikas et al. 2018). Such as nuclear factor kappa B (NF-κB) facilitates the expression of p62 molecule and mediates the removal of injured mitochondria (Li et al. 2019). Previously, it has been shown that increased LPS has been regarded as one of the most important contributors to the progression of ERS (Nakayama et al. 2010; Mohamed et al. 2019) and mitophagy (Yun et al. 2019). The toll-like receptor (TLR) signalling as receptors of LPS leads to the synergistic induction of inflammatory cytokine and immune-related genes expression (Takeuchi et al. 2001). In addition, other studies have also observed that the mitophagy-related gene PARK2 expression which was led by LPS-induced oxidative stresses in the early stage must be related to inflammatory pathways (Yun et al. 2019).

The role of thiamine in animal physiology has received substantial attention (Pan et al. 2016). The benefits of the dietary supplementation of thiamine include a better rumen function in ruminants (Pan et al. 2018), enhancing antioxidant status (Tolstyk and Khmelevskii 1991) and decreasing apoptosis response (Chornyy et al. 2007) in rats, and relieving rumen inflammation in goats (Zhang, Meng, et al. 2019). Besides, a study has been reported that thiamine deficiency is relevant to oxidative stress, ERS, autophagy in mice (Liu et al. 2017). Thiamine deficiency triggers the expression of inflammatory cytokines via regulating NF-κB pathways (Jhala et al. 2014). Studies have demonstrated the fact that an increase of high concentrate diet can decrease the concentration of thiamine in the rumen and may cause thiamine deficiency (Schwab et al. 2006). However, the roles of thiamine in mitophagy and ERS functions in goats remain unclear during HC diet feeding. This study hypothesised that the thiamine supplementation with HC diet would exert protective effects and consequently it would alleviate the HC diet-induced ERS and mitophagy in rumen epithelium. Thus, the objective of this study was to investigate the influences of the dietary supply of thiamine on mitophagy and ERS during HC diet feeding. This work might provide a novel target for repairing ruminal epithelium injury and a new strategy for preventing long-term HC diet-induced ruminal epithelium injury for use in clinical and livestock production.

Materials and methods
Animals, diets and experimental design
Before the beginning of the experiment, twenty-four Boer goats (average weight = 35.62 ± 2.4 kg, body condition score = 3.15 ± 0.14, where 0 = emaciated and 5 = obese; Russel et al. 1969) were drenched against parasites via ivermectin (0.2 mg of ivermectin/kg), adapted to the experimental environment, and fed a basal experimental diet (concentrate: forage 30:70) for 2 weeks. After 2 weeks of prefeeding period, the goats in parity 1 or 2 were allotted to three diets for 12 weeks. The treatments included a
low-concentrate diet (CON; \( n = 8 \); concentrate: forage 30:70), a high-concentrate diet (HC; \( n = 8 \); concentrate: forage 70:30) and high-concentrate diet with 200 mg thiamine/kg DMI (HCT; \( n = 8 \); concentrate: forage 70:30). The animals were housed in individual tie stalls (1.5 \( \times \) 1.5 m) at the Lintang Experimental Station, Yangzhou City (Yangzhou, Jiangsu, China) and fed ad libitum with free access to fresh tap water throughout the entire experimental period. The diet composition (Supplementary Table S1) was formulated to meet or exceed the energy requirements of goats according to NRC (1985). The thiamine dose was clarified according to previous dose-response experiments in goats (Zhang, Ma, et al. 2020; Zhang et al. 2021; Ma, Wang, et al. 2021). Goats were fed at 7:00 AM and 6:00 PM, one-half of daily ration at each feeding. One-half of the proposed thiamine (thiamine hydrochloride, purity \( \geq 99\% \); Aladdin, Shanghai, China) dose was fully mixed with the HC diet for HCT goats at each feeding. Goats were euthanized with an intravenous injection of sodium pentobarbital (200 mg/kg BM) at the end of the experiment.

### Sample collection

Immediately after slaughter, the ruminal fluid sample (50 mL) was collected from the ruminal ventral sac and after measurement of the pH, the samples were filtered through 4 layers of cheesecloth, centrifuged at 10,000 \( \times g \) for 15 min at 4 \( ^\circ \)C and then stored at \(-20\) \( ^\circ \)C for subsequent analysis. Two blood samples were collected from jugular vein in 5 mL vacuum tubes coated with sodium heparin, one was preserved as whole blood for leucocytes assessment and the other was centrifuged at 3000 \( \times g \) at 4 \( ^\circ \)C for 15 min to collect plasma for biochemicals analysis. Blood physiological and biochemical analyses were performed using an automated haematological analyser (Sysmex K-1000D; Sysmex, Tokyo, Japan) and a Vitros 250 Chemistry System (Ortho Clinical Diagnostics, Markham, Canada), respectively. Representative rumen ventral tissue samples were collected within 5 min of slaughtering and then washed 3 times in precooled phosphate-buffered saline (PBS) (Lesmeister et al. 2004; Steele et al. 2011). Approximately 10 g of smaller pieces rumen epithelium tissues with RNALater\textsuperscript{TM} preservation buffer (Beyotime Institute of Biotechnology, Beijing, China) were put into freezing tube and transferred into liquid nitrogen for RNA analysis. For histological (ultrastructural) analysis, the intact rumen tissue (2 cm\(^2\)) was sampled from ventral rumen and fixed in 2\% glutaraldehyde (Sigma-Aldrich, Saint Louis, US).

### Ultrastructural evaluation of rumen epithelium

Based on a prior study (Graham and Simmons 2005), rumen epithelium samples were fixed with ice-cold 2\% glutaraldehyde in 5 mL for the ultrastructural analysis. In brief, samples were rinsed two times for 15 mins with Sorenson’s buffer (41.3 g of Na\(_2\)HPO\(_4\)-7H\(_2\)O, 6.41 g of NaH\(_2\)PO\(_4\), pH 7.2, distilled water to 1.0 L), then sequential dehydration was conducted via 25, 50, 75\% acetone (30 min steps), and 100\% acetone (2 changes of 1 h duration) before impregnation with epoxy resin via 25, 50, 75\% acetone-resin (1 h duration) steps to 100\% resin for 3 h before a curing step for 24 h at 60 \( ^\circ \)C. Subsequently, survey sections were cut, stained, and finally viewed using a transmission electron microscope (Tecnai G230, FEI NanoPorts, Hillsboro, US) with an accelerating voltage of 80 kV.

### Mitochondrial isolation from rumen epithelium

The mitochondria of rumen epithelium were extracted using a specific kit (Invent Biotechnologies. Inc. Beijing, China). All procedures were carried out at 4 \( ^\circ \)C according to the manufacturer’s protocol. Briefly, the rumen epithelium tissues were homogenised in MSH buffer (10 mmol/L HEPES, pH 7.5), containing 200 mmol/L mannitol, 70 mmol/L sucrose, 1.0 mmol/L egtazic acid and 2.0 mg/mL serum albumin. Then the homogenate was centrifuged at 1000 \( \times g \) for 10 min and then the supernatant was further centrifuged at 3500 \( \times g \) for 10 min to obtain the mitochondrial pellet (Pintana et al. 2014).

### Determination of the ROS generated by rumen epithelium

Isolated rumen epithelial mitochondria were disposed of with 2’,7’-dichlorohydro-fluorescein diacetate (DCFH-DA), which can cross the mitochondrial membrane and degraded via intracellular esterase. This non-fluorescent molecule accumulates intracellularly, and consequent oxidation yields the highly fluorescent product DCF. DCF may be determined by an enhancement in fluorescence at 528 nm. The isolated mitochondria were treated with 2 mmol/L DCFH-DA and incubated at 24 \( ^\circ \)C for 20 min. Subsequently, the fluorescence intensity was evaluated via a fluorescence microplate reader as previously described (Pipatpiboon et al. 2012).
**Mitochondrial membrane potential (ΔΨm)**

Changes in the ΔΨm values were determined using a mitochondrial membrane potential assay kit (Amyjet Scientific, Wuhan, China) with the cyanine dye JC-1 (5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolyl carbocyanine iodide) according to the manufacturer’s particular protocols. Briefly, isolated mitochondria were suspended in a 0.5 mL medium containing 5 mmol/L JC-1. The OD values of samples were measured by an automatic fluorescence microplate reader (FLx800, Bio-Tek, Vermont, US) at 590 nm and 530 nm, respectively. As the ΔΨm is proportional to the ratio of OD_{590} to OD_{530}, the ΔΨm was calculated as OD_{590}/OD_{530} (Li et al. 2011).

**Mitochondrial adenosine triphosphate (ATP) determination**

The ATP concentration in rumen epithelial tissues was determined by ATP determination kits (Beyotime Institute of Biotechnology, Beijing, China) following previous studies (Xing et al. 2016). All values were shown as fold changes relative to the CON diet.

**Mitochondrial DNA (mtDNA) content measurement in rumen epithelium**

With genomic DNA being used as loading control variable, the concentration of mtDNA was determined by amplifying the Mitochondria DNA loop and the nuclear-encoded β-actin genes using real-time PCR as described previously (Huang et al. 2017). Total DNA was separated using a QIAamp DNA Mini Kit (Vazyme Biotech Co, Nanjing, China), following the manufacturer’s protocols. Primer sequences of both genes are: for GAPDH: 5’-GGTCTCATCCTCTGCACT-3’ (forward), 5’-GGTCTATGTCCTCCAGA-3’ (reverse); for mitochondria DNA loop: 5’-ACGTCAATACCCGCGACT-3’ (forward), 5’-CTATCATGCGGGCGGAC-3’ (reverse). The relative copy number of mtDNA was calculated based on the ratio of mtDNA to nuclear DNA (Cao et al. 2018; Zhang, Peng, et al. 2020).

**Determination of the activities of respiratory chain complexes I–IV within the rumen epithelium mitochondria**

The mitochondria respiratory chain complexes activities quantitative determination kits (Suzhou Comin Biotechnology Co., Ltd., Suzhou, Jiangsu, China) were used to measure the enzyme activities of complexes I (NADH-CoQ reductase), II (succinate-CoQ reductase), III (CoQ-cytochrome c reductase), and IV (cytochrome c oxidase) according to the manufacturer’s instructions (Medja et al. 2009; Hargreaves et al. 2018). Briefly, the isolated mitochondria were subjected to lysis for 30 times employing 3s each time on ice by the Ultrasonic Processor (Branson, MO, USA). The samples were centrifuged at 12,000 × g for 15 min at 4°C, and then protein concentration was detected using the Bicinchoninic Acid protein assay kit (Vazyme Biotech Co, Nanjing, China). Ultimately, each estimated value was homogenised compared with the concentration of the total protein in each sample (Zhang, Li, et al. 2019).

**Total RNA extraction and real-time PCR**

Total RNA was isolated with Total RNA Isolation Kit V2 (Vazyme Biotech Co., Ltd). Then RNA integrity was evaluated using Nano Drop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.) and gel electrophoresis. Equal concentrations of RNA were reverse transcribed into cDNA using HiScript III-RT SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech Co., Ltd) according to the manufacturer’s protocol. The target primers were synthesised by Sangon Biotech (Shanghai, China), which shown in Table 1. The real-time PCR reactions were conducted using the ChamQTM SYBR® qPCR Master Mix Kit (Vazyme, Nanjing, China) with the 7500 Fast Real-Time PCR System (Applied Bio-systems, CA, USA) as previously described by Zhang, Ma, et al. (2020). All processes were measured in triplicate. The GAPDH was identified as a housekeeping gene according to screening method of Gao et al. (2013). The quantitative PCR results were calculated using the 2^–ΔΔCt method compared with housekeeping gene (Livak and Schmittgen 2001). All experiments were performed six times.

**Statistical analysis**

The results were expressed in the form of means and pooled standard error of means (SEMs) (Pan et al. 2017). Statistical data were carried out using SPSS 21.0 software (SPSS Inc., Chicago, IL). The sex effect was involved in the original statistical model, giving no significant result (p > .05). Thus, the sex effect was eliminated from the final model in which treatment was only the fixed effect. Differences between groups were determined with one-way ANOVA with Tukey’s post hoc test. Significance was set at p < .05.
Table 1. Primers for quantitative real-time PCR.

| Gene Name | Sequences (5′→3′)** | Product Size (bp) | Gene ID |
|-----------|---------------------|-------------------|---------|
| ATM       | F: GACCAATTCGAGTGATGCT R: AGCCAAAGACCCACCAAA | 164 | XM_018059835.1 |
| BRCa1     | F: TGGCCCTTACCCATACAC R: CACCTGGCTGATGTCCCTT | 265 | XM_018065165.1 |
| HSbp1     | F: GTGGTCAGACACTCCCTAACAA R: CGTCATGAGGTCGCTGATG | 130 | XM_018061771.1 |
| ULK1      | F: GAGCACTAAGCGCCCAAGAC R: ATGGTGCCGATGTCCTAG | 220 | XM_013970555.1 |
| LC3       | F: TGTCACACATGCAGGGATGTG R: GCTGTCAGATGTCCGCGATG | 131 | XM_013968932.1 |
| ATG5      | F: AGACACAATAGCAACGCTT R: CCAGGACGACTGCAAGAGA | 117 | XM_005684610.2 |
| ATG7      | F: TGAACTTCCAGCAGTCACCC R: TGATGGCCTGGTCTCTTCAG | 223 | XM_013973644.1 |
| Beclin1   | F: CGGACGCAAGAATGCTGAAAG R: GGTCGTCAGATGTCCGCGATG | 270 | XM_005693865.2 |
| PINK1     | F: CATCGGCCTACAGTGGCTTT R: GCCTTCGTTGTGCTGATC | 124 | XM_018055041.1 |
| PERK      | F: GCAGAGGACGAGGTTTCTT R: TAAGGTCGCACTGTGGTACG | 170 | NM_004836.7 |
| ATF4      | F: GTCTCTCTGGCAGACTGCTA R: TCATCACTGGCTGCGAAAG | 213 | XM_018048794.1 |
| ATF6      | F: TGGACCCGAGACCTGATGTTG R: TCAGTGAGCAGCAGTAGG | 192 | XM_018046547.1 |
| XBP1      | F: GCCGGCTAAPGTACTGCTG R: TGAGCCTGACTCGTTT | 216 | XM_018061044.1 |
| HSPA5     | F: ATCTAGCCGACCAGTACGG | 255 | NM_001287571.1 |
| Ddit3     | F: CCTTACCACCTCTCGACC R: TGGCCACTGTGTTTCGGT | 175 | NM_001287323.1 |
| Ddit4     | F: CTTTTGGGATGCTCGTCTGT R: GACACCCCTCAGGATGTC | 213 | XM_005699173.3 |

**ATM = ataxia telangiectasia mutated; BRCa1 = breast cancer 1; HSbp1 = heat shock factor binding protein 1; ULK1 = unc-51 like autophagy activating kinase 1; LC3 = microtubule associated protein 1 light chain 3; ATG5 = autophagy related 5; PINK1 = PTEN induced putative kinase 1; PERK = eukaryotic translation initiation factor 2 alpha kinase 3; ATF4 = activating transcription factor 4; XBP1 = X-box binding protein 1; HSPA5 = heat shock 70 kDa protein 5; Ddit3 = DNA damage-inducible transcript 3.**

Results

Growth performance

As Table 2 shown that the final weight, net weight gain, average daily dry matter intake (ADMI), and ADG of the HC group were lower (p < .05) than that in the CON group. In contrast, the HCT group demonstrated higher (p < .05) final weight, net weight gain, and ADG than in the HC group.

Rumen epithelial ultrastructure

Transmission electron micrographs of rumen epithelium cross-sections within the CON group showed integrity and normal nuclei, mitochondria, and intercellular junctions (Figure 1(A,B)). For the HC-fed goats, the cells were swollen and seriously damaged, which most of the desmosomes degenerate to form hemidesmosomes, in which the mitochondria were swollen and enlarged obviously, and the endoplasmic reticulum was severely expanded (Figure 1(C,D)). However, the HCT group showed high (p < .05) activities of complex I-IV, ∆Ψm, relative mtDNA content and ATP contents in the rumen epithelium.

Blood physiological and biochemical parameters

Compared with the CON group, the HC diets led a significant increase (p < .05) in lymphocytes and leukocytes but total protein, globulin and monocytes were reduced (p < .05) (Table 3). In contrast, the HCT group expressed a lower (p < .05) content of lymphocytes and leukocytes but a higher (p < .05) total protein and monocytes than the HC group. Furthermore, the content of globulin increased in the HCT group compared with the HC group, although the change was statistically insignificant.

Mitochondrial ROS production, ∆Ψm, mitochondrial DNA and ATP content, and mitochondrial complex activities in the rumen epithelium

The activities of complex I-IV, ∆Ψm, relative mtDNA content and ATP contents were decreased (p < .05) in the HC diet compared with that of the CON group but the mtROS production was increased (p < .05) (Table 4). However, the HCT group showed high (p < .05) activities of complex I-IV, ∆Ψm, relative mtDNA content and ATP contents and a low (p < .05) mtROS production compared with that of the HC group.
Relative to the CON groups, the ULK1, LC3, ATG5, ATG7, Beclin1, PINK1, PERK, ATF4, ATF6, XBP1, HSPA5, DDIT3 and DDIT4 mRNA levels were increased ($p < .05$) but the BRCA1, ATM, HSBP1 were decreased ($p < .05$) in rumen epithelium of the HC group (Table 5). Compared with the HC group, dietary thiamine supplementation reversed the expression levels for the above-mentioned genes ($p < .05$).

The expression levels of apoptotic, inflammation and oxidation-related genes of the HC group were higher ($p < .05$) than that in the CON group (Unpublished data, Supplementary Figure S1). In contrast, the HCT group showed lower ($p < .05$) expression levels for the above-mentioned genes than that in the HC group.

**Discussion**

Our previous study showed that dietary thiamine supplementation enhanced the rumen epithelial development and reduced inflammatory response in ruminants during the HC feeding (Zhang, Peng, et al. 2020; Pan et al. 2017). We assume that this effect might be associated with the changes in epithelial barrier function, mitochondrial function, and ERS. Consequently, the current study was performed to verify the as-proposed hypothesis.

Multiple studies uncovered from different aspects that a single mild episode of SARA does not affect ruminal development in the short term (Penner et al. 2010; Hook et al. 2011). Hence, to investigate the HC diet-induced metabolic disorders in ruminal epithelium comprehensively, we fed the goats HC diet (70% concentrate) which lasted for a long time (12 weeks). The
low rates of weight gain in the HC goats emphasised the negative effect of long-term HC diet feeding. The positive effect on feed intake (as proved by a high ADMI) indicated that dietary thiamine improved the animal growth with the HC diet, but the change was statistically insignificant. However, the enhancement of growth rate is independent of feed efficiency (feed conversion ratio, FCR) in the current study.

The ruminal epithelial barrier plays a significant role in the immune system of ruminants (Penner et al. 2011), promoting high-efficiency absorption of nutrients and restraining the invasion of microbes and toxins from the rumen into the blood circulation system. The rumen epithelium has a particularly strict separation mechanism between selective absorption via the transcellular route, whereas the paracellular pathway exhibits rather tightly sealed (Aschenbach et al. 2019). The short-chain fatty acid (SCFA) are almost completely transported into the rumen epithelium, resulting in a enormously risk of intracellular pH homeostasis (Gäbel et al. 2002; Aschenbach et al. 2011). The impairment of ruminal epithelial barrier function is frequently detected in HC-fed ruminants (Plaizier et al. 2012). The studies confirmed the view that not only pH, but parakeratosis from prolonged exposure to the HC diet in the rumen can result in epithelial inflammation (Kleen et al. 2003; Penner et al. 2010). Furthermore, it is believed that the low pH triggers a vast release of LPS from gram-negative bacteria (Emmanuel et al. 2007). Previous studies indicated that LPS-induced oxidative stress leads to mitophagy which is related to inflammatory pathways (Yun et al. 2019). Besides, many researchers have reported that LPS induces ERS in different cell types (Shi et al. 2017; Lim et al. 2018).

Closer examination of the ruminal epithelial strata of HC-fed goats using transmission electron microscopy demonstrated a clear degree of damage in cell layers. Initially, it seems to be an alteration of the capability of cells to synthetise lysosomes. It was followed by the degradation of some cellular organelles by hydrolytic enzymes of lysosomes. The cells in the HC group were swollen and damaged seriously, which most of the desmosomes degenerate to form hemidesmosomes, the mitochondria were swollen and enlarged obviously, and the endoplasmic reticulum was severely expanded. On the contrary, the structure of the cells in the HCT group was more integrated compared with the HC group, with minimum degree of damage to mitochondria and endoplasmic reticulum. This suggests that dietary thiamine supplementation protects the integrity of rumen epithelial cells.

Thiamine identifies to be a potent factor whose deficiency can induce disorders of carbohydrate metabolism (Martin et al. 2003), energy failure, excess release of free radicals (Todd and Butterworth 1999; Pannunzio et al. 2000), ERS (Wang et al. 2007). The anti-inflammatory mechanism of thiamine in rats (Shoeb and Ramana 2012) and humans (González-Ortiz et al. 2011) has been verified that exogenous thiamine decreased LPS concentration and inhibited NFkB activation by reducing the release of cytokines.

### Table 4. Effects of feeding high-concentrate diet with thiamine supplementation on mtROS production, mitochondrial complex activity, ATP content, relative mtDNA content and ΔΨm in rumen epithelium of goats.

| Item                          | CON  | HC   | HCT  | SEM  | p-value |
|-------------------------------|------|------|------|------|---------|
| mtROS Production, fold change | 1.00 | 5.63 | 2.49 | 0.24 | .008    |
| Complex I, nmol/min/mg protein| 326.87 | 237.64 | 281.02 | 14.24 | .016    |
| Complex II, nmol/min/mg protein| 358.21 | 292.06 | 321.47 | 8.32 | .027    |
| Complex III, nmol/min/mg protein| 636.47 | 513.65 | 618.79 | 12.48 | .005    |
| Complex IV, nmol/min/mg protein| 415.54 | 387.96 | 402.33 | 10.54 | .037    |
| ATP content, fold change      | 1.00 | 0.53 | 0.81 | 0.07 | .007    |
| Relative mtDNA content, %     | 100.00 | 61.29 | 91.48 | 6.25 | .018    |
| ΔΨm, fold change              | 1.00 | 0.56 | 0.79 | 0.08 | .009    |

**a,b,c** Means values within a row with different letters differ significantly (p < .05).

### Table 5. Effects of thiamine supplementation on the mRNA abundance of rumen epithelium in goats challenged with a high-concentrate diet.

| Item                          | CON  | HC   | HCT  | SEM  | p-value |
|-------------------------------|------|------|------|------|---------|
| DNA damage, repair            |      |      |      |      |         |
| BRCA1                         | 1.00 | 0.54 | 0.79 | 0.09 | .013    |
| ATM                           | 1.00 | 0.73 | 1.32 | 0.07 | .008    |
| HSBP1                         | 1.00 | 0.64 | 0.92 | 0.06 | .025    |
| Mitophagy                     |      |      |      |      |         |
| ULK1                          | 1.00 | 2.56 | 1.74 | 0.13 | .009    |
| LC3                           | 1.00 | 3.13 | 1.63 | 0.16 | .007    |
| ATG5                          | 1.00 | 2.26 | 1.33 | 0.07 | .008    |
| ATG7                          | 1.00 | 1.93 | 1.13 | 0.09 | .012    |
| Beclin1                       | 1.00 | 1.82 | 1.13 | 0.15 | .008    |
| PINK1                         | 1.00 | 2.53 | 1.58 | 0.15 | .008    |
| Endoplasmic reticulum stress  |      |      |      |      |         |
| PERK                          | 1.00 | 3.57 | 2.18 | 0.13 | .006    |
| ATF4                          | 1.00 | 1.79 | 1.15 | 0.06 | .026    |
| ATF6                          | 1.00 | 1.92 | 1.33 | 0.07 | .014    |
| XBP1                          | 1.00 | 2.08 | 1.46 | 0.11 | .009    |
| HSP5A                         | 1.00 | 1.89 | 1.79 | 0.14 | .024    |
| DDIT3                         | 1.00 | 1.38 | 1.17 | 0.12 | .036    |
| DDIT4                         | 1.00 | 3.31 | 2.04 | 0.17 | .008    |

**a,b** Means values within a row with different letters differ significantly (p < .05).

**CON** = low-concentrate diet; **HC** = high-concentrate diet; **HCT** = high-concentrate diet supplemented with 200 mg of thiamine/kg of DMI. n = 8 goats/treatment.
As a result, the decreased LPS concentrations of blood and rumen fluid in goats treated with thiamine are related to such effects during the HC feeding. Blood immune typing is a crucial method that can be used to diagnose immunological deficiencies (Cao et al. 2016). In the current study, the blood physiological and biochemical parameters were observed to define whether dietary thiamine supplementation alleviates the systemic inflammation in goats during the HC feeding. The results showed that the HCT group expressed a lower content of lymphocytes and leukocytes but a higher total protein and monocytes than the HC group. These data hints that thiamine supplementation plays a positive role in the down-regulation of the inflammatory response. Similar findings have been reported in goats (Zhang, Ma, et al. 2020) and cows (Pan et al. 2017) fed the high concentrated diet and rumen fluid in goats treated with thiamine are regulated down-regulated the expression level of ERS-related genes including PERK, ATF4, ATF6, XBP1, HSPA5, DDIT3, DDIT4 in the rumen epithelium of the HCT group. The present data suggest that thiamine, at least in part, exerts the anti-ERS effects and protects the ruminal epithelium from HC diet-induced damages. It has been revealed that ERS modulates the expressions of ER chaperons, ER-associated degradation (ERAD) genes such as XBP1, ATF4, HSPA5 (also known as BIP), DDIT3, DDIT4 etc. which induce apoptosis through activation of C/EBP homologous protein (CHOP) and caspases (Gessner et al. 2014). It is well-established that that LPS induces the expression of IRE1 in cells (Mohamed et al. 2019). The activity of IRE1 is involved in RNA degradation to keep lower protein synthesis, which activates the unconventional splicing of the XBP1 and leads to the expression of the genes related to the recovery of ER folding potential (Hollien and Weissman 2006). The CHOP, XBP1, and ATF4 are highly significant for cell viability, and XBP1 is a crucial monitor of the UPR because it can activate a mass of genes related to restoring ER folding capacity (Acosta-Alvear et al. 2007). The truth that the ERS also causes an expression of nuclear factor erythroid-2 related factor 2 (Nrf2), probably as a measure to the decrease of oxidative stress provoked under ERS conditions (Cullinan et al. 2003). The gene Nrf2 plays a significant role in regulating the activation of the defensive system, which leads to the protection of cells against the adverse impacts of oxidative stress and suppresses apoptosis (Manna et al. 2014). Interestingly, dietary thiamine supplementation up-regulated expression of the Nrf2 gene (Supplementary Figure S1), for which the reason may be the fact that thiamine relieved oxidative lesions in ruminal epithelium for the HC feeding. In addition, our results also showed that dietary thiamine supplementation was able to alleviate inflammatory damage, which is consistent with previous findings (Zhang, Peng, et al. 2020; Pan et al. 2017). The positive effect for down-regulated ERS may be related to relief of inflammation.

In the immunity response, an optimal mitochondrial state is a requirement for inflammation and pathogen resistance. Under normal physiological conditions, these ROS generated with mitochondria are regulated by a positive mechanism of endogenous antioxidants. Nevertheless, mitochondrial impairment, leading to the loss of $\Delta \Psi m$, can result in the release of ROS, which can cause damages to proteins and DNA (Harris et al. 2018). In this regard, this study surveyed the ROS contents in mitochondria and the exvalues compared pression of DNA damage, repair-related genes and uncovered that dietary thiamine supplementation controlled the elevated ROS contents induced by the HC diet. On the other side, the increased ROS concentration breaks the $\Delta \Psi m$ through the change of permeability (Wang et al. 2017). Dietary thiamine supplementation reduced the ROS contents, thus, we speculated that it might also affect the rumen epithelium $\Delta \Psi m$ level. Our data showed that the HCT group had higher $\Delta \Psi m$ with the HC group. The fact has been demonstrated that ROS injures the production of mitochondrial ATP (Cole-Ezea et al. 2012). On the current results, goats with the HC-treated experienced a decline in ATP contents but dietary thiamine treatment ascending mitochondrial ATP contents. Such discoveries certified that thiamine relieved oxidative stress and elevating the functions of mitochondria.
Previous studies showed that the damage of mitochondrial DNA may cause damage to the complexes of the electron transport chain (Kowaltowski et al. 2009). So far, information regarding mtDNA content in LPS-induced rumen epithelium injury of ruminants is unavailable. According to our data, dietary thiamine treatment of HC feeding goats restrained the decline in rumen epithelial mtDNA levels. A fact stressed by statistics reported that LPS treatment decreased ATP and mitochondrial complex I–IV activities (Liu et al. 2015). Besides, the increased mtDNA impairs complexities of the respiratory chain and subsequently reduces the generation of ATP. The present study findings uncovered that dietary thiamine supplementation could alleviate prolonged HC diet-induced rise in the rumen epithelial activities in goats. We assumed that thiamine supplementation during a long-term HC diet in goat may enhance rumen epithelial function by suppressing the excessive inflammatory response, superoxide over-production and rumen epithelial mitochondrial depolarisation. As a result, keep both complexes I-IV and mtDNA, thereby preserve rumen epithelium integrity and energy production.

As a self-protection mechanism of cells, mitophagy exerts a pivotal role in the degradation of the dysfunctional mitochondrion and the regulation of cellular homeostasis (Kim and Lee 2014; Hirotta et al. 2011). LPS-induced undue ROS exacerbates cellular environmental homeostasis, particularly by inducing the disabled metabolism of mitochondria, which subsequently caused the negative storage of defective mitochondria. Inversely, the surplus injured mitochondria generate superfluous mtROS and cause the loss of ΔΨm, these events could, in turn, induce mitophagy (Wang et al. 2019). According to our results, dietary thiamine supplementation down-regulated the expression of mitophagy related-genes including ULK1, LC3, ATG5, ATG7, Beclin1, PINK1. In line with preceding study, mitophagy can be activated at multiple stimulus conditions (Kim and Ho 2010). Mitophagy potentially exerts a positive role in the unbinding pathway in exciting situations. On the counter, it is detrimental when it is immoderately triggered, which therefore provokes apoptosis-mediated cellular death. Our findings suggested that dietary thiamine supplementation suppressed apoptosis of rumen epithelium during the HC feeding (Supplementary Figure S1). Parkin and PINK1 have defined as essential proteins of inter coordination for keep mitochondrial homeostasis. Parkin was recruited to damaged mitochondria in the optimal condition to mediate mitophagy (Mortiboys et al. 2008). Tran et al. (2011) indicated that LPS-induced NFκB binds to the parkin protein to suppress transcriptional activity, that inducing an exaggerated inflammatory response. Based on the above results, it speculated that thiamine subdued LPS-induced mitochondrial disorder. In short, thiamine could protect from the damaged mitochondria-induced cell apoptosis, and this positive role is related to decrease of inflammatory response.

Conclusions

These results demonstrated that dietary thiamine could enhance rumen epithelial integrity by suppressing the responses of ERS and mitophagy during long-term HC diet feeding. This also shows that thiamine exerts a positive role in the treatment of gastrointestinal tract diseases that involved high energy diet in humans and goats.

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Ethical approval

The authors confirm that they have adhered standards of the protection of animals and feed legislation used for scientific purposes. The trials procedures endorsed by the Ethics Committee of Yang Zhou University (SYXX2013-0057) were carried out according to the protocols for Experimental Animals of the Ministry of Science and Technology (2006, Beijing, China).

Disclosure statement

The authors declare that there is no conflict of interest associated with the paper. The authors alone are responsible for the content and writing of this article.

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Data availability statement

The datasets generated or analysed during the current study are available from the corresponding author by request. All data that support the findings in of this study are included in the article.

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