JAST (Journal of Animal Science and Technology) TITLE PAGE

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ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Comparative effects of arginine, tryptophan, and threonine on cellular antioxidant responses and barrier function in porcine intestinal epithelial cells
Running Title (within 10 words)	Amino acids and barrier protection in porcine intestinal epithelial cells
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Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	Not applicable.
Acknowledgements	Not applicable.
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions	Conceptualization: Park JY, Lee SY, Kim JH

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Please specify the authors' role using this form.	Formal analysis: Park JY, Kim YB, Lee SY, Kim JH Methodology: Park JY, Kim YB, Park GY, Shin JW, Lee SY, Kim JH Validation: Park JY, Kim JH Investigation: Park JY, Kim YB, Park GY, Shin JW, Lee SY, Kim JH Writing - original draft: Park JY, Lee SY, Kim JH Writing - review & editing: Park JY, Kim YB, Park GY, Shin JW, Lee SY, Kim JH
Ethics approval and consent to participate	This article does not require IACUC approval because there are no human and animal participants.
Declaration of generative AI	No AI tools were used in this article.

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Abstract

The objective of this study was to investigate the comparative effects of arginine (Arg), tryptophan (Trp), and threonine (Thr) on cellular antioxidant responses and barrier function in porcine intestinal epithelial cells. IPEC-J2 cells were treated with 250 or 500 μM Arg, Trp, or Thr for 24 h and subsequently exposed to 1 mM hydrogen peroxide (H_2O_2) for 6 h. Cell viability, total antioxidant capacity (TAC), superoxide dismutase (SOD) activity, fluorescein isothiocyanate (FITC)-dextran permeability, transepithelial electrical resistance (TEER), tight junction-related gene expression, and ZO-1 localization were evaluated. Results indicated that H_2O_2 exposure reduced ($p < 0.05$) cell viability and SOD activity, increased ($p < 0.05$) paracellular permeability, decreased ($p < 0.05$) TEER, and downregulated ($p < 0.05$) *ZO-1* and *OCLN* expression. Both concentrations of Arg and Thr attenuated the reduction ($p < 0.05$) in cell viability, whereas Trp had no significant effect. Both concentrations of Arg and 500 μM Trp or Thr maintained SOD activity, whereas TAC was unaffected by amino acid treatment. All amino acid treatments reduced ($p < 0.05$) FITC-dextran permeability and increased ($p < 0.05$) TEER compared to the H_2O_2-treated control. The greatest TEER responses were observed with 500 μM Arg and Thr at 24 h and with 500 μM Arg at 48 h. *ZO-1* expression was increased ($p < 0.05$) by all amino acid treatments except 500 μM Arg, whereas *OCLN* expression was increased ($p < 0.05$) only by 500 μM Arg and Thr. Immunofluorescence observations also indicated improved junctional ZO-1 localization, particularly following treatment with 500 μM Arg or Thr. These findings indicate that Arg and Thr attenuated the reduction in cell viability, whereas all three amino acids improved epithelial barrier function, as reflected by reduced FITC-dextran permeability and increased TEER. Although no significant differences among amino acids were detected in permeability at the same concentration, treatment-dependent differences were observed in TEER.

Keywords: Arginine; Oxidative stress; Porcine intestinal epithelial cell; Threonine; Tryptophan

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INTRODUCTION

Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the capacity of cellular antioxidant defense systems, resulting in oxidative damage to lipids, proteins, and nucleic acids [1]. The intestinal epithelium is particularly susceptible to oxidative injury because it is continuously exposed to dietary, microbial, environmental, and inflammatory challenges [2,3]. Excessive oxidative stress can reduce intestinal epithelial cell viability, impair antioxidant defenses, and disrupt tight junction integrity, thereby increasing paracellular permeability and compromising epithelial barrier function [4-6]. In pigs, disruption of the intestinal epithelial

barrier can interfere with nutrient utilization and increases susceptibility to intestinal inflammation and other physiological disturbances [2,7]. Therefore, identifying nutritional strategies that enhance the antioxidant responses and barrier function of porcine intestinal epithelial cells is important for maintaining intestinal health under oxidative stress.

Among potential nutritional strategies, arginine (Arg), tryptophan (Trp), and threonine (Thr) are functional amino acids that contribute to intestinal homeostasis through roles extending beyond protein synthesis [8,9]. Among these, Arg is a conditionally essential amino acid involved in nitric oxide and polyamine metabolism, antioxidant defense, and epithelial repair [10]. Trp, in turn, is an essential aromatic amino acid whose metabolic pathways are associated with the regulation of cellular redox balance, immune responses, and epithelial barrier function [11]. Finally, Thr is an essential amino acid that is extensively utilized by intestinal tissues and contributes to the synthesis of mucins and other proteins required for epithelial maintenance [12]. Previous studies have shown that these amino acids can attenuate oxidative damage or improve intestinal barrier function under various experimental conditions [10,12,13,14]. However, most studies have evaluated them individually or under different experimental designs, and their direct comparative effects on oxidatively stressed porcine intestinal epithelial cells remain unclear.

We hypothesized that Arg, Trp, and Thr would differentially modulate cellular antioxidant responses and epithelial barrier function in hydrogen peroxide (H_2O_2)-induced IPEC-J2 cells, with their effects varying according to the amino acid and concentration. The objective of the present study was therefore to compare the effects of 250 and 500 μM Arg, Trp, and Thr on cell viability, antioxidant responses, and epithelial barrier function in H_2O_2-treated porcine intestinal epithelial cells.

MATERIALS AND METHODS

Cell cultivation

The IPEC-J2 cells (ACC 701, DSMZ, Braunschweig, Germany) originally derived from the jejunum of newborn piglets were used in this study. The cells were seeded on calf skin collagen-coated dishes (Sigma-Aldrich) and cultured at 37°C in a humidified incubator with 5% CO_2. The culture medium was Dulbecco's Modified Eagle Medium (DMEM; Welgene Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco). The medium was replaced every two to three days under identical incubation conditions.

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Establishment of oxidative stress conditions

To determine optimal oxidative stress conditions, cells were seeded into 96-well plates at a density of 5×10^4 cells/mL and exposed to H_2O_2 (Daejungchem) at final levels of 0.01, 0.1, or 1 mM for 6 h. After treatment, the medium was removed and the cells were washed with phosphate-buffered saline (PBS; Welgene Inc.). Cell viability was subsequently assessed using the EZ-Cytox assay kit (DoGenBio Co., Ltd.) according to the manufacturer's instructions. Briefly, 20 μL of reagent was added to each well, and the plates were incubated for 3 h. Absorbance was measured at 450 nm using a microplate reader (LTek).

Amino acid treatment

Cells were seeded in 96-well plates (5×10^4 cells/mL) and cultured until reaching confluency. Treatments with L-Arg, L-Trp, or L-Thr (≥ 98%, Sigma-Aldrich Inc.) were applied at concentrations of 0, 250, or 500 μM for 24 h (six replicates per treatment). Following amino acid treatment, the cells were incubated for 6 h with either fresh culture medium (positive control, PC) or 1 mM H_2O_2 (negative control, NC), along with the amino acid-treated groups. Cells were then harvested for subsequent analyses. The concentrations of 250 and 500 μM were as physiologically relevant cellular exposure concentrations and were consistent with concentrations used our previous studies in chicken intestinal epithelial cells [4-6, 15]. Because in vitro concentrations cannot be directly translated into dietary inclusion levels, corresponding practical dietary levels require validation in vivo.

Cell viability assay

Cells were seeded in 96-well plates (5×10^4 cells/mL) and cultured at 37°C with 5% CO_2 for 24 h. The cells were then treated with 0, 250, or 500 μM of each amino acid for 24 h. Afterward, the NC and amino acid-treated groups were exposed to 1 mM H_2O_2 for 6 h. Following incubation, 20 μL of the EZ-Cytox assay reagent was added to each well, and the cells were further incubated for 3 h according to the manufacturer's instructions. Cell viability was determined by measuring absorbance at 450 nm using a microplate reader (LTek).

Antioxidant responses

IPEC-J2 cells were seeded at a density of 5×10^4 cells/mL in 6-well plates with 6 replicates per treatment. The cells were pretreated with 250 or 500 μM of each amino acid for 24 h and then exposed to 1 mM H_2O_2 for 6 h, excluding the PC. After treatment, the cells were washed twice with ice-cold PBS. Cell lysis was performed using a lysis buffer containing 98% RIPA, 1% protease inhibitor, and 1% phosphatase inhibitor (GenDEPOT) for 15

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min at 4°C, following the method of Wang et al. [16] with minor modifications. Lysates were centrifuged at 15,000 × g for 10 min at 4°C, and supernatants were collected. Total antioxidant capacity (TAC) and superoxide dismutase (SOD) activity were determined using commercial kits (DoGenBio Co., Ltd.) according to the manufacturer's instructions. Antioxidant indices were normalized to the total protein concentration of each sample, determined with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Paracellular permeability assay

Paracellular permeability was assessed using 4 kDa fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich), as described by Park et al. [17]. IPEC-J2 cells were seeded at a density of 5×10^4 cells/mL onto the 0.4-μm pore apical chamber of Transwell inserts (SPL Life Sciences Co., Ltd.) and cultured for 2 d. The cells were then cultured for an additional 10 d to reach confluence and promote differentiation. On d 12, the cells were treated with 250 or 500 μM of Arg, Trp, or Thr for 24 h, followed by exposure to 1 mM H_2O_2 for 6 h. The PC group was neither pretreated with amino acids nor exposed to H_2O_2, whereas the NC group was exposed to H_2O_2 without amino acid pretreatment. After treatment, FITC-dextran was dissolved in DMEM at a final concentration of 2.2 mg/mL and added to the apical chamber, while DMEM alone was added to the basolateral chamber. The cells were incubated for 1 h. After incubation, 200 μL of basolateral medium was collected, and fluorescence was measured at 490/535 nm (excitation/emission) using a fluorescence microplate reader (LTek).

Transepithelial electrical resistance (TEER)

Cells were seeded at a density of 5×10^4 cells/mL onto 0.4-μm pore apical chambers (SPL Life Sciences Co., Ltd.) and cultured for 10 d. During this period, both apical and basolateral media were renewed every 2-3 d until the cells reached full confluence and differentiation was established. Cells were pretreated with amino acids for 24 h and then exposed to 1 mM H_2O_2 for 6 h. After the 6 h exposure, the H_2O_2-containing medium was removed and replaced with fresh DMEM, and TEER was measured at 0, 24, and 48 h thereafter. Thus, the 24 and 48 h values represent recovery after removal of H_2O_2 rather than continued exposure. Following treatment with amino acids and H_2O_2, the plates were placed on a hot plate maintained at 37°C. Prior to TEER measurement, the apical and basolateral media were replaced with DMEM, and TEER was measured at 0, 24, and 48 h using an EVOM3 Epithelial Volt/Ohm Meter (World Precision Instruments Inc), according to the manufacturer's protocol. The procedure was based on the methods of Du et al. [18] and Vergauwen et al. [7], with minor modifications. To prevent contamination between measurements, electrodes were rinsed with distilled water after each use. TEER

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was recorded at four equidistant positions (north, south, east, and west) around the insert, and the mean of these values was considered the TEER for each well. TEER values ($\Omega \cdot cm^2$) were calculated by multiplying the measured resistance with the surface area of the apical chamber (0.33 cm^2).

Tight junction-related gene expression

Total RNA was extracted from cells cultured in 6-well plates using TRIzol reagent (Invitrogen) according to the manufacturer's protocol with minor modifications. Cells were lysed directly in 1 mL of TRIzol, and the lysates were transferred to 2 mL microcentrifuge tubes. Subsequently, 200 μL of chloroform (Sigma-Aldrich) was added and vortexed thoroughly. Following centrifugation at 12,000 rpm for 15 min at 4°C, the aqueous phase was carefully collected, and mixed with 500 μL of isopropanol, and incubated on ice for 10 min. The RNA pellet was obtained by centrifugation at 12,000 rpm for 10 min at 4°C, washed with 1 mL of 75% ethanol, and centrifuged again at 8,000 rpm for 5 min at 4°C. After removal of the supernatant, the pellet was briefly air-dried and dissolved in 50 μL of nuclease-free water. To ensure complete solubilization, the RNA solution was heated at 60°C for 15-20 min in heating block (Labtron Equipment Ltd.). The concentration and purity (260/280 ratio) of the extracted RNA were quantified using a NanoDrop spectrophotometer (LTek).

To analyze the mRNA expression of tight junction-related genes, including *zonula occludens-1* (*ZO-1*) and *occludin* (*OCLN*), cDNA was synthesized from total RNA using a commercial cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Primers were designed using Primer-BLAST and synthesized by Xenotech. Primer sequences are listed in Table 1. Quantitative real-time PCR was performed using TOPreal™ SYBR Green Master Mix (Enzynomics Co., Ltd.) on a QuantStudio 1 Real-Time PCR System (Thermo Fisher Scientific). Each reaction was prepared in a 20 μL volume containing 1 μL of cDNA, 10 μL of SYBR Green Master Mix, 0.5 μL each of forward and reverse primers (10 μM), and nuclease-free water. Thermal cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, annealing at gene-specific temperatures for 30 sec, and extension at 72°C for 20 sec. Amplification specificity was confirmed by melting curve analysis. Relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method, with *GAPDH* as the reference gene.

Immunofluorescence staining

Cells were seeded into 96-well plates at a density of 5×10^4 cells/mL and cultured under standard conditions. As described above, cells were treated with Arg, Trp, or Thr for 24 h, followed by exposure to 1 mM H_2O_2 for 6 h.

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After treatment, the cells were washed twice with PBS and subsequently fixed with 4% paraformaldehyde at 37°C for 45 min. Following fixation, the cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 40 min and blocked with 2% bovine serum albumin (Roche Diagnostics**)** for 3 h at room temperature to prevent nonspecific binding. Subsequently, PBS washes were performed between each step to remove unbound reagents. The cells were then incubated overnight at 4°C with an anti-ZO-1 rabbit polyclonal antibody (1:100, BS-1329R, Bioss), followed by incubation with a FITC-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:2000, Invitrogen) for 30 min at room temperature in the dark. Nuclei were counterstained with DAPI (Invitrogen), and fluorescence images were captured using an EVOS M5000 fluorescence microscope (Thermo Fisher Scientific). Immunofluorescence images were acquired and evaluated by an observer blinded to the treatment groups.

Statistical analysis

Statistical analyses were performed using the PROC MIXED procedure of SAS (SAS Institute Inc.), with treatment as a fixed effect and each biological replicate as the experimental unit. For each assay, cells of six different passages were seeded, and each treatment was represented once within each of the six independent passages, resulting in six biological replicates per treatment (n = 6). For the one-way analyses, no random effects were included in the model, and variation among independent passages was retained within the residual error of the model. The eight experimental groups were analyzed as distinct treatment levels, and amino acid type and concentration were not analyzed as factorial main effects or interaction. For TEER, treatment effects at each time point (0, 24, and 48 h) were analyzed using the one-way model described above. In addition, for each amino acid (Arg, Trp, and Thr), the data were analyzed using a repeated-measures model in the MIXED procedure of SAS, with treatment level (NC, 250 μM, and 500 μM), time (0, 24, and 48 h), and the treatment × time interaction included as fixed effects. The repeated measurements obtained from the same Transwell insert at 0, 24, and 48 h were modeled using an unstructured covariance structure, with insert nested within treatment specified as the subject, and the Kenward-Roger method was used to approximate the denominator degrees of freedom. Outliers were assessed using the UNIVARIATE procedure together with model-based diagnostics from the GLM procedure of SAS. Studentized residuals were obtained from the fitted model, and observations with an absolute studentized residual greater than 3 were considered potential outliers [19]. On this basis, a total of 7 observations across all assays were identified as outliers and excluded, and the analyses were performed on the remaining data. The LSMEANS statement was used to calculate treatment means, and the PDIFF option was applied for pairwise comparisons only when the overall F-test for the treatment effect was significant, corresponding to Fisher's

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protected least significant difference approach. No additional multiple-comparison adjustment was applied. Significance was established at $p < 0.05$.

RESULTS

Determination of optimal oxidative stress

The effects of H_2O_2 concentration (0, 0.01, 0.1, and 1 mM) on cell viability in IPEC-J2 are shown in Figure 1. Exposure to H_2O_2 for 6 h resulted in a significant decrease ($p < 0.05$) in cell viability. Cells treated with 0.01 and 0.1 mM H_2O_2 exhibited less ($p < 0.05$) cell viability than the 0 mM concentration, whereas exposure to 1 mM H_2O_2 significantly decreased ($p < 0.05$) cell viability compared to 0.01 and 0.1 mM concentrations. Therefore, 1 mM H_2O_2 was selected as the challenge concentration for subsequent experiments based on the reproducible reduction in cell viability.

Cell viability

The effects of Arg, Trp, and Thr on the viability of IPEC-J2 exposed to oxidative stress are shown in Figure 2. The NC group significantly decreased ($p < 0.05$) cell viability compared to the PC group. The amino acid groups with Arg and Thr showed significantly greater cell viability than the NC group ($p < 0.05$). Among the amino acid groups, both levels of Arg and Thr groups showed greater cell viability compared with the NC and Trp group ($p < 0.05$). Both levels of Trp group showed no significant difference in cell viability compared with the NC group in IPEC-J2.

Antioxidant responses

The effects of Arg, Trp, and Thr on antioxidant responses in IPEC-J2 exposed to oxidative stress are shown in Figure 3. No significant differences in TAC were observed among treatments. However, SOD activity was significantly decreased ($p < 0.05$) in the NC group compared to the PC group. Except for the 250 μM Trp and 250 μM Thr groups, all amino acid-treated groups exhibited significantly greater ($p < 0.05$) SOD activity than the NC group. Among amino acids, 500 μM Thr showed the greatest SOD activity ($p < 0.05$), while both levels of Arg also demonstrated significantly greater ($p < 0.05$) activity than the NC group. In addition, 500 μM Trp group significantly increased ($p < 0.05$) SOD activity compared to the NC group.

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Paracellular permeability

The effects of Arg, Trp, and Thr on paracellular permeability in IPEC-J2 are shown in Figure 4. The NC group exhibited significantly increased ($p < 0.05$) FITC-dextran compared to the PC group. However, all amino acid-treated groups showed significantly less FITC-dextran than the NC group ($p < 0.05$). No significant differences in permeability were observed among the amino acid treatments at the same concentration level.

Trans-epithelial electrical resistance

The effects of Arg, Trp, and Thr on TEER in IPEC-J2 are presented in Figure 5. At 0 h, no significant difference in TEER was observed among all groups. At 24 h, the NC group showed a significantly lower ($p < 0.05$) TEER value than the PC group. Similarly, all amino acid-treated groups exhibited greater ($p < 0.05$) TEER values compared with the NC group. Among these, cells treated with 500 μM Arg and Thr showed significantly greater ($p < 0.05$) TEER values than the NC and other amino acid-treated groups. At 48 h, the NC group showed a significantly less ($p < 0.05$) TEER value than the PC. All amino acid-treated groups exhibited significantly greater ($p < 0.05$) TEER values compared with the NC group. Consistent with the results observed at 24 h, the 500 μM Arg group showed the greatest ($p < 0.05$) TEER value among all treatments. For each amino acid, the interaction between treatment and time was significant ($p < 0.05$). The main effect of treatment was significant for Arg, Trp, and Thr ($p < 0.05$). The main effect of time was also significant for Arg and Thr ($p < 0.05$).

Tight junction-related gene expression

The NC group showed a significantly reduced ($p < 0.05$) *ZO-1* gene expression compared to the PC group (Figure 6). Except for cells treated with 500 μM Arg, all amino acid-treated groups exhibited significantly greater ($p < 0.05$) *ZO-1* gene expression than the NC group. Among the amino acid treatments, 500 μM Thr resulted in a significant increase ($p < 0.05$) in *ZO-1* expression. Similarly, *OCLN* gene expression was significantly less ($p < 0.05$) in the NC group than in the PC group. Treatment with 500 μM Arg and Thr significantly increased ($p < 0.05$) *OCLN* gene expression compared to the NC group.

Immunofluorescence

Representative immunofluorescence images showed visually different patterns of *ZO-1* localization among treatments (Figure 7). In the PC group, *ZO-1* staining appeared relatively continuous along the cell borders, whereas the NC group showed a more discontinuous staining pattern. Amino acid-treated cells generally appeared

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to exhibit more continuous *ZO-1* staining than the NC group. In particular, cells treated with 500 μM Arg and Thr showed a *ZO-1* staining pattern that appeared more similar to those of the PC group. In contrast, cells treated with Trp appeared to show less continuous and less clearly defined junctional staining than cells treated with Arg or Thr. Because fluorescence intensity and junctional continuity were not quantitatively analyzed, these observations should be interpreted qualitatively.

DISCUSSION

Oxidative stress is an important contributor to intestinal epithelial dysfunction in pigs [20]. Excessive production of ROS can overwhelm cellular antioxidant defenses, impair epithelial cell viability, and disrupt tight junction integrity, thereby increasing paracellular permeability [21]. Therefore, maintaining antioxidant responses and intestinal barrier function under oxidative stress represents an important nutritional target [22,23]. In the present study, IPEC-J2 cells were used as an in vitro model of the porcine intestinal epithelium. This non-transformed cell line, derived from the jejunum of an unsuckled piglet, forms polarized monolayers and retains several functional characteristics of intestinal epithelial cells; therefore, it is suitable for studies of intestinal barrier function [7,24]. Oxidative stress was induced using H_2O_2, a membrane-permeable oxidant commonly used to model ROS-mediated epithelial damage. Exposure to 0.01 and 0.1 mM H_2O_2 reduced cell viability, with a further decrease observed at 1 mM. Accordingly, treatment with 1 mM H_2O_2 for 6 h was selected to establish a robust oxidative challenge while retaining sufficient viable cells for subsequent analyses. Previous studies have shown that H_2O_2 exposure increases intracellular ROS production, disrupts the organization of ZO-1, decreases TEER, and increases paracellular permeability in IPEC-J2 monolayers [25,26]. Consistent with these findings, H_2O_2 treatment in the present study decreased cell viability and SOD activity, increased FITC-dextran permeability, and reduced TEER and tight junction-related gene expression, supporting the suitability of the model for evaluating the protective effects of amino acids. In this context, Arg, Trp, and Thr were selected because these amino acids have functional roles in the intestine beyond serving as substrates for protein synthesis [9]. Arg is involved in antioxidant defense, nitric oxide metabolism, and epithelial barrier regulation, whereas Trp contributes to immune and cellular stress responses through its metabolites [11,27]. Thr is extensively utilized by intestinal epithelial cells for mucin and mucosal protein synthesis and is therefore important for maintaining epithelial integrity [12]. Evaluating the three amino acids under an identical H_2O_2-induced oxidative challenge enabled a direct comparison of their effects on cell viability, antioxidant responses, and epithelial barrier function.

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Cell viability was evaluated as an integrated indicator of cellular damage and protection under oxidative stress [28]. In the current study, treatment with 250 or 500 μM Arg or Thr attenuated the H_2O_2-induced reduction in cell viability, resulting in values comparable to those of the untreated control group. However, no significant difference was observed between the two concentrations of either Arg or Thr within the tested range. The protective effect of Arg is consistent with previous studies reporting that Arg attenuates oxidative damage and preserves epithelial integrity in oxidatively stressed IPEC-J2 cells [29]. This response may be related to the involvement of Arg in nitric oxide and polyamine metabolism, antioxidant defense, and cellular repair; however, these pathways were not directly evaluated in the present study. Thr is extensively utilized by the intestine for the synthesis of mucins and other mucosal proteins and may therefore support epithelial cell maintenance and repair during oxidative stress [30,31]. Nevertheless, because mucin synthesis, cell proliferation, and apoptosis-related pathways were not measured, the mechanisms underlying the protective effect of Thr remain to be clarified. In contrast, cell viability did not differ significantly between the Trp-treated groups and the H_2O_2-treated control. This finding suggests that Trp was less effective than Arg and Thr in preserving overall cellular viability within the concentration range evaluated. Notably, Trp improved several measures of barrier function despite its lack of effect on cell viability, suggesting that the responses to individual amino acids may differ depending on the parameter evaluated.

SOD activity and TAC were measured to evaluate different aspects of the antioxidant response. SOD is an important enzymatic antioxidant that converts superoxide radicals into H_2O_2 and molecular oxygen and thereby limits the accumulation of superoxide-derived reactive species [32]. In the present study, H_2O_2 exposure decreased SOD activity, whereas both concentrations of Arg and 500 μM Trp or Thr attenuated this reduction. In contrast, 250 μM Trp and Thr did not significantly affect SOD activity compared with the H_2O_2-treated control. A significant effect on SOD activity was observed only at 500 μM and not at 250 μM within the evaluated range. Additional concentrations would be required to establish a dose-response relationship. The effect of Arg may be associated with its involvement in nitric oxide metabolism, glutathione synthesis, and the regulation of antioxidant pathways such as Nrf2 signaling [33]. Similarly, previous studies have reported that Thr supplementation enhances antioxidant enzyme activity in intestinal tissues [34-36]. In contrast to SOD activity, TAC did not differ among treatments. TAC provides an integrated measure of antioxidant potential and may not directly reflect changes in individual antioxidant enzymes. In the present study, SOD activity changed in response to H_2O_2 and amino acid treatments, whereas TAC remained unchanged. This discrepancy may reflect differences in the aspects of antioxidant defense assessed by the two measurements. Moreover, the possibility that the TAC assay was

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insufficiently sensitive to detect relatively small treatment-related changes under the present experimental conditions cannot be excluded. Therefore, the effects of the amino acids on antioxidant defense were more clearly reflected in SOD activity than in TAC under the present experimental conditions.

Intestinal barrier function was evaluated using the FITC-dextran flux assay, which measures the passage of a macromolecular marker across the epithelial monolayer and is commonly used as an indicator of paracellular permeability [37]. H_2O_2 exposure increased FITC-dextran passage, indicating disruption of epithelial barrier function. Treatment with Arg, Trp, or Thr at either concentration reduced FITC-dextran passage compared with the H_2O_2-treated control, although no significant differences were observed among the three amino acids at the same concentration. Notably, Trp reduced paracellular permeability despite having no significant effect on cell viability. This finding indicates that the effects of amino acids on epithelial barrier function may not strictly parallel their effects on overall cellular viability. It also suggests that Trp may influence junctional permeability through mechanisms that are not fully reflected by the cell viability assay [38].

TEER was measured as a complementary indicator of ionic resistance across the epithelial monolayer and allowed serial assessment of barrier integrity [37]. No significant differences in TEER were observed among treatments at 0 h, indicating comparable barrier resistance before the subsequent changes became evident [39]. At 24 and 48 h, the H_2O_2-treated control showed lower TEER than the untreated control, whereas all amino acid-treated groups exhibited greater TEER values than the H_2O_2-treated control. At 24 h, 500 μM Arg and Thr produced the greatest values among the amino acid treatments. At 48 h, 500 μM Arg produced the greatest TEER value among all treatments. These results indicate that all three amino acids attenuated the H_2O_2-induced reduction in epithelial resistance, with the higher concentrations of Arg and Thr tending to produce greater responses among the two concentrations tested. Together with the reduced FITC-dextran permeability, these findings support the protective effects of Arg, Trp, and Thr on epithelial barrier function under oxidative stress, although the magnitude of the response differed according to the amino acid, concentration, and parameter evaluated.

The expression patterns of *ZO-1* and *OCLN* were generally consistent with the functional changes in epithelial barrier integrity, although mRNA levels may not fully reflect tight junction protein abundance or function [40]. H_2O_2 exposure reduced the expression of both genes, whereas amino acid treatment partially attenuated these reductions. *ZO-1* expression was greater than that in the H_2O_2-treated control in all amino acid-treated groups except the 500 μM Arg group, with the greatest increase observed in cells treated with 500 μM Thr among the treatments evaluated. In contrast, *OCLN* expression was increased only by 500 μM Arg and Thr, indicating that the transcriptional responses of tight junction-related genes depended on both the amino acids and their

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concentration. ZO-1 serves as a cytoplasmic scaffolding protein that links transmembrane tight junction proteins to the actin cytoskeleton, whereas OCLN contributes to the regulation of paracellular permeability and junctional stability [40]. Therefore, the increased expression of these genes, particularly treatment with 500 μM Arg and Thr, is consistent with the greater TEER and lower FITC-dextran permeability observed under the corresponding treatment conditions. However, the gene expression responses did not fully correspond to the functional barrier measurements, indicating that transcriptional regulation alone may not account for the protective effects of these amino acid.

The immunofluorescence images provided additional spatial information on ZO-1 distribution at the cell boundaries. H_2O_2 exposure resulted in a less continuous and less clearly defined junctional staining pattern whereas cells treated with 500 μM Arg or Thr appeared to exhibit more continuous ZO-1 localization along the intercellular border. These observations were generally consistent with the recovery of TEER and the reduction in FITC-dextran permeability. Notably, the apparent improvement in ZO-1 localization after treatment with 500 μM Arg occurred despite the absence of a significant increase in ZO-1 mRNA expression. This discrepancy raises the possibility that Arg influences epithelial barrier function through post-transcriptional mechanisms, such as protein stabilization, intracellular trafficking, redistribution to the plasma membrane, or cytoskeletal reorganization [41]. However, because tight junction protein levels were not measured in the present study, this interpretation remains speculative and requires confirmation by protein-level analyses, such as western blotting for ZO-1 and OCLN. Likewise, Trp improved TEER and reduced paracellular permeability despite its relatively modest effects on *ZO-1* and *OCLN* expression, suggesting that its barrier-protective effects may involve post-transcriptional regulation, altered tight junction protein localization, or metabolite-mediated signaling pathways that are not fully captured by the mRNA expression of these genes [11,40]. Collectively, these findings suggest that Arg, Trp, and Thr may preserve epithelial barrier function through distinct regulatory processes involving both tight junction gene expression and junctional protein organization [8,42]. Nevertheless, the immunofluorescence findings should be regarded as qualitative observations complementary to the functional and transcriptional results. Despite these findings, several limitations should be considered. An amino-acid-only control without H_2O_2 was not included, so specific cytoprotection could not be fully distinguished from general metabolic stimulation. In addition, ZO-1 and OCLN protein abundance was not quantitatively assessed, limiting the interpretation of the relationship between transcript-level changes and functional barrier responses. Further studies incorporating this control are warranted to confirm these findings.

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CONCLUSION

In conclusion, Arg, Trp, and Thr differentially attenuated H2O2-induced oxidative stress and epithelial barrier dysfunction in IPEC-J2 cells under the conditions tested. Arg and Thr attenuate the reduction in cell viability, whereas both concentrations of Arg and 500 μM Trp and Thr preserve SOD activity despite the absence of significant changes in TAC. All three amino acids reduce FITC-dextran permeability and improve TEER, with the higher concentrations of Arg and Thr tending to produce greater barrier-protective responses among the two concentrations tested. These functional improvements were accompanied by the recovery of ZO-1 and OCLN expression and by qualitative changes in junctional ZO-1 staining observed in representative immunofluorescence images, which were not quantitatively assessed. Notably, Trp reduces paracellular permeability and improves TEER without significantly affecting cell viability, indicating that amino acid-mediated cytoprotection and barrier preservation may involve distinct regulatory mechanisms. Overall, these findings indicate that, under the acute H_2O_2-induced oxidative stress applied in this study, Arg, Trp, and Thr protect IPEC-J2 cells to differing degrees, with Arg and Thr acting on both antioxidant and barrier endpoints and Trp acting primarily on barrier endpoints, although the underlying molecular mechanisms warrant further investigation.

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REFERENCES

1. Ji LL, Yeo D. Oxidative stress: an evolving definition. Fac Rev. 2021;10:13. https://doi.org/10.12703/r/10-13
2. Liao SF. Invited review: maintain or improve piglet gut health around weanling: the fundamental effects of dietary amino acids. Animals. 2021;11:1110. https://doi.org/10.3390/ani11041110
3. Kim HW, Lee SY, Kim YB, Kim JH. Influence of functional nutrients on poultry performance and health by reducing stress response under environmental stress. Worlds Poult Sci J. 2025;81:879-915. https://doi.org/10.1080/00439339.2025.2506399
4. Kim HW, Lee SY, Hur SJ, Kil DY, Kim JH. Effects of functional nutrients on chicken intestinal epithelial cells induced with oxidative stress. J Anim Sci Technol. 2023;65:1040-52. https://doi.org/10.5187/jast.2023.e22
5. Lee SY, Jang HG, Lee HW, Kim DB, Lee HJ, Park JH, et al. Mitigation of oxidative stress in chicken intestinal epithelial cells by functional nutrients. J Anim Sci Technol. 2026;68:795-813. https://doi.org/10.5187/jast.2025.e25
6. Park JY, Kim YB, Lee HN, Park GY, Shin JW, Lee SY, et al. Protective roles of arginine, threonine, and tryptophan against oxidative stress in chicken intestinal epithelial cells. J Anim Sci Technol. 2026. https://doi.org/10.5187/jast.2500486. Available at (verified 20 Jul 2026)
7. Vergauwen H, Tambuyzer B, Jennes K, Degroote J, Wang W, De Smet S, et al. Trolox and ascorbic acid reduce direct and indirect oxidative stress in the IPEC-J2 cells, an in vitro model for the porcine gastrointestinal tract. PLoS One. 2015;10:e0120485. https://doi.org/10.1371/journal.pone.0120485
8. Mou Q, Yang HS, Yin YL, Huang PF. Amino acids influencing intestinal development and health of the piglets. Animals. 2019;9:302. https://doi.org/10.3390/ani9060302
9. Luise D, Chalvon-Demersay T, Correa F, Bosi P, Trevisi P. A systematic review of the effects of functional amino acids on small intestine barrier function and immunity in piglets. Animal. 2023;17:100771. https://doi.org/10.1016/j.animal.2023.100771
10. Zheng P, Yu B, He J, Yu J, Mao X, Luo Y, et al. Arginine metabolism and its protective effects on intestinal health and functions in weaned piglets under oxidative stress induced by diquat. Br J Nutr. 2017;117:1495-502. https://doi.org/10.1017/S0007114517001519

ACCEPTED

11. Liu G, Gu K, Wang F, Jia G, Zhao H, Chen X, et al. Tryptophan ameliorates barrier integrity and alleviates the inflammatory response to enterotoxigenic Escherichia coli K88 through the CaSR/Rac1/PLC-γ1 signaling pathway in porcine intestinal epithelial cells. Front Immunol. 2021;12:748497. https://doi.org/10.3389/fimmu.2021.748497
12. Zhang H, Chen Y, Li Y, Zhang T, Ying Z, Su W, et al. L-threonine improves intestinal mucin synthesis and immune function of intrauterine growth-retarded weanling piglets. Nutrition. 2019;59:182-7. https://doi.org/10.1016/j.nut.2018.07.114
13. Wei X, Li D, Feng C, Mao H, Zhu J, Cui Y, et al. Effects of hydrogen peroxide and L-tryptophan on antioxidative potential, apoptosis, and mammalian target of rapamycin signaling in bovine intestinal epithelial cells. J Dairy Sci. 2022;105:10007-19. https://doi.org/10.3168/jds.2022-21869
14. Kim YB, Park JY, Lee HN, Yeom GL, Park GY, Shin JW, et al. Effects of dietary threonine and arginine on growth performance, stress indicators, and gut health in broiler chickens under multiple stress. J Anim Sci Technol. 2026. https://doi.org/10.5187/jast.2500450. Available at (verified 20 Jul 2026)
15. Wu G. Amino acids: metabolism, functions, and nutrition. Amino Acids. 2009;37:1-17. https://doi.org/10.1007/s00726-009-0269-0
16. Wang R, Li K, Sun L, Jiao H, Zhou Y, Li H, et al. L-Arginine/nitric oxide regulates skeletal muscle development via muscle fibre-specific nitric oxide/mTOR pathway in chickens. Anim Nutr. 2022;10:68-85. https://doi.org/10.1016/j.aninu.2022.04.010
17. Park SH, Kim J, Kim D, Moon Y. Mycotoxin detoxifiers attenuate deoxynivalenol-induced pro-inflammatory barrier insult in porcine enterocytes as an in vitro evaluation model of feed mycotoxin reduction. Toxicol In Vitro. 2017;38:108-16. https://doi.org/10.1016/j.tiv.2016.10.003
18. Du Z, Kim KH, Kim J, Moon Y. Fungal deoxynivalenol-induced enterocyte distress is attenuated by adulterated adlay: in vitro evidences for mucoactive counteraction. Front Immunol. 2018;9:186. https://doi.org/10.3389/fimmu.2018.00186
19. Steel RGD, Torrie JH, Dickey DA. Principles and procedures of statistics: a biometrical approach. 3rd ed. New York: McGraw-Hill; 1997.
20. Chang S, Lee J, Jung S, Song D, Park S, Jeon K, et al. Effects of illite or bentonite on cytotoxicity, antibacterial and adsorption capacity in porcine intestinal epithelial cells. J Anim Sci Technol. 2026;68:487-98. https://doi.org/10.5187/jast.2024.e107

ACCEPTED

21. Zhu LH, Zhao KL, Chen XL, Xu JX. Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs. J Anim Sci. 2012;90:2581-9. https://doi.org/10.2527/jas.2011-4444
22. Awad WA, Hess C, Hess M. Enteric pathogens and their toxin-induced disruption of the intestinal barrier through alteration of tight junctions in chickens. Toxins. 2017;9:60. https://doi.org/10.3390/toxins9020060
23. Ortega ADSV, Szabó C. Adverse effects of heat stress on the intestinal integrity and function of pigs and the mitigation capacity of dietary antioxidants: a review. Animals. 2021;11:1135. https://doi.org/10.3390/ani11041135
24. Schierack P, Nordhoff M, Pollmann M, Weyrauch KD, Amasheh S, Lodemann U, et al. Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. Histochem Cell Biol. 2006;125:293-305. https://doi.org/10.1007/s00418-005-0067-z
25. Paszti-Gere E, Barna RF, Kovago C, Szauder I, Ujhelyi G, Jakab C, et al. Changes in the distribution of type II transmembrane serine protease, TMPRSS2 and in paracellular permeability in IPEC-J2 cells exposed to oxidative stress. Inflammation. 2015;38:775-83. https://doi.org/10.1007/s10753-014-9988-9
26. Liang J, Zhou Y, Cheng X, Chen J, Cao H, Guo X, et al. Baicalin attenuates H2O2-induced oxidative stress by regulating the AMPK/Nrf2 signaling pathway in IPEC-J2 cells. Int J Mol Sci. 2023;24:9435. https://doi.org/10.3390/ijms24119435
27. Alican I, Kubes P. A critical role for nitric oxide in intestinal barrier function and dysfunction. Am J Physiol Gastrointest Liver Physiol. 1996;270:G225-37. https://doi.org/10.1152/ajpgi.1996.270.2.G225
28. Stoddart MJ. Cell viability assays: introduction. Methods Mol Biol. 2011;740:1-6. https://doi.org/10.1007/978-1-61779-108-6_1
29. Chapman JC, Liu Y, Zhu L, Rhoads JM. Arginine and citrulline protect intestinal cell monolayer tight junctions from hypoxia-induced injury in piglets. Pediatr Res. 2012;72:576-82. https://doi.org/10.1038/pr.2012.137
30. Law GK, Bertolo RF, Adjiri-Awere A, Pencharz PB, Ball RO. Adequate oral threonine is critical for mucin production and gut function in neonatal piglets. Am J Physiol Gastrointest Liver Physiol. 2007;292:G1293-301. https://doi.org/10.1152/ajpgi.00221.2006

ACCEPTED

31. Mao X, Zeng X, Qiao S, Wu G, Li D. Specific roles of threonine in intestinal mucosal integrity and barrier function. Front Biosci (Elite Ed). 2011;3:1192-200. https://doi.org/10.2741/e322
32. Ighodaro OM, Akinloye OA. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. Alex J Med. 2018;54:287-93. https://doi.org/10.1016/j.ajme.2017.09.001
33. Liang M, Wang Z, Li H, Cai L, Pan J, He H, et al. L-arginine induces antioxidant response to prevent oxidative stress via stimulation of glutathione synthesis and activation of Nrf2 pathway. Food Chem Toxicol. 2018;115:315-28. https://doi.org/10.1016/j.fct.2018.03.029
34. Hong Y, Jiang W, Kuang S, Hu K, Tang L, Liu Y, et al. Growth, digestive and absorptive capacity and antioxidant status in intestine and hepatopancreas of sub-adult grass carp Ctenopharyngodon idella fed graded levels of dietary threonine. J Anim Sci Biotechnol. 2015;6:34. https://doi.org/10.1186/s40104-015-0032-1
35. Chen YP, Cheng YF, Li XH, Yang WL, Wen C, Zhuang S, et al. Effects of threonine supplementation on the growth performance, immunity, oxidative status, intestinal integrity, and barrier function of broilers at the early age. Poult Sci. 2017;96:405-13. https://doi.org/10.3382/ps/pew240
36. Ji S, Qi X, Ma S, Liu X, Min Y. Effects of dietary threonine levels on intestinal immunity and antioxidant capacity based on cecal metabolites and transcription sequencing of broiler. Animals. 2019;9:739. https://doi.org/10.3390/ani9100739
37. Keser Y, Boulant S, Stanifer ML. Assessment of epithelial barrier integrity by TEER and FITC-dextran permeability assays. Bio Protoc. 2026;16:e5673. https://doi.org/10.21769/BioProtoc.5673
38. Chen M, Liu Y, Xiong S, Wu M, Li B, Ruan Z, et al. Dietary L-tryptophan alleviated LPS-induced intestinal barrier injury by regulating tight junctions in a Caco-2 cell monolayer model. Food Funct. 2019;10:2390-8. https://doi.org/10.1039/c9fo00123a
39. Kannapin F, Schmitz T, Hansmann J, Schlegel N, Meir M. Measurements of transepithelial electrical resistance (TEER) are affected by junctional length in immature epithelial monolayers. Histochem Cell Biol. 2021;156:609-16. https://doi.org/10.1007/s00418-021-02026-4
40. Zihni C, Mills C, Matter K, Balda MS. Tight junctions: from simple barriers to multifunctional molecular gates. Nat Rev Mol Cell Biol. 2016;17:564-80. https://doi.org/10.1038/nrm.2016.80
41. Xia M, Ye L, Hou Q, Yu Q. Effects of arginine on intestinal epithelial cell integrity and nutrient uptake. Br J Nutr. 2016;116:1675-81. https://doi.org/10.1017/S000711451600386X

ACCEPTED

42. Stamatovic SM, Johnson AM, Sladojevic N, Keep RF, Andjelkovic AV. Endocytosis of tight junction proteins and the regulation of degradation and recycling. Ann N Y Acad Sci. 2017;1397:54-65. https://doi.org/10.1111/nyas.13346

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496 **Table 1.** The sequence of the primers used in quantitative real-time PCR.

Gene[1)]	Primer sequence (5'-3')	Size for PCR product (bp)	Accession no.
GAPDH	Forward: TCGGAGTGAACGGATTTGGC	189	NM_001206359.1
	Reverse: TGACAAGCTTCCCGTTCTCC		
ZO-1	Forward: TCAAGGTCTGCCGAGACAAC	140	XM_021098896.1
	Reverse: ATCACAGTGTGGTAAGCGCA		
OCLN	Forward: TATACAGATCCACGAGCAGC	160	NM_001163647.2
	Reverse: CACCCAGGATAGCACTCATT		

497 [1)] *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *ZO-1*, zonula occludens-1; *OCLN*, occludin.

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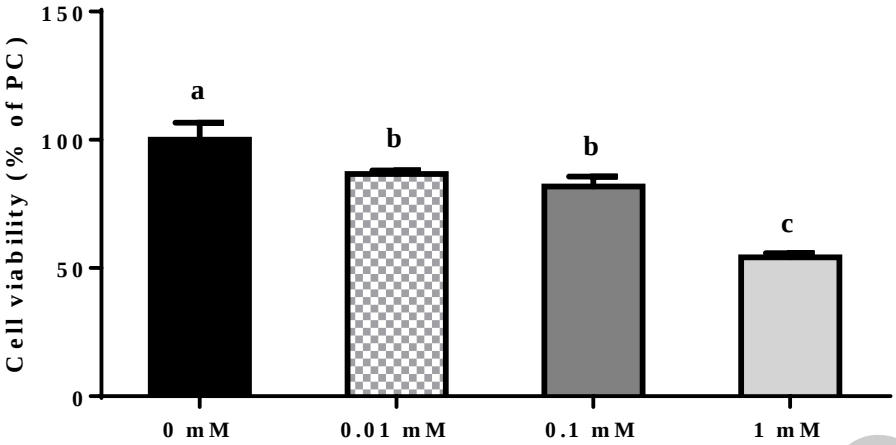
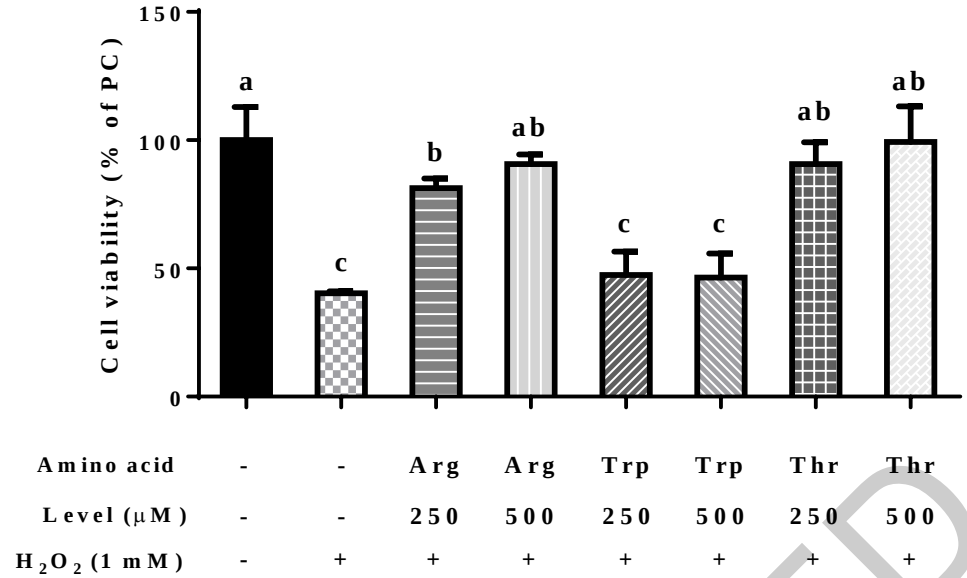


Figure 1. Determination of optimal H_2O_2 concentration for inducing oxidative stress in IPEC-J2 cells. Cells were treated with different concentrations of H_2O_2 (0, 0.01, 0.1, or 1 mM) for 6 h. All data are presented as means ± SE (n = 6). $^{a\text{-}c}$ Different letters indicate significant differences among treatments ($p < 0.05$).

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505 **Figure 2. Effects of Arg, Trp, and Thr on cell viability of IPEC-J2 cells under oxidative stress.** Cells were
506 pretreated with Arg, Trp, or Thr at levels of 250 or 500 μM for 24 h, followed by exposure to 1 mM H_2O_2 for 6
507 h. All data are presented as means ± SE (n = 6). [a-c] Different letters indicate significant differences among
508 treatments ($p < 0.05$).

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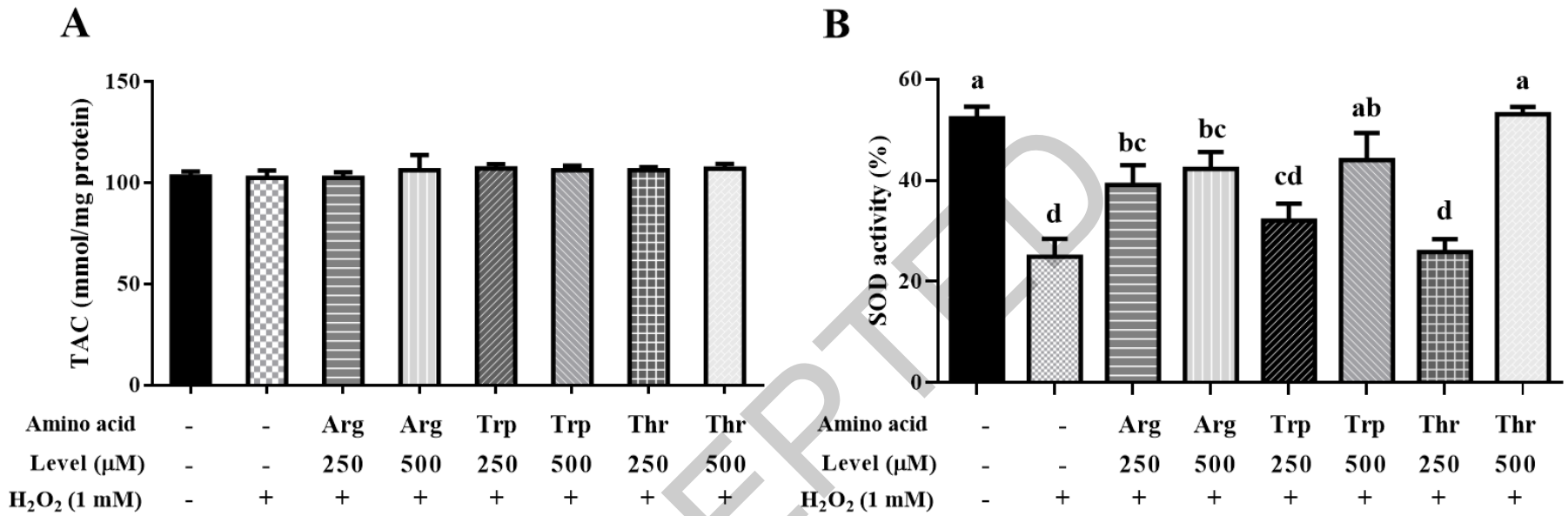


Figure 3. Effects of Arg, Trp, and Thr on the antioxidant capacity of IPEC-J2 cells under oxidative stress. Cells were pretreated with Arg, Trp, or Thr at levels of 250 or 500 μM for 24 h, followed by exposure to 1 mM H_2O_2 for 6 h. (A) Total antioxidant capacity (TAC). (B) Superoxide dismutase (SOD) activity. All data are presented as means ± SE (n = 6). $^{a-d}$ Different letters indicate significant differences among treatments ($p < 0.05$).

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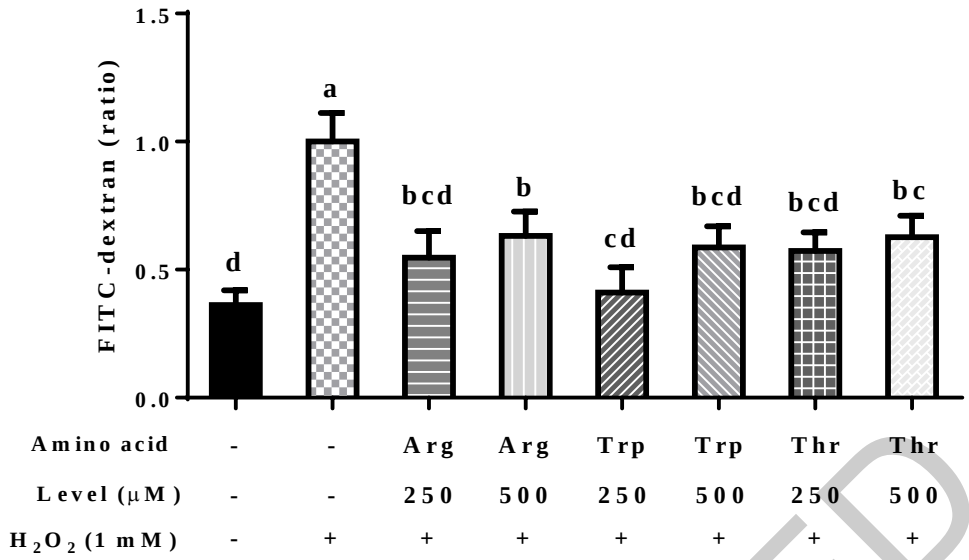


Figure 4. Effects of Arg, Trp, and Thr on paracellular permeability of IPEC-J2 cells under oxidative stress. Cells were pretreated with Arg, Trp, or Thr at levels of 250 or 500 μM for 24 h, followed by exposure to 1 mM H_2O_2 for 6 h. All data are presented as means ± SE (n = 6). [a-d] Different letters indicate significant differences among treatments ($p < 0.05$).

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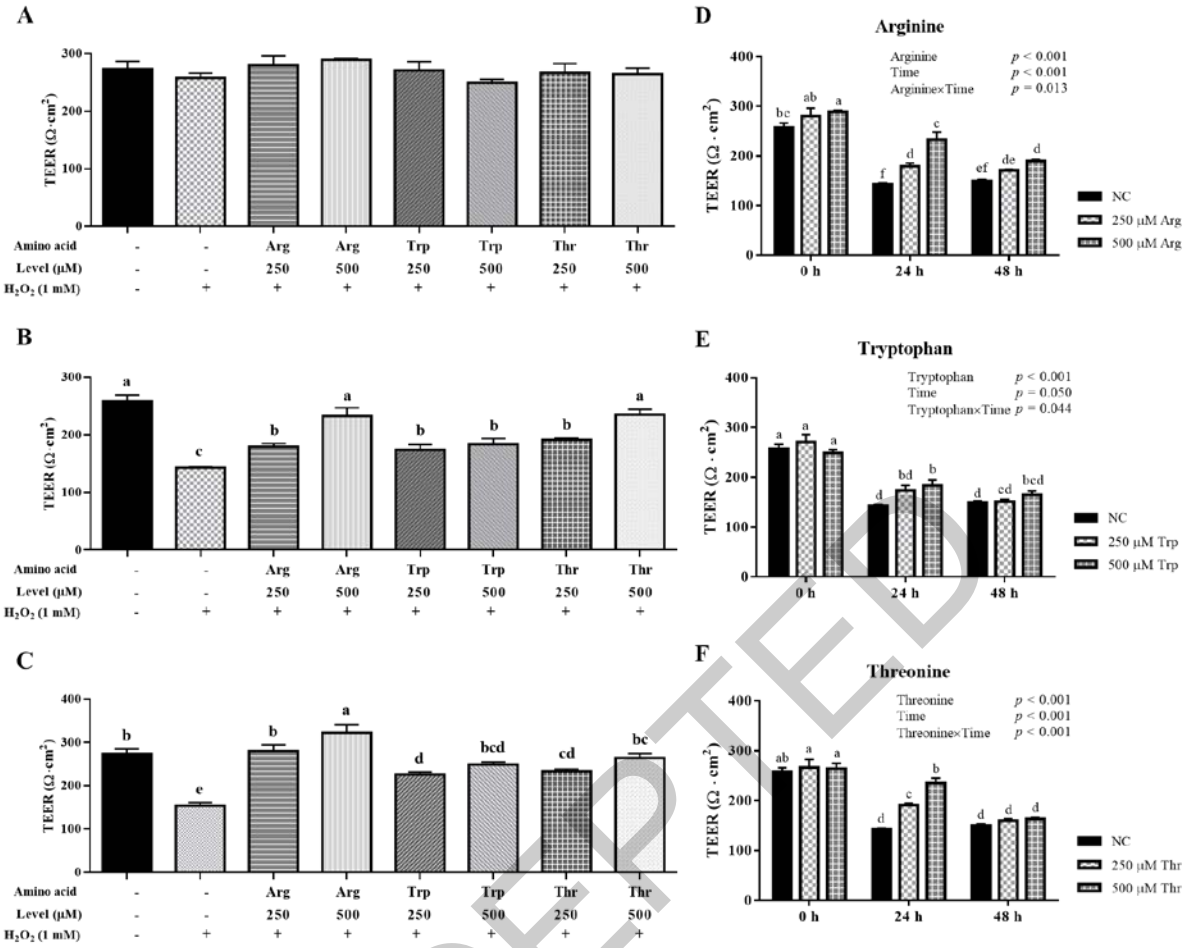
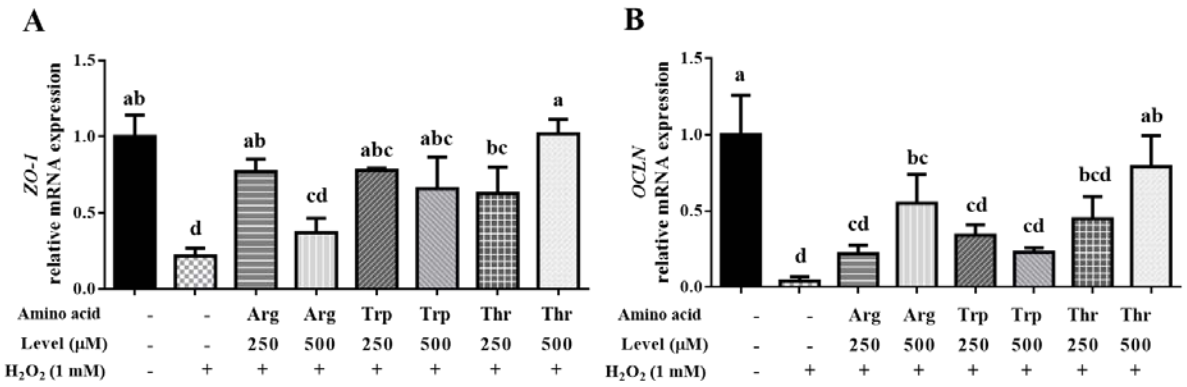


Figure 5. Effects of Arg, Trp, and Thr on transepithelial electrical resistance in IPEC-J2 cells under oxidative stress. Cells were pretreated with Arg, Trp, or Thr at levels of 250 or 500 μM for 24 h, followed by exposure to 1 mM H_2O_2 for 6 h. (A) TEER was measured at 0 h. (B) TEER was measured at 24 h. (C) TEER was measured at 48 h. (D) Arginine, (E) Tryptophan, and (F) Threonine at 0, 24, and 48 h. All data are presented as means ± SE (n = 6). $^{a-e}$ Different letters indicate significant differences among treatments ($p < 0.05$).

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531 **Figure 6. Effects of Arg, Trp, and Thr on trans mRNA expression related to tight junction in IPEC-J2**
532 **cells under oxidative stress.** Cells were pretreated with Arg, Trp, or Thr at levels of 250 or 500 μM for 24 h,
533 followed by exposure to 1 mM H_2O_2 for 6 h. (A) *ZO-1*. (B) *OCLN*. All data are presented as means ± SE (n =
534 6). $^{a-d}$ Different letters indicate significant differences among treatments ($p < 0.05$).

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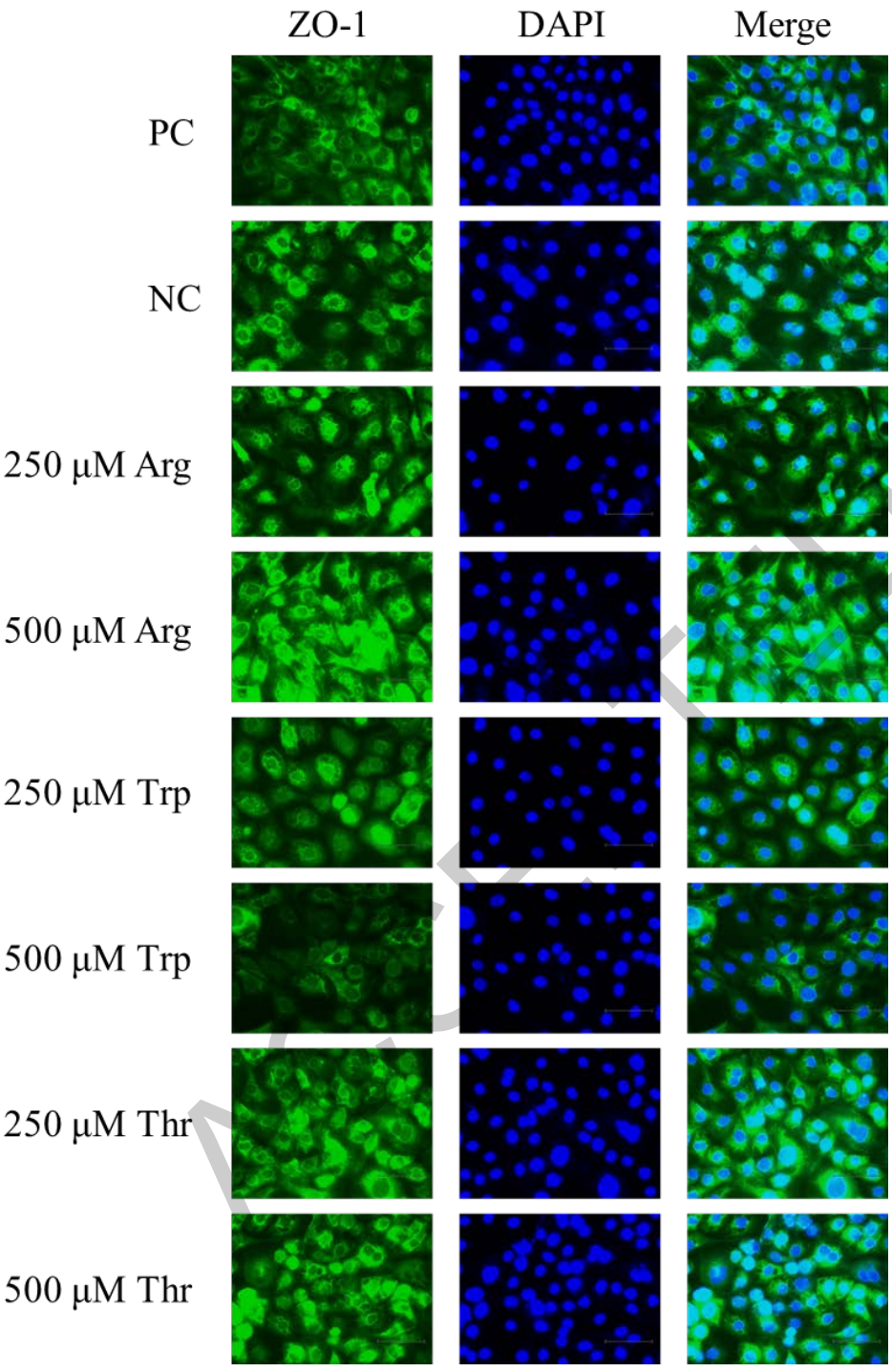


Figure 7. Effects of Arg, Trp, and Thr on ZO-1 localization in IPEC-J2 cells under oxidative stress, assessed by immunofluorescence staining. Cells were pretreated with Arg, Trp, or Thr at levels of 250 or 500 μM for 24 h, followed by exposure to 1 mM H_2O_2 for 6 h. ZO-1 is shown in green, and nuclei were counterstained with DAPI (blue). Representative images are shown, and all images were acquired under identical microscope settings. Scale bars = 75 μm.