ABSTRACT

Amino acids are primarily absorbed in the ruminant small intestine, and the small intestine is a target organ prone to oxidative stress, causing intestinal dysfunction. Previous study suggested that L-Trp could benefit intestinal function and production performance. This study aimed to explore the effects of L-Trp on hydrogen peroxide (H₂O₂)-induced oxidative injury in bovine intestinal epithelial cells (BIEC) and the potential mechanism. The effects of L-Trp on cell apoptosis, antioxidative capacity, AA transporters, and the mammalian target of rapamycin (mTOR) signaling pathway were evaluated in BIEC treated with 0.8 mM L-Trp for 2 hours combined with or without H₂O₂ induction. In addition, to explore whether the effects of 0.8 mM L-Trp on oxidative stress were related to mTOR, an mTOR-specific inhibitor was used. The percentage of apoptosis was measured using flow cytometry. The relative gene abundance and protein expression in BIEC were determined using real-time PCR and Western blot assay, respectively. Results showed L-Trp at 0.4 and 0.8 mM enhanced the cell viability, and it was inhibited by L-Trp at 6.4 mM. L-Tryptophan at 0.4, 0.8, and 1.6 mM remarkably decreased the percentage of apoptosis and enhanced antioxidative capacity in H₂O₂-mediated BIEC. Moreover, L-Trp at 0.8 mM increased the relative gene abundance and protein expression of antioxidative enzymes and AA transporters, and the mTOR signaling pathway. The mTOR inhibitor lowered the protein expression of large neutral amino acid transporter 1, but the inhibition of mTOR did not alter the activities of catalase and superoxide dismutase or protein expression of alanine-serine-cysteine transporter 2 with or without H₂O₂ induction. L-Tryptophan increased catalase and superoxide dismutase activities in H₂O₂-mediated BIEC, although not with a present mTOR inhibitor. L-Tryptophan increased the protein expression of large neutral amino acid transporter 1 and alanine-serine-cysteine transporter 2 in H₂O₂-mediated BIEC with or without the presence of an mTOR inhibitor. The present work suggested that L-Trp supplementation could alleviate oxidative injury in BIEC by promoting antioxidative capacity and inhibiting apoptosis, and the mTOR signal played vital roles in the alleviation.

Key words: bovine intestinal epithelial cell, L-tryptophan, antioxidant enzyme, transporter of amino acid

INTRODUCTION

The small intestine is not only the main site for digestion and absorption of rumen undegradable starch and protein for ruminants, but also a defensive barrier (Suzuki, 2020). However, it is susceptible to damage caused by reactive oxygen species (ROS), inducing apoptosis and reducing antioxidant capacity, and further intestinal disorders (Sottero et al., 2018; Lan et al., 2021). Weaning is a potent stressor for dairy calves because of the extreme dietary shift, which likely leads to intestinal barrier dysfunction and damage (Wood et al., 2015; Li et al., 2018), further increasing morbidity and mortality. Elevated blood ROS production was found in weaning dairy calves (Bordignon et al., 2019), and when the balance between defensive potential (ability) (enzymatic system and non-enzymatic antioxidants) and ROS production is upset, oxidative stress occurs (Lykkesfeldt and Svendsen, 2007). Hence, protecting small intestine from oxidative stress induced by weaning is crucial to maintaining intestinal function. Identifying an ideal nutritional candidate, which has antioxidant capacity, is urgently needed.
Tryptophan, an essential aromatic α-amino acid, is a substrate for protein synthesis and a precursor of molecules crucial for whole-body homeostasis (Bender, 1983; Oxenkrug, 2007). Yeste et al. (2020) reported that supplementing 4.5 g/d of Trp via milk replacer might suggest some benefits in Trp uptake or implications in oxidative defenses in calves during weaning. Relatively little is known about the effects of L-Trp on oxidative stress and intestinal function in ruminants, although studies did suggest that L-Trp had remarkable benefits on intestinal barrier function in nonruminants (Shen et al., 2012a,b; Wang et al., 2015). Whether supplying L-Trp could help with scavenging ROS and protect the small intestine from oxidative injury in ruminants is unknown, as is the underlying mechanism.

Based on these considerations, we hypothesized that L-Trp could alleviate oxidative injury and maintain intestinal function. Because enterocytes are key components of the intestinal mucosa epithelium and major targets of ROS, bovine intestinal epithelial cells (BIEC) were used to explore the effect on intestinal function of oxidative stress in vitro. In addition, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) is an abundant and stable ROS and widely used to induce oxidative injury (Zhang et al., 2021; Mao et al., 2022). Therefore, to test the hypothesis, we investigated the effects of L-Trp on antioxidative capacity, cell apoptosis and expression of AA transporters in BIEC mediated by H\textsubscript{2}O\textsubscript{2}. We also explored the underlying mechanism by which L-Trp alleviated oxidative stress.

**MATERIALS AND METHODS**

**Cell Culture**

The primary BIEC were isolated and cultured completely as described previously (Zhan et al., 2017). The primary BIEC were then immortalized, and BIEC, a cell line, were used in this study (Zhan et al., 2020). The cells were seeded and cultured in DMEM/F12 complete medium (11330057, Gibco) supplemented with 10% fetal bovine serum (10099141, Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (10378016, Gibco), 4 mM glutamine (G8540, Sigma-Aldrich), 15 ng/mL epidermal growth factor (E4127, Sigma-Aldrich), 1 μg/mL hydrocortisone (H0888, Sigma-Aldrich), and 1% NEAA [containing glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, and L-serine, 10 mM each (TMS-001, Sigma-Aldrich)] and seeded on 10-cm\textsuperscript{2} plastic dishes (351092, Corning). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO\textsubscript{2}, and the medium was exchanged every 24 h. When the confluence was between 85% and 95%, cells were passaged routinely. The BIEC purified in 20–25 passages were used for experimental assays. In this study, every experiment was repeated three times, and within each date, three wells were performed in each treatment.

**Establishing an Oxidative Stress Model of BIEC Induced by H\textsubscript{2}O\textsubscript{2}**

The damage caused by H\textsubscript{2}O\textsubscript{2} is considered a simple and reproducible model for inducing oxidative stress. The BIEC were treated with H\textsubscript{2}O\textsubscript{2} (applied at the Pharmaceutical Distribution Center of Zhejiang Agricultural and Forestry University) at concentrations of 0, 0.5, 1.0, 1.5, and 2.0 mM for 2 and 4 h, respectively. The H\textsubscript{2}O\textsubscript{2} dose was selected according to previous studies on bovine intestinal and mammary epithelial cells (Sun et al., 2021; Mao et al., 2022) and our pilot study.

**Cell Viability**

The effect of H\textsubscript{2}O\textsubscript{2} on cell viability was determined using cell counting kit-8 (CCK-8) assay (C0042, Beyotime Biotechnology) in accordance with the manufacturer’s protocol. In brief, BIEC were plated within 96-well culture plates (Corning) at the density of 10\textsuperscript{4} cells/well and cultured for 24 h before treatment. After culturing for 2 and 4 h, respectively, the CCK-8 solution was added and incubated at 37°C for another 2 h. The absorbance was tested with a microplate reader at 450 nm. All values were expressed as a proportion of the control.

**Determination of Lactate Dehydrogenase**

After stimulating by H\textsubscript{2}O\textsubscript{2} at different concentrations for 2 and 4 h, the lactate dehydrogenase (LDH), an important indicator of cell membrane integrity, was determined using a commercial LDH assay kit (A020–2-2, Jiancheng Bioengineering Institute). The cell supernatant was gathered and added with solutions sequentially, and then incubated according to the instructions. After standing at room temperature for 5 min, the samples were tested at 450 nm using a microplate reader.

**Antioxidative Status**

After incubation, the BIEC were rinsed with ice-cold PBS and lysed with RIPA Lysis Buffer R2220. Cells were centrifuged at 1,500 × g and 4°C for 15 min to remove cellular debris, and the cell supernatant was collected. Bicinchoninic acid protein assay reagent was used to determine cellular protein concentration (A045–4-2, Jiancheng Bioengineering Institute). The
samples were added with solutions, incubated at 37°C for 30 min and tested at 450 nm. In addition, the intracellular total antioxidant capacity (T-AOC), the activities of superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) were determined as detailed in our previous study (Wei et al., 2019). All data presented were normalized to protein concentration.

**Flow Cytometry**

To measure the percentage of apoptosis of BIEC, the flow cytometry (FACS Calibur, Becton, Dickinson and Company) and Annexin V-FITC apoptosis assay kits (K101–100, Univ Biotech Company) were used. The BIEC were washed with cold PBS then fixed in binding buffer mixed with 5 μL of FITC Annexin V and 5 μL of PI. After being incubated in the dark at room temperature for 15 min, the cells were added with 400 μL of binding buffer then analyzed within 30 min. The cells located at right 2 quadrants of all plots were defined as the apoptotic cells, and the percentage of apoptotic cells were analyzed using the FlowJo-V10 software (BD).

**Effect of L-Trp on BIEC Challenged by H2O2**

The L-Trp (purity ≥98.5%, A601911, Sango Biotech) was dissolved with medium. Doses and treatment time were selected based on following considerations: First, previous studies have shown that serum Trp concentration in dairy calves ranged from 0.04 to 0.09 mM (Yeste et al., 2018). Due to the lack of experiment with Trp supplementation in calves, it is unknown how much serum Trp concentration in calves could be increased by Trp supplementation. Second, in our pilot study (details shown in supplementary experiment), L-Trp concentrations lower than 6.4 mM did not affect cell viability after 2 h of incubation (Supplemental Figure S1, https://doi.org/10.6084/m9.figshare.21202088.v1). In addition, after 12 h of incubation, L-Trp at 0.05, 0.1, and 0.2 mM did not alter cell viability or LDH, and L-Trp at 0.4 and 0.8 mM increased cell viability with no effect on LDH (Supplemental Figures S2A and S2B, https://doi.org/10.6084/m9.figshare.21202088.v1). Thus, the conditions of L-Trp at 0.4, 0.8, 1.6, and 3.2 mM for 2 h of incubation were chosen for use in the next experiment.

This experiment aimed to explore the effect of L-Trp on oxidative stress in BIEC and its potential mechanism. The BIEC were incubated with 1 mM H2O2 and several concentrations of L-Trp for 2 h. The treatments were H2O2, H2O2 + 0.4 mM L-Trp, H2O2 + 0.8 mM L-Trp, H2O2 + 1.6 mM L-Trp, H2O2 + 3.2 mM L-Trp. The cell viability, percentage of apoptosis, LDH concentration of cell supernatant, and intracellular T-AOC were measured as described previously.

Based on the results, L-Trp at 0.8 mM was used for the next experiment. The treatments were CON, H2O2, L-Trp, and H2O2 + L-Trp. The relative mRNA expression and protein expression of antioxidant enzymes [CAT, manganese superoxide dismutase (SOD2), glutathione peroxidase 1 (GPX1)], transporters of AA [large neutral amino acid transporter 1 (LAT1), and alanine-serine-cysteine transporter2 (ASCT2)], and mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase 1 (S6K1)/eukaryotic initiation factor 4E-binding protein (4E-BP1) were measured.

**Inhibition of mTOR in BIEC**

To explore the association between mTOR and oxidative stress in BIEC with or without L-Trp supply, mTOR inhibition assays were carried out. The specific mTOR inhibitor rapamycin (Rapa, Selleck Chemicals) was dissolved in dimethylsulfoxide (Sigma-Aldrich) to produce a 10 mM stock solutions and stored at −80°C. Before applications in cell treatments, stock solutions were diluted to working concentrations. The treatments were control, Rapa, H2O2, H2O2 + Rapa, H2O2 + L-Trp, and H2O2 + Rapa + L-Trp. After 2 h treatments, the total RNA and proteins were separately collected for further analyses. Based on the study described previously, the dose of L-Trp used was 0.8 mM, and the Rapa used was 10 nM, which was selected based on a previous study (Guo et al., 2018) and a previous dose response experiment in our laboratory.

**RNA Extraction and Real-Time PCR Analysis**

Total RNA was extracted from BIEC using FavorPrep Blood/Cultured Cell Total RNA Mini Kit (Favorgen). The concentration and purity of RNA samples were determined by measuring the absorbance at 260 and 280 nm using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc.). RNA samples with an optical density ratio at 260/280 nm >1.8 were then reverse transcribed for cDNA synthesis using a Prime Script RT Reagent Kit (Takara). The quantitative PCR was performed by SYBR Premix Ex Taq II Kit (Takara) in the CFX96 (Bio-Rad). Table 1 shows primer sequences. The 20-μL reaction system (containing 6.4 μL of nuclease-free water, 0.8 μL of forward primer, 0.8 μL of reverse primer, 2 μL of cDNA, and 10 μL of SYBR Premix Ex Taq) were amplified at 95°C for 60 s, followed by 40 cycles at 95°C for 30 s and 58°C for 30 s.
for 60 s. The PCR reaction efficiencies were between 90 and 100%. The relative stability of two candidate genes (GAPDH and β-actin) was calculated by GeNorm software. We found that β-actin was expressed in comparable abundance and not affected by experimental factors (Vandesompele et al., 2002); thus, the β-actin was chosen as an internal normalization control. The relative mRNA abundances of genes were calculated using the 2−ΔΔCt method and normalized to β-actin. Three replicates were performed in each sample.

**Protein Preparation and Western Blot**

At the end of the incubation period, the BIEC were collected and lysed in ice-cold RIPA Lysis Buffer (P0013C, Beyotime Biotechnology). The protein concentration was determined by BCA protein assay kit (P0012S, Beyotime Biotechnology). Samples (10 μg/lane) were loaded and separated by 10% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes (IPVH00010, Millipore). The membranes were blocked in 5% BSA for 1 h at room temperature and then incubated with primary antibodies at 4°C, including SOD2 (1:1,000, catalog number ab68155, CST), CAT, GPX1, LAT1, ASCT2, P-mTOR, mTOR, P-S6K1, S6K1, P-4EBP1, 4EBP1 (1:3,000, catalog number A11780; 1:1,000, catalog number A1248; respectively, ABclonal) and β-actin (1:1,000, Hua’an Biotechnology). Then, membranes were incubated with the goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody (1:3000, catalog number D110058; Sangon Biotech) for 1 h at room temperature. The blots were visualized with enhanced chemiluminescence (Beyotime Biotechnology) immunoblotting detection system. The relative quantity of protein bands was determined by Image J (National Institutes of Health) and normalized to β-actin protein in each sample. Three replicates were performed in each sample.

**Statistical Analysis**

The distribution of normality and homogeneity of variances was evaluated before the analysis. Next, the data were analyzed by one-way ANOVA, and Duncan’s multiple comparison tests were used to compare group differences if a significant treatment effect was observed using SPSS 20.0 (SPSS Inc.). Data were expressed as mean ± the standard error of the mean. The significance level was \( P \leq 0.05 \).

**RESULTS**

**Effect of H2O2 on Oxidative Injury of BIEC**

Figure 1A shows the effects of H2O2 on cell viability, LDH concentration of supernatant and intracellular T-AOC. Cell viability was decreased in 1, 1.5, and 2 mM H2O2 treatment groups for 2 and 4 h incubation compared with the control group (\( P < 0.05 \), and the

| Table 1. The primers sequences used in this study (F = forward; R = reverse) |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| **Gene** | **Accession no.** | **Primer sequence (5′-3′)** | **Product length (bp)** | **Efficiency (%)** | **R²** |
| ACTG1 | NM_173979.3 | F: CGGGAATATCGTCCGTGAC | 277 | 96 | 0.996 |
| CAT | NM_001035386.1 | F: AAGACCTCCAAGGCGAAGGTG | 120 | 98 | 0.992 |
| SOD2 | NM_174076.3 | F: CGGCTTCTGAGGGCTCCACAC | 179 | 93 | 0.999 |
| LAT1 | NM_201527.2 | F: CGTGACCTTGGTTCCCTTGKC | 108 | 100 | 0.998 |
| ASCT2 | NM_174601.2 | F: TCATCCAGATCGGGAAGGT | 321 | 92 | 0.991 |
| MTOR | NM_001081514.1 | F: AGTGGTACGCTTGCCGA | 175 | 91 | 0.989 |
| 4EBP1 | NM_001077893.2 | F: AAGATCATCTCTGACCAGAA | 109 | 92 | 0.993 |
| S6K1 | NM_205816.1 | F: ATGCTCCTTCTGCTTGTG | 227 | 95 | 0.992 |

1 β-actin, actin gamma 1 (ACTG1); catalase (CAT); glutathione peroxidase (GPX1); superoxide dismutase 2 (SOD2); large neutral amino acid transporter 1 (LAT1); alanine-serine-cysteine transporter 2 (ASCT2); mammalian target of rapamycin (MTOR); eukaryotic initiation factor 4E-binding protein (4EBP1); ribosomal protein S6 kinase 1 (S6K1).
cell viability in 0.5 mM H$_2$O$_2$ treatment did not change either for 2 or 4 h. The LDH concentration increased in all treatment groups (0.5, 1, 1.5, and 2 mM H$_2$O$_2$) for both 2 and 4 h incubation compared with the control group (P < 0.05). Intracellular T-AOC decreased in 1, 1.5, and 2 mM H$_2$O$_2$ treatment groups for 2 h, and decreased in 0.5, 1, 1.5, and 2 mM H$_2$O$_2$ treatment groups for 4 h (P < 0.05). Figure 1B shows the results of enzyme activities of SOD, GPX, and CAT for 2 h incubation. The enzyme activities of SOD and GPX of BIEC decreased in 1, 1.5, and 2 mM H$_2$O$_2$ treatment groups compared with the control group (P < 0.05). The CAT activity decreased with the increase of H$_2$O$_2$ (P < 0.05). Figure 1C shows representative charts of flow cytometry analyses of apoptosis and the analyses results. At 0.5, 1, 1.5, and 2 mM, H$_2$O$_2$ increased the percentage of apoptosis of BIEC (P < 0.05). Based on these results, H$_2$O$_2$ at 1 mM for 2 h incubation was chosen as the condition to trigger the oxidative status in next experiments.

**L-Trp Regulated the Oxidative Stress of BIEC Mediated by H$_2$O$_2$**

**Cell Viability, LDH Concentration, T-AOC, and Percentage of Apoptosis.** Figure 2A shows the effect of L-Trp on cell viability, LDH concentration of supernatant, and intercellular T-AOC, and Figure 2B shows the percentage of apoptosis. Cell viability increased in all L-Trp groups, and the 0.8 mM L-Trp treatment group had the highest cell viability (P < 0.05). Compared with control group, the LDH concentration decreased in all L-Trp treatment groups, and the LDH in 0.4, 0.8, and 1.6 mM L-Trp groups was lower than that in 3.2 mM L-Trp group (P < 0.05). The T-AOC of BIEC was elevated in 0.4, 0.8, and 1.6 mM L-Trp groups (P < 0.05) as compared with control group, and the T-AOC in 3.2 mM L-Trp group was unaffected. Compared with control group, the percentage of apoptosis decreased in all L-Trp groups, and the rate in 0.8, 1.6, and 3.2 mM L-Trp groups was lower than that in 0.4 mM L-Trp group (P < 0.05). Based on these results and considering the effectiveness and efficiency of L-Trp, L-Trp at 0.8 mM was chosen for use in the next experiment.

**Antioxidant Enzymes.** Figures 3A, 3B, and 3C show relative mRNA abundance and protein expression of CAT, SOD2, and GPX1, respectively. The relative mRNA abundance and protein expression of CAT and SOD2 decreased in H$_2$O$_2$ group, and increased in the L-Trp group as compared with control (P < 0.05). The relative mRNA abundance of GPX1 in the L-Trp group was higher than in the control (P < 0.05), and the protein expression was unchanged. Moreover, the relative mRNA abundance and protein expression of CAT, GPX1, and SOD2 in the H$_2$O$_2$ + L-Trp group was higher than in the H$_2$O$_2$ group (P < 0.05).

**Transporters of AA.** Figures 3D and 3E show the relative mRNA abundance and protein expression of LAT1 and ASCT2, respectively. As compared with the control, the relative mRNA abundance and protein expression of LAT1 were lower in the H$_2$O$_2$ group, and the relative mRNA expression of LAT1 in the L-Trp group was higher (P < 0.05). Moreover, the relative mRNA abundance and protein expression of ASCT2 decreased in the H$_2$O$_2$ group as compared with control (P < 0.05). The H$_2$O$_2$ + L-Trp group had higher relative mRNA abundance and protein expression of ASCT2 than the H$_2$O$_2$ group (P < 0.05).

**mTOR-S6K1/4EBP1.** As compared with the control, the relative mRNA expression and the ratio of the phosphorylated to total mTOR, 4EBP1, and S6K1 were decreased in H$_2$O$_2$ treatment (P < 0.05), as Figures 4A, 4B, and 4C show, and the L-Trp treatment had higher relative mRNA expression of S6K1 (P < 0.05). In addition, the relative mRNA expression and the ratio of the phosphorylated to total mTOR, 4EBP1, and S6K1 increased in H$_2$O$_2$ + L-Trp treatment compared with H$_2$O$_2$ treatment (P < 0.05).

**Inhibition of mTOR Affected the L-Trp-Mediated mTOR Phosphorylation and Antioxidant Enzymes in BIEC**

The ratio of the phosphorylated to total mTOR was depressed with inhibitor treated (P < 0.05), as Figure 5A shows. Although L-Trp increased the ratio of the phosphorylated to total mTOR in H$_2$O$_2$ mediated BIEC (P < 0.05), L-Trp did not change that ratio in H$_2$O$_2$ mediated BIEC with an inhibitor. The mTOR inhibitor did not affect the enzyme activities of CAT or SOD (Figures 5B and 5C, respectively). CAT and SOD enzyme activities decreased with or without the inhibitor in H$_2$O$_2$ mediated BIEC (P < 0.05). In addition, L-Trp increased the enzyme activities of CAT and SOD in H$_2$O$_2$ mediated BIEC (P < 0.05), and L-Trp did not increase the enzyme activities of CAT and SOD with the presence of an inhibitor in H$_2$O$_2$ mediated BIEC.

Figures 5D and 5E show the effects of the mTOR inhibitor on relative protein expression of LAT1 and ASCT2, respectively. The mTOR inhibitor depressed the LAT1 expression as compared with the control (P < 0.05). The mTOR inhibitor depressed LAT1 expression...
Figure 1. Hydrogen peroxide (H$_2$O$_2$)-induced oxidative injury in bovine intestinal epithelial cells (BIEC). (A) The cell viability, lactate dehydrogenase (LDH) concentration of supernatant, and intracellular total antioxidant capacity (T-AOC) of BIEC. Cells were treated with different concentrations of H$_2$O$_2$ (0, 0.5, 1, 1.5, and 2 mM) for 2 and 4 h, respectively. The results of cell viability are shown as the proportion of control. The result of T-AOC was normalized to protein expression. gprot = grams of protein; mgprot = milligrams of protein. (B) The activities of superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) of BIEC. Cells were treated with different concentrations of H$_2$O$_2$ (0, 0.5, 1, 1.5, and 2 mM) for 2 h. The intracellular T-AOC, the activities of SOD, GPX, and CAT were normalized to protein concentration. (C) The percentage of apoptosis of BIEC. Cells were treated with different concentrations of H$_2$O$_2$ (0, 0.5, 1, 1.5, and 2 mM) for 2 h and determined by annexin V-fluorescein isothiocyanate/propidium iodide staining and flow cytometry. The cells located in the right 2 quadrants (Q2 and Q3) of all plots were defined as the apoptotic cells, and the percentage of apoptotic cells was analyzed using the FlowJo-V10 software (BD). The data are shown as the mean ± SEM. The values with different lowercase letters are significantly different (P ≤ 0.05).
in H₂O₂-mediated BIEC with L-Trp ($P < 0.05$), but not in H₂O₂-mediated BIEC. L-Trp increased LAT1 expression in the presence of the inhibitor in H₂O₂-mediated BIEC ($P < 0.05$). With or without H₂O₂ mediation, the mTOR inhibitor did not change ASCT2 expression, and L-Trp increased ASCT2 expression in H₂O₂-mediated BIEC with or without the present of an inhibitor ($P < 0.05$).

**DISCUSSION**

This study used immortalized BIEC to investigate the effects of L-Trp supplementation on antioxidative capacity, cell apoptosis, expression of AA transporters, and mTOR signal in BIEC mediated by H₂O₂. In addition, it explored the underlying mechanism by which L-Trp alleviated oxidative stress.
Figure 3. L-Tryptophan affected the expression of antioxidant enzymes and amino acid transporters in bovine intestinal epithelial cells (BIEC) mediated by hydrogen peroxide (H$_2$O$_2$). Cells were treated with L-Trp (0 and 0.8 mM) combined with or without H$_2$O$_2$ (1 mM) for 2 h. The relative mRNA abundance and protein expression of antioxidant enzymes and amino acid transporters were measured using real-time PCR and Western blot, respectively. (A, B, and C) The relative mRNA abundance and protein expression of catalase (CAT), manganese superoxide dismutase (SOD2), and glutathione peroxidase 1 (GPX1). (D and E) The relative mRNA abundance and protein expression of large neutral amino acid transporter 1 (LAT1) and alanine-serine-cysteine transporter 2 (ASCT2). The data are shown as mean ± SEM. Values with different lowercase letters are significantly different (P ≤ 0.05).
Usually, the generation and elimination of free radicals, mainly ROS, are in dynamic equilibrium. Oxidative stress is an imbalance between defensive potential (ability), both enzymatic system and nonenzymatic antioxidants, and the free radicals. H2O2 has been widely used to induce oxidative stress in bovine epithelial cells (Sun et al., 2021; Mao et al., 2022). Cell viability, concentration of LDH in supernatant, and percentage of apoptosis, together with intracellular T-AOC and SOD, GPX, and CAT activities, are key and common indicators to evaluate cells’ oxidative status. In this study, H2O2–induced oxidative stress caused decreased cell viability, increased the percentage of apoptosis, and depressed antioxidant capacity, as we expected.

Based on our observation, the effect of L-Trp on cell viability in BIEC was relevant to the concentration of L-Trp. Wang et al. (2015) found a similar trend, in which the cell viability of intestinal porcine epithelial cell line 1 was increased by L-Trp treatments (0.4 and 0.8 mM). Epithelial cells and lumen microbes break down dietary Trp into metabolites for use by the gastrointestinal tract (Dai et al., 2011; Grifka-Walk et al., 2021), although we did not measure the concentrations of L-Trp or metabolites in medium and cells. We presumed the effect on cell viability would be associated with the activation of mTOR, which proved to play important roles in cell proliferation, protein synthesis, and so on (Dowling et al., 2010). Our study also found that L-Trp elevated the mTOR.

In addition, L-Trp supplementation decreased the percentage of apoptosis, enhanced the antioxidant capacity, and elevated the expression of AA transporters in BIEC challenged by H2O2, indicating that L-Trp might alleviate oxidative injury by decreasing apoptosis and enhancing antioxidant capacity, and thus maintain the intestinal function. Previous studies have demonstrated that Trp could act as an effective antioxidant (Nayak and Buttar, 2016; Xu et al., 2018). The amino group in the molecular structure of Trp could be combined with H2O2 for deaminated oxidation, thereby hindering the occurrence of oxidation reactions to a certain extent and reducing the content of free radicals (Wu, 2009). Amino acid uptake is conducted by various transporters, and net uptake was affected by the number and affinity of transporters. The transporters can be regulated by hormones, physiological conditions, substrates, and so

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**Figure 4.** Effects of L-Trp on mammalian target of rapamycin (mTOR) signal pathway in bovine intestinal epithelial cells (BIEC) mediated by hydrogen peroxide (H2O2). Cells were treated with L-Trp (0 and 0.8 mM) combined with or without H2O2 (1 mM) for 2 h. The relative mRNA abundance and ratio of the phosphorylated (P) to total (T) mTOR signal pathway factors were measured using real-time PCR and Western blot, respectively. (A) The relative mRNA abundance of mTOR and the ratio of the phosphorylated to total mTOR. (B) The relative mRNA abundance of ribosomal protein S6 kinase 1 (S6K1) and the ratio of the phosphorylated to total S6K1. (C) The relative mRNA abundance of eukaryotic initiation factor 4E-binding protein (4EBP1) and the ratio of the phosphorylated to total 4EBP1. The data are shown as mean ± SEM. The values with different lowercase letters are significantly different ($P \leq 0.05$).
Figure 5. l-Tryptophan enhanced antioxidative capacity through mammalian target of rapamycin (mTOR) in bovine intestinal epithelial cells (BIEC) mediated by hydrogen peroxide (H₂O₂). Cells were incubated with rapamycin (Rapa), H₂O₂, and L-Trp as the following treatments for 2 h. The treatments were control, Rapa, H₂O₂, H₂O₂ + Rapa, L-Trp, L-Trp + H₂O₂, L-Trp + H₂O₂ + Rapa. The concentrations of L-Trp, H₂O₂, and Rapa used were 0.8, 1, and 10 nM, respectively. (A) The ratio of the phosphorylated (P) to total (T) mTOR was measured using Western blot. The results were normalized to protein expression. mgprot = milligrams of protein. (B and C) The activities of catalase (CAT) and superoxide dismutase (SOD). (D and E) The relative protein expression of large neutral amino acid transporter 1 (LAT1) and alanine-serine-cysteine transporter 2 (ASCT2). The activities of CAT and SOD were normalized to protein concentration. The data are shown as mean ± SEM. The values with different lowercase letters are significantly different ($P \leq 0.05$).
on. AA transporters were summarized and presented in Kandasamy et al. (2018).

In this study, we measured the transporters LAT1 and ASC2 in BIEC, which were elevated by L-Trp treatment. The increased expression of transporters might be partly a result of substrate stimulation. The results of mTOR inhibition suggested that the elevation of transporter LAT1 would be partly because of mTOR activation, hence the protein synthesis. We also measured mTOR signaling pathway to confirm our speculation. The discussion is presented below. In addition, the mTOR inhibition results indicated that transporter ASC2 elevation by L-Trp would be irrelevant to the mTOR. Nevertheless, the increased expression of AA transporters (LAT1 and ASC2) by L-Trp suggested that L-Trp could protect the BIEC from oxidative damage, thereby maintaining the function of AA transporters and further supporting the supplementation of L-Trp in BIEC.

The serine-threonine protein kinase mTOR is mainly affected by stress, energy status, and oxygen signals, thereby coordinating autophagy, growth, and macromolecule synthesis. The process outputs were S6K1 and 4EBP1, and so on (Ma and Blenis, 2009). Oxidative stress ordinarily inhibits mTOR, and this study also found decreases in the ratio of the phosphorylated to total 4EBP1 and S6K1, which was in accordance with Kimball et al. (2008) and Rohowetz et al. (2018). Persistent activation of mTOR could initiate protein synthesis and protect the mitochondria from oxidative stress in β cells, thus strengthening mitochondrial capacity and function (Wang et al., 2016). Under oxidative stress, the mTOR was elevated by L-Trp, but no difference was found under normal conditions, suggesting the elevation of mTOR might be a result of alleviated oxidative stress and substrate stimulation. Consistent with the mTOR activation, L-Trp also increased the ratio of the phosphorylated to total 4EBP1 under oxidative stress. In addition, L-Trp increased S6K1 with or without oxidative stress. The depressed mTOR signaling pathway because of oxidative stress was activated by L-Trp, further suggesting that the rate of protein synthesis in BIEC might be enhanced, and the rate of intracellular proteolysis might be reduced (Wang et al., 2015). Moreover, as mentioned in Shen et al. (2012a), the small intestinal epithelial cells have a high metabolic rate and a short half-life, so the regulatory effect of Trp on protein turnover in intestine is highly effective, further enhancing the expression of tight junction proteins and improving feed efficiency and growth performance (Shen et al., 2012a,b; Wang et al., 2015).

As we discussed previously, L-Trp contributed to the enzymatic antioxidants in alleviating oxidative stress in BIEC. The interesting finding in this study was that the alleviation by L-Trp might be relevant to mTOR activation. When the mTOR was inhibited by the specific inhibitor, the L-Trp could not activate the mTOR, and the enzymatic antioxidants were unchanged. The enzymatic antioxidants, such as SOD, GPX, and CAT, were mainly regulated by nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway (Kensler et al., 2007), and play a significant role in cellular defense systems against oxidative stress. This finding was in accordance with Jiang et al. (2016), in which Trp enhanced GSH and GPX levels, and the increased antioxidant capacity was shown to be associated with Nrf2/ARE and mTOR pathways. Inhibiting the mTOR in mouse embryonic fibroblasts, suppressed the phosphorylation of p62 and the expression of the Nrf2 targeted HO-1 (Ichimura et al., 2013). Some studies have presented that the activation of mTOR can lead to the activation of the Keap-Nrf2/ARE pathway in a p62-dependent manner (Bae et al., 2013). In addition, the downregulation of Nrf2 caused the ATP deficit, leading to the activation of AMPK and negatively regulating mTOR (Jia et al., 2016; Guriev et al., 2020). The Nrf2 can directly regulate the promoter of the mTOR gene where the ARE sequence was identified (Bendavit et al., 2016). Accordingly, we presumed L-Trp could activate the mTOR and Nrf2/ARE pathways in BIEC challenged by oxidative stress, which needed further validation.

The ROS was shown to activate protein kinase B (Akt), further leading to mTOR phosphorylation and autophagy suppression (Dai et al., 2017; Rohowetz et al., 2018), and the autophagy activation through Akt-mTOR signaling contributed to the anti-apoptotic effect to combat oxidative stress (Shi et al., 2020). As we mentioned previously, the percentage of apoptosis was decreased with L-Trp treatment under oxidative stress. These findings might suggest that the L-Trp could alleviate oxidative stress through autophagy activation. Further study is warranted to verify the inference.

CONCLUSIONS

The present study demonstrated that L-Trp alleviated oxidative injury in H2O2-mediated BIEC via promoting antioxidative capacity and depressing apoptosis, thus maintaining intestinal function. L-Tryptophan could also regulate the mTOR signaling pathway. Furthermore, this study provides insights into nutritional regulation mechanism of L-Trp in body metabolism.

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