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Abstract

Chicken intestinal epithelial cells (cIECs) play a crucial role in nutrient absorption and maintaining barrier integrity but are highly susceptible to oxidative stress. This study investigated the efficacy of arginine (Arg), tryptophan (Trp), and threonine (Thr) in protecting cIECs from hydrogen peroxide (H_2O_2) induced oxidative stress. The experimental design consisted of 8 treatment groups: a positive control (PC, cells incubated with fresh medium), a negative control (NC, cells exposed to 1 mM H_2O_2), and 6 additional groups pretreated with either 250 or 500 mM Arg, Trp, or Thr for 24 h (6 replicates per treatment) prior to 6 h incubation with 1 mM H_2O_2. The results demonstrated that all amino acid treatments significantly improved ($p < 0.05$) cell viability compared to NC. Total antioxidant capacity (TAC) was markedly lower ($p < 0.05$) in NC than in PC; however, 250 μM Arg and 250 μM Trp significantly enhanced ($p < 0.05$) TAC compared to NC. Paracellular permeability was significantly lower ($p < 0.05$) in PC compared with NC. Treatments with 500 μM Arg, 250 μM Trp, and both Thr levels reduced ($p < 0.05$) paracellular permeability compared to NC and other amino acid groups. At all time points, transepithelial electrical resistance was consistently greater ($p < 0.05$) in PC than in NC, with notable effects observed for 500 μM Arg and Thr treatments, which exhibited greater ($p < 0.05$) values than the other amino acid treatments. Expression of tight junction-related genes, including *zonula occludens-1*, *claudin*, *occludin*, and *junctional adhesion molecule 2*, was greater ($p < 0.05$) in PC than in NC. All amino acid treatments increased ($p < 0.05$) the expression of *claudin* and *occludin* compared with NC. In conclusion, Arg, Trp, and Thr attenuate oxidative stress in cIECs by enhancing cell viability, antioxidant capacity, and barrier function, primarily through upregulation of tight junction-related gene expression.

Keywords: arginine, chicken intestinal epithelial cell, oxidative stress, threonine, tryptophan

INTRODUCTION

The intestinal epithelium functions as a selective barrier that enables the uptake of digested nutrients and simultaneously provides physical and chemical protection against the passage of pathogenic microorganisms, toxins, and other detrimental substances from the intestinal lumen into the host, thereby facilitating efficient nutrient absorption and supporting barrier integrity [1,2]. The maintenance of this barrier function relies on the coordinated activities of intestinal epithelial cells (IECs), which form tight junction complexes and secrete mucus [3,4]. In broiler chickens, the intestinal tract is crucial for overall health, animal welfare, and achieving optimal growth performance [5]. Effective nutrient absorption and a robust intestinal barrier are not only fundamental for supporting growth and maximizing feed conversion ratio but also play a key role in reducing the incidence of intestinal infections [6]. Nevertheless, compromise of intestinal epithelial integrity leads to heightened paracellular permeability, which triggers inflammation, stimulates epithelial cell death, and consequently diminishes nutrient utilization, growth efficiency, and health [7,8]. Thus, maintaining intestinal barrier integrity is especially critical in poultry production system. In commercial production settings, broiler chickens are routinely subjected to various environmental, nutritional, and pathogenic stressors, such as heat stress, high stocking density, contamination of feed with mycotoxins, and exposure to infectious agents [9,10]. These conditions often lead to increased oxidative stress, disturb redox balance, and predispose cells susceptible to oxidative damage [11-13].

Oxidative stress occurs when the generation of reactive oxygen species (ROS) exceeds the capacity of the cellular antioxidant system, resulting in oxidative damage to cellular lipids, proteins, and nucleic acids [14]. Accumulation of excess ROS within IECs depletes antioxidant reserves, disrupts cytoskeletal integrity, and damages the structure and function of tight junctions, thus elevating paracellular permeability [13,15]. Among different ROS, hydrogen

peroxide (H_2O_2) acts as a key mediator in redox signaling and regulation [16]. While physiological levels of H_2O_2 are important for cell signaling, higher levels result in cell injury [1,17]. Therefore, oxidative stress induced by H_2O_2 in IECs is frequently utilized as an in vitro experimental model to elucidate the mechanisms governing intestinal barrier function and to assess potential protective interventions.

Previous studies have demonstrated that amino acids are crucial for maintaining intestinal function [18,19]. Among these, Arg has been shown to enhance nitric oxide (NO) production via the NO synthase pathway, thereby facilitating ROS scavenging, modulating antioxidant enzyme activities, and influencing gene expression through the NF-κB and MAPK signaling pathways [11,20]. Furthermore, Arg acts as a precursor for polyamine synthesis, which is vital for cellular proliferation and differentiation [21]. In a similar manner, Trp provides antioxidant benefits mainly via metabolites derived from the kynurenine and serotonin/melatonin pathways, which possess free radical scavenging abilities [22,23]. Additionally, Thr is an essential amino acid required for the synthesis of mucin glycoproteins that compose the mucus layer, providing both physical and chemical defense to preserve intestinal epithelial integrity [24,25]. The beneficial roles of Arg, Trp, and Thr in supporting intestinal health have been elucidated, especially in vivo studies. However, most previous studies have focused on whole-organism models, where interactions among multiple physiological systems make it difficult to ascertain direct cellular actions. As a result, there is a paucity of comparative evidence regarding their effects on chicken intestinal epithelial cells (cIECs) under oxidative stress. To bridge this knowledge gap, the current study utilized an in vitro cIECs model to assess the specific effects of Arg, Trp, and Thr on epithelial function.

Therefore, the objective of this study was to investigate the protective roles of Arg, Trp, and Thr against H_2O_2-induced oxidative stress in cIECs. In particular, this research examined their effects on cell viability, morphology, antioxidant capacity, paracellular permeability,

transepithelial electrical resistance (TEER), and tight junction-related gene expression to elucidate their roles in maintaining epithelial barrier integrity under oxidative stress.

MATERIALS AND METHODS

Cell cultivation

The cIECs used in this study were initially isolated and described by Kim et al. [26] and Lee et al. [27]. Primary cells were isolated from 16-d-old chick embryos derived from specific pathogen-free birds (VALO Biomedia GmbH, Osterholz-Scharmbeck, Germany). The isolated cells were distributed onto culture dishes coated with fibronectin (fibronectin, Corning, Corning, NY, USA) and incubated at 37°C in a humidified environment containing 5% CO_2 for 24 h. Cells maintenance was performed using Dulbecco's modified Eagle medium (DMEM; Welgene Inc., Gyeongsan, Republic of Korea) supplemented with 2.5% fetal bovine serum (Gibco, Grand Island, NY, USA), 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA), 2,500 μg/mL gentamicin (Gibco, Grand Island, NY, USA), 10 μg/mL insulin (Welgene Inc., Gyeongsan, Republic of Korea), and 20 ng/mL epidermal growth factors (Welgene Inc., Gyeongsan, Republic of Korea). The culture medium was replaced every two to three days while maintaining the same incubation settings.

Determination of optimal oxidative stress

To establish the optimal oxidative stress conditions for cIECs, cells were seeded in 96-well plates at a density of 5×10^4 cells/mL and exposed to H_2O_2 (Daejungchem, Siheung, Republic of Korea) at levels of 0.01, 0.1, or 1 mM for 6 h to induce oxidative stress. After treatment, the supernatant was carefully aspirated, and cells were rinsed with PBS (Welgene

Inc., Gyeongsan, Republic of Korea). Cell viability was then assessed using the EZ-Cytox assay (DoGenBio Co., Ltd., Seoul, Republic of Korea) according to the manufacturer's protocol. Following this, 20 μL of the EZ-Cytox assay reagent was added to each well, and incubation continued for 3 h. Absorbance was subsequently measured at 450 nm using a microplate reader (LTek, Seongnam, Republic of Korea), and cell viability was determined accordingly.

Cell treatment

Cells were seeded in 96-well plates at a density of 5×10^4 cells/mL and incubated until confluency. Afterward, the cells received treatments with 0, 250, or 500 μM of L-Arg, L-Trp, and L-Thr for 24 h, with 6 replicates per group (≥ 98%, Sigma-Aldrich Inc., St. Louis, MO, USA). Following the amino acid treatment, the cells were incubated with either fresh culture medium (positive control, PC) or 1 mM of H_2O_2 (negative control, NC), alongside amino acid-treated groups, for a further 6 h. After the final treatment, the cells were collected for subsequent analytical procedures.

Cell viability and morphology

Cell morphology was examined according to the procedure outlined by Lin et al. [28], incorporating minor modifications. After the respective treatments, cell morphology was visualized using phase-contrast microscopy (EVOS M5000 Imaging System, Thermo Fisher Scientific, Bothell, WA, USA), and images were acquired at 100× magnification. Cells were seeded in 96-well plates at a density of 5×10^4 cells/mL and incubated at 37°C in a humidified environment containing 5% CO_2 for 24 h. Cell viability was determined with the EZ-Cytox assay adhering the manufacturer's protocol. Briefly, cells underwent 24 h treatment with 0, 250, or 500 μM of Arg, Trp, or Thr, excluding the PC and NC groups from treatment. Subsequently,

1 mM H_2O_2 was administered to the NC and each amino acid treatment group for 6 h. After the incubation period, 20 μL of EZ-Cytox assay reagent was dispensed into each well, and the cells were incubated for an additional 3 h, according to the manufacturer's guidelines. The cell viability was quantified by measuring absorbance at 450 nm using a microplate reader (LTek, Seongnam, Republic of Korea).

Antioxidant capacity

The cIECs were seeded at a density of 5×10^4 cells/mL in 6-well plates with 6 replicates per treatment. Cells were pretreated with either 250 or 500 μM of Arg, Trp, or Thr for 24 h, with the PC and NC groups excluded from pretreatment. Subsequently, the NC group and Arg, Trp, and Thr groups were exposed to 1 mM H_2O_2 for 6 h. After treatment, the supernatant was removed, and the cells were washed twice with ice-cold PBS. Cell lysis was performed using a lysis buffer containing 98% RIPA buffer, 1% protease inhibitor, and 1% phosphatase inhibitor cocktail (GenDEPOT, Barker, TX, USA) for 15 min at 4°C as outlined by Wang et al. [29] with slight adjustments. The lysates were centrifuged at 15,000 × g for 10 min at 4°C, and the supernatant was collected. The collected resulting supernatant was then analyzed to determine total antioxidant capacity (TAC) and superoxide dismutase (SOD) activity using commercial assay kits (DoGenBio Co., Ltd., Seoul, Korea), as instructed by the manufacturer.

Paracellular permeability

Paracellular permeability was assessed using 4 kDa fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich, St. Louis, MO, USA) following the method detailed by Park et al. [30]. The cIECs were seeded at a density of 5×10^4 cells onto the apical chamber of Transwell inserts (SPL Life Sciences Co., Ltd., Pocheon, Republic of Korea) and cultured for 2 d. Subsequently,

the cells were cultured for a further 10 d to ensure confluence and to facilitate differentiation. On d 11, cells received treatment with 250 or 500 μM of Arg, Trp, or Thr for 24 h, after which they were exposed to 1 mM H_2O_2 for 6 h. The PC group underwent neither amino acid pretreatment nor H_2O_2 exposure, while the NC group was subjected to H_2O_2 without prior amino acid treatment. Following these procedures, FITC-dextran was prepared in DMEM at a final concentration of 2.2 mg/mL and introduced into the apical chamber; DMEM alone was placed in the basolateral chamber. The incubation period lasted 1 h. After incubation, 200 μL from the basolateral chamber was collected and placed into a black 96-well plate (SPL Life Sciences Co., Ltd., Pocheon, Republic of Korea) for fluorescence evaluation. Fluorescence was quantified using a spectrophotometer (LTek, Seongnam, Republic of Korea) with an excitation wavelength of 490 nm and an emission wavelength of 535 nm.

Transepithelial electrical resistance (TEER)

Cells were seeded at a density of 5×10^4 cells/mL on 0.4-μm pore apical chambers (SPL Life Sciences Co., Ltd., Pocheon, Republic of Korea) and cultured for 10 d, with the medium in both the apical and basolateral compartments replaced every 2- to 3-day interval. The cells were maintained under these conditions until both confluency and differentiation were confirmed. After treatment with amino acids and H_2O_2, the culture plates were placed on a hot plate held at 37°C. Before TEER assessment, both chambers had their media replaced with DMEM, and TEER was assessed at 0, 24, and 48 h using the EVOM3 Epithelial Volt/Ohm Meter (World Precision Instruments Inc., Sarasota, FL, USA), according to the manufacturer's protocol. The measurement of TEER was performed according to the methods described by Du et al. [31] and Vergauwen et al. [32], with slight modifications. Electrodes were thoroughly rinsed with distilled water between samples to prevent cross-contamination affecting resistance values. TEER readings for each well were obtained at four distinct positions (north, south, east,

and west) within the apical chamber to address membrane heterogeneity. An average of the four measurements represented the final TEER value. TEER ($\Omega \cdot cm^2$) was calculated by multiplying recorded resistance by the apical chamber surface area (0.33 cm^2).

Tight junction-related gene expression

Total RNA was extracted from cells cultured in 6-well plates using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol, incorporating slight modifications. Briefly, cells were lysed with 1 mL of TRIzol and transferred to 2 mL microcentrifuge tubes. Subsequently, 200 μL of chloroform (Sigma-Aldrich, St. Louis, MO, USA) was added, followed by thorough vortexing to ensure adequate mixing. After centrifugation at 12,000 rpm for 15 min at 4°C, the upper aqueous phase was carefully separated and combined with 500 μL of isopropanol by gentle inversion, then incubated on ice for 10 min. The RNA was precipitated by centrifuging at 12,000 × g for 10 min at 4°C, and the supernatant was cautiously discarded. The RNA pellet was washed with 1 mL of 75% ethanol, vortexed briefly, and centrifuged at 8,000 rpm for 5 min at 4°C. The remaining ethanol was removed, and the pellet was air-dried for a short period before being dissolved in 50 μL of nuclease-free water. Dissolution was completed by heating the RNA solution at 60°C for 15-20 min in a heating block. RNA concentration and purity were measured using a NanoDrop spectrophotometer (LTek, Seongnam, Republic of Korea). For mRNA expression analysis of tight junction-related genes, including *zonula occludin-1* (*ZO-1*), *claudin* (*CLDN*), *occludin* (*OCLN*), and *junctional adhesion molecule 2* (*JAM2*), and cDNA synthesis was performed from total RNA using a commercial cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Primer pairs were designed using Primer-BLAST and synthesized by Xenotech (Daejeon, Republic of Korea). The specific primer sequences for each gene are detailed in Table 1. Quantitative real-time PCR was conducted with

TOPreal™ SYBR Green Master Mix (Enzynomics Co., Ltd., Daejeon, Republic of Korea) on a QuantStudio 1 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was carried out as described in the manufacturer's instructions from Thermo Fisher Scientific, incorporating slight modifications. Each PCR reaction was prepared in a total volume of 20 μL, including 1 μL of cDNA, 10 μL of SYBR Green Master Mix, 0.5 μL each of forward and reverse primers (10 mM), and nuclease-free water. The thermal profile consisted of an initial denaturation step at 95°C for 10 min, followed by 40 amplification cycles comprising denaturation at 95°C for 10 sec, gene-specific annealing at the appropriate temperature for 30 sec, and extension at 72°C for 20 sec. Specificity of amplification was verified by performing a melting curve analysis. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method with normalization to the housekeeping gene (*GAPDH*).

Statistical analysis

Statistical analysis was conducted using the PROC MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA). Each replicate was considered as an experimental unit. Outliers were identified using the UNIVARIATE procedure of SAS [33]. The LSMEANS procedure was used to calculate treatment means, and the PDIFF option was applied to separate them for significance testing. All data are presented as mean ± SEM (n = 6). Significance for statistical tests was established at $p < 0.05$.

RESULTS

Determination of optimal oxidative stress

To identify the optimal oxidative stress condition, cIECs were incubated with 0.01, 0.1, or 1 mM H_2O_2 for 6 h, followed by evaluation of cell viability (Fig. 1). All H_2O_2-treated groups displayed a significant reduction ($p < 0.05$) in cell viability compared to the untreated group. Importantly, 1 mM H_2O_2 resulted in the lowest ($p < 0.05$) cell viability among the groups. Therefore, 1 mM H_2O_2 was chosen as the optimal dose to induce oxidative stress in subsequent experiments based on these results.

Cell viability and morphology

Cell viability was significantly lower ($p < 0.05$) in the NC group than in the PC group (Fig. 2). Exposure to H_2O_2 caused extensive cell death and disruption of intercellular junctions (Fig. 3), differing from the morphology observed in the PC group. Pretreatment with Arg, Trp, or Thr led to partial mitigation of H_2O_2-induced morphological damage. Notably, 500 μM Thr largely maintained cell structure and minimized morphological disruption.

Antioxidant capacity

The TAC activity decreased ($p < 0.05$) in the NC group compared to the PC group (Fig. 4). Pretreatment with 250 μM Arg and Trp significantly improved ($p < 0.05$) TAC activity compared to the NC group, while other amino acid treatments showed no effect. The SOD activity was similarly suppressed ($p < 0.05$) in the NC group. Nevertheless, none of the amino acid-treated groups demonstrated a significant difference from the NC group regarding SOD activity.

Paracellular permeability

Paracellular permeability, assessed by FITC-dextran, was examined to evaluate changes in epithelial barrier function during oxidative stress (Fig. 5). The PC group exhibited significantly less ($p < 0.05$) paracellular permeability than the NC group. Although none of the amino acid-treated groups completely recovered permeability to the level observed in the PC group, paracellular permeability was significantly reduced ($p < 0.05$) with 500 μM Arg, 250 μM Trp, and both 250 and 500 μM Thr compared to the NC group.

Transepithelial electrical resistance (TEER)

To assess the protective effects of amino acids on transcellular barrier function under oxidative stress, TEER values were measured at 0, 24, and 48 h after H_2O_2 exposure (Fig. 6). Cells exposed to H_2O_2 showed significantly decreased ($p < 0.05$) TEER compared to the PC group at all measured time points. At 0 h, all amino acid-treated groups showed significantly increased ($p < 0.05$) TEER values compared to the NC group, whereas the 500 μM Arg and Thr groups induced significantly greater ($p < 0.05$) values than the other amino acid groups. After 24 h, both 250 and 500 μM Arg and 500 μM Thr exhibited significantly greater ($p < 0.05$) TEER values compared with the NC group. After 48 h, TEER values in both levels of Arg and Thr groups were significantly greater ($p < 0.05$) than those in the NC and Trp groups.

Tight junction-related gene expression

To determine the impact of amino acids under oxidative stress conditions, the expression of tight junction-related genes, including *ZO-1*, *CLDN*, *OCLN*, and *JAM2*, were analyzed (Fig. 7). The expressions of *ZO-1*, *OCLN*, and *JAM2* were significantly decreased ($p < 0.05$) in the NC group compared to the PC group. All the amino acid treatments increased ($p < 0.05$) the expression of *CLDN* and *OCLN* compared to the NC group.

DISCUSSION

In chickens, IECs are essential for nutrient absorption and for preserving the structural integrity of the intestinal barrier [34]. Under various rearing conditions, chickens are frequently exposed to environmental and nutritional stressors that can induce oxidative stress, which subsequently has a direct impact on IECs [10,14]. Oxidative damage to IECs results in reduced antioxidant capacity, compromises tight junctions, and eventually causes cell death, collectively impairing intestinal health [14,26,35]. Thus, mitigating oxidative stress in IECs is essential for maintaining intestinal health and improving overall growth performance in chickens. Among the amino acids, Arg facilitates NO synthesis, which influences cellular redox status by regulating ROS [36,37]. Additionally, Trp serves as a precursor of melatonin and mediates antioxidant activity through the regulation of redox homeostasis [22,23]. Furthermore, Thr encourages cell proliferation by influencing protein synthesis via the mammalian target of rapamycin pathway [22,25]. Therefore, in this study, we established an oxidative stress model in cIECs to investigate the protective properties of Arg, Trp, and Thr against oxidative stress.

In this experiment, H_2O_2 was utilized to induce oxidative stress in cIECs, which is recognized as a primary ROS involved in the regulation of redox homeostasis in biological systems. Unlike low or physiological levels of H_2O_2, elevated levels cause oxidative modifications of proteins, changes in cellular reaction pathways, both reversible and irreversible damage to biomolecules, suppression of cell proliferation, and cell death, culminating in oxidative stress [16,38]. We therefore assessed the level of H_2O_2 needed to induce oxidative stress in cIECs. Previous research similarly has used H_2O_2 to induce oxidative stress in diverse IECs models, such as Caco-2, IPEC-J2, and IEC-6, and has shown that H_2O_2 levels ranging from 0.1 to 1 mM with exposure times of 30 min to 24 h effectively induce oxidative stress [28,39,40]. Consistent with these findings, the current study found that

317 exposure to 1 mM H_2O_2 markedly decreased cell viability compared with other levels and
318 resulted in considerable morphological disturbances and functional impairment of the barrier.
319 Therefore, using 1 mM H_2O_2 was adequate for inducing oxidative stress without causing
320 complete cell loss, which allowed for the subsequent analysis of amino acid treatment effects.

321 Cell viability is a crucial parameter for assessing healthy cell cultures and is commonly
322 utilized as an indicator in most cell-based experiments [41]. In this study, cell viability
323 decreased notably in the oxidative stress group; however, all amino acid treatments
324 demonstrated partial reversal of this decline. Notably, supplementation with 500 μM Thr
325 yielded the most substantial improvement in cell viability under oxidative stress conditions. To
326 further investigate epithelial responses, we also assessed cell morphology, which is a primary
327 visual indicator of the health of the intestinal epithelium [42]. In the present study, significant
328 cell death and loss of intercellular junctions were detected following H_2O_2 treatment. Relative
329 to the spindle-shaped appearance of untreated cIECs, cells exposed to H_2O_2 became smaller,
330 lost their typical spindle morphology, and turned rounded. Oxidative stress led to an overall
331 reduction in cell size, induced irregular or rounded cell shapes, and visibly compromised or
332 broke down intercellular contacts, resulting in gaps between adjacent cells. These
333 morphological alterations are indicative of structural damage induced by oxidative stress and
334 impairment of barrier function [43]. Such visible changes are likely linked to the marked
335 decrease in cell viability seen after H_2O_2 exposure. Conversely, amino acid supplementation
336 alleviated both the reduction in viability and the morphological alterations, with 500 μM Thr
337 demonstrating the most effective preservation of epithelial structure. Thr, as a principal
338 substrate for mucin production in the intestinal epithelium, is essential for the mucus layer
339 formation, which serves to strengthen both the physical and chemical barriers and thereby
340 inhibits the attachment of pathogens and toxic agents [24,25,44]. The mucus layer further
341 shields the epithelial surface from direct oxidative damage and contributes the stabilization of
342 intercellular junctions [45]. The present findings corroborate that 500 μM Thr supports

maintenance of cytoskeletal integrity, preserves intercellular adhesion, and sustains epithelial cell survival under oxidative stress conditions, which together promote barrier maintenance. Additionally, Arg and Trp treatments reduced oxidative stress-induced declines in cell viability and morphological integrity. Arg, acting as a precursor for ornithine, is metabolized into polyamines and proline, biomolecules known to stimulate cell proliferation, control the cell cycle regulation, and regulate gene transcription [46,47].

In the current study, Arg treatment increased TAC activity, reinforcing the antioxidant defense system, decreasing cellular oxidative damage, and thus supporting better cell viability. Likewise, Trp is metabolized into compounds exhibiting both free radical scavenging and anti-inflammatory effects, which help inhibit reactions mediated by ROS [23,48]. Consistent with this mechanism, Trp treatment was found to increase TAC activity in the present study, which likely enhances cellular defenses against ROS, reduces oxidative damage, and supports improved cell viability [22,49]. Therefore, the results suggest that Arg, Trp, and Thr ameliorate impairments in cIECs caused by oxidative stress, with 500 μM Thr providing the greatest benefits by preserving epithelial morphology, maintaining intercellular adhesion, and improving cell viability. The antioxidant defense system, which mediated by both enzymatic and non-enzymatic antioxidant mechanisms, is essential for cellular protection against damage induced by ROS [50]. Enzymatic components, such as SOD, directly degrade or detoxify ROS, while non-enzymatic components, including amino acid metabolites, eliminate free radicals and help sustain redox homeostasis [51,52]. This defense mechanism is especially crucial in IECs, which are frequently exposed to external stimuli, ensuring the maintenance of barrier integrity and cellular viability [53]. In this study, exposure to oxidative stress resulted in significant decreases in both TAC and SOD activity, reflecting an imbalance in the redox state. Treatment with 250 μM Arg and Trp resulted in noteworthy increases in TAC activity, though SOD activity remained unaltered, indicating that these amino acids primarily contribute to the non-enzymatic antioxidant defense. These results align with previous findings showing that Arg

supplementation enhances TAC activity in IECs exposed to oxidative stress [54]. This effect may be attributed to Arg's action through the L-Arg-NO pathway, generating NO that indirectly alleviates excessive ROS and modulates the intracellular redox state [55]. In addition, Trp metabolism via the kynurenine and serotonin pathways produces metabolites with properties that scavenge free radicals and chelate metals, thus reducing oxidative processes [22,49]. In agreement with the present results, previous studies have demonstrated NO-mediated modulation of redox balance by Arg as well as the radical-scavenging action of Trp-derived metabolites. Collectively, these findings indicate that Arg and Trp play important roles in maintaining redox balance in cIECs under oxidative stress.

Paracellular permeability is assessed by quantifying the translocation of the high molecular marker FITC-dextran across the cell monolayer, which directly indicates a loss in the sealing capacity between adjacent cells [56,57]. In this study, the NC group showed elevated FITC-dextran levels, aligning with previous studies that reported increased paracellular permeability in IECs under oxidative stress [26,54,58]. Furthermore, these results confirm previous findings that Arg treatment attenuates FITC-dextran permeability in IECs when subjected to oxidative stress, and that Thr supplementation produces a parallel reduction in FITC-dextran [26,27,54]. Similarly, Trp supplementation reduced FITC-dextran, suggesting an improvement in epithelial barrier integrity under oxidative challenge. Oxidative stress promotes apoptosis or anoikis in cIECs, resulting in the development of intercellular voids at the sites of cellular loss, which consequently raises paracellular permeability [59,60]. Arg supports cell viability and proliferation through polyamine biosynthesis, while Thr is crucial for mucin synthesis, enhancing barrier protection against cell loss [21,25]. Trp may further contribute to barrier preservation by modulating cellular redox balance through its antioxidant metabolites like serotonin, thereby limiting oxidative stress–induced epithelial cell death [22,49]. Overall, supplementation with Arg and Thr is proposed to counteract oxidative stress-induced cIECs death, thereby preserving cIECs structural integrity and limiting paracellular permeability.

TEER measures the electrical resistance across the epithelial monolayer, serving as an indirect metric of intercellular adhesion and the selective permeability of the epithelial barrier [61,62]. In the current study, TEER was significantly decreased in cIECs exposed to oxidative stress. With the exception of 500 μM Trp, which increased TEER, oxidative stress consistently led to decreased TEER measurements at all intervals. Thr supplementation exhibited the lowest paracellular permeability at each measured amino acid levels, and TEER values at 48 h were significantly greater following both Arg and Thr treatments. Previous research demonstrated that exposing Caco-2 cells to Arg under heat stress resulted in decreased TEER [63]. Upon production of Arg-derived NO, it activates guanylate cyclase within IECs, leading to increased cGMP, stimulation of PKG, and initiation of cytoskeletal remodeling [64]. Similarly, Thr supplementation improved TEER in cIECs under oxidative stress or lipopolysaccharide challenge [26,27]. Thr is involved in supporting cytoskeletal stabilization [65]. Therefore, Arg or Thr supplementation alleviates oxidative stress in cIECs and enhances TEER.

Analysis of tight junction-related gene expression provides molecular evidence that substantiates protein-level alterations and verifies the maintenance of tight junction structure at the transcriptional level [66,67]. The key transmembrane proteins CLDN, OCLN, and JAM2 interact with the actin cytoskeleton via ZO-1 and are recognized as core indicators of tight junction stability [68]. Importantly, the expression of tight junction genes, including *ZO-1*, *CLDN*, *OCLN*, and *JAM2*, declined in cells subjected to oxidative stress. This decrease may result from the upregulation of the *NF-κB*, *MAPK*, and protein kinase signaling pathways, which negatively regulate the transcription of tight junction-related genes [68,69]. With the exception of Trp, all amino acid interventions alleviated oxidative stress and led to elevated expression of tight junction genes. Previous studies have demonstrated that improved TEER values and reduced paracellular permeability are strongly linked to the conservation of tight junction integrity at the molecular scale [70,71]. This effect is probably due to Arg's capacity to suppress the activation of *NF-κB* and *MAPK* inflammatory pathways and thus limit tight

junction impairment, while Thr maintains barrier integrity by supporting mucin synthesis [20,24,72]. Therefore, the evidence indicates that Arg and Thr treatment in cIECs promotes intestinal barrier integrity by increasing tight junction gene expression, lowering paracellular permeability, and sustaining electrical resistance.

In conclusion, oxidative stress diminishes cell viability and antioxidant capacity, alters morphology, and weakens barrier integrity in cIECs, while treatment with Arg, Trp, and Thr partially mitigates these adverse outcomes. Specifically, 250 μM Arg and Trp contribute to improved antioxidant capacity. Additionally, 500 μM Arg and all levels of Thr decrease paracellular permeability, elevate TEER, and stimulate the expression of tight junction-related genes, demonstrating strengthened barrier function. Hence, the amino acids evaluated in this study improve key functional properties of cIECs by providing protection against oxidative stress.

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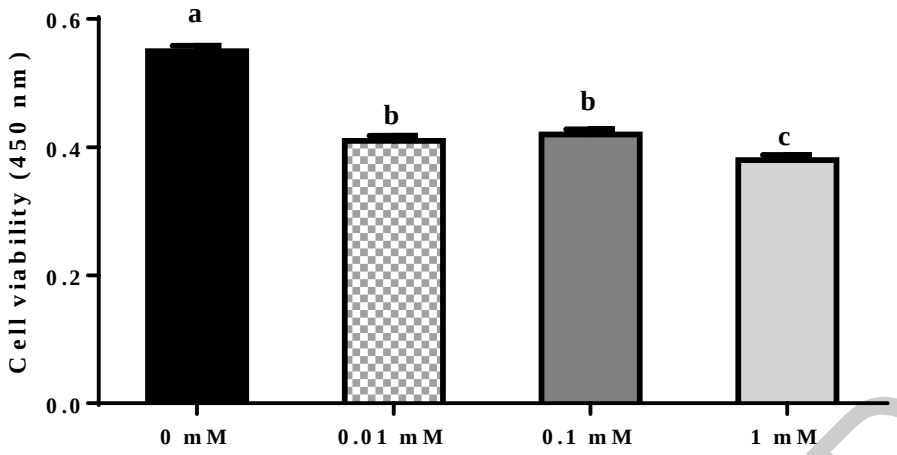
Table 1. The sequence of the primers used in quantitative real-time PCR

RNA target	Primer sequence (5'-3')	Size for PCR product (bp)	Accession no.
GAPDH	F: ATGGCATCCAAGGAGTGAGC	130	NM_204305.2
	R: GGGAACAGAACTGGCCTCTC		
ZO-1	F: AGGTGAAGTGTTTCGGGTTG	188	XM_015278975.1
	R: AGAAATCCGCTCGATCTCCT		
CLDN1	F: GCTGACCTGTACTTGAGCTG	171	NM_001013611.2
	R: TGGCACAGGGTTAATGCAAA		
OCLN	F: GTGGAGTCCAGTGATGAGCG	142	NM_205128.1
	R: TGTCCATCTCAGCACAGAGC		
JAM2	F: GTGAATTTACAGTTCCTCCC	187	NM_001006257.2
	R: GTTATGTTGGCTGTTCTAGC		

F, forward primer; R, reverse primer; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *ZO-1*, zonula occludens-1; *CLDN1*, claudin-1; *OCLN*, occludin; *JAM2*, junctional adhesion molecule 2.

661 **Figure captions**

662



663

664 **Fig. 1**. Determination of optimal H_2O_2 concentration for oxidative stress induction in cIECs.
665 Cells were treated with different concentrations of H_2O_2 (0, 0.01, 0.1, or 1 mM) for 6 h, and
666 cell viability was measured. All data are presented as means ± SEM (n = 6). Different letters
667 indicate significant differences among treatments ($p < 0.05$).

668

669

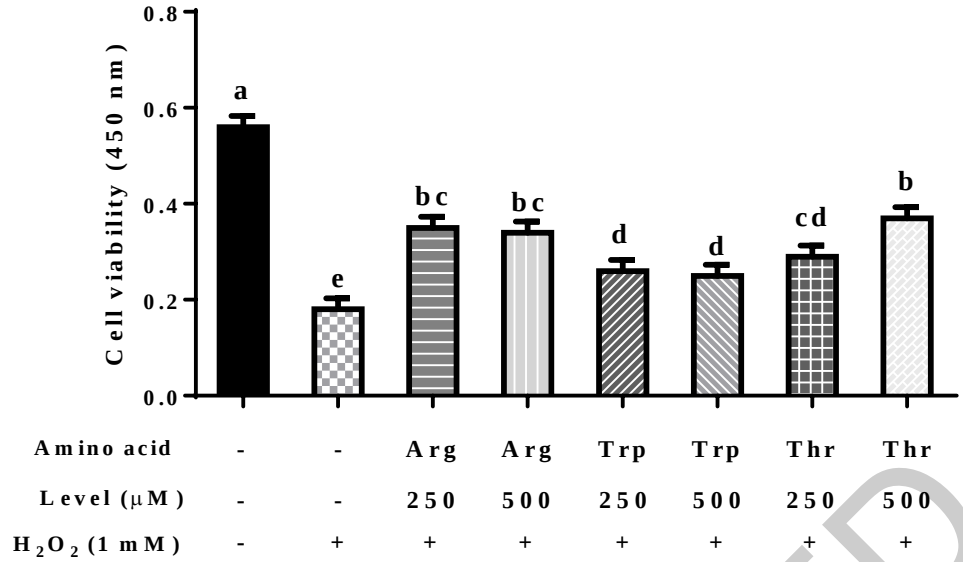
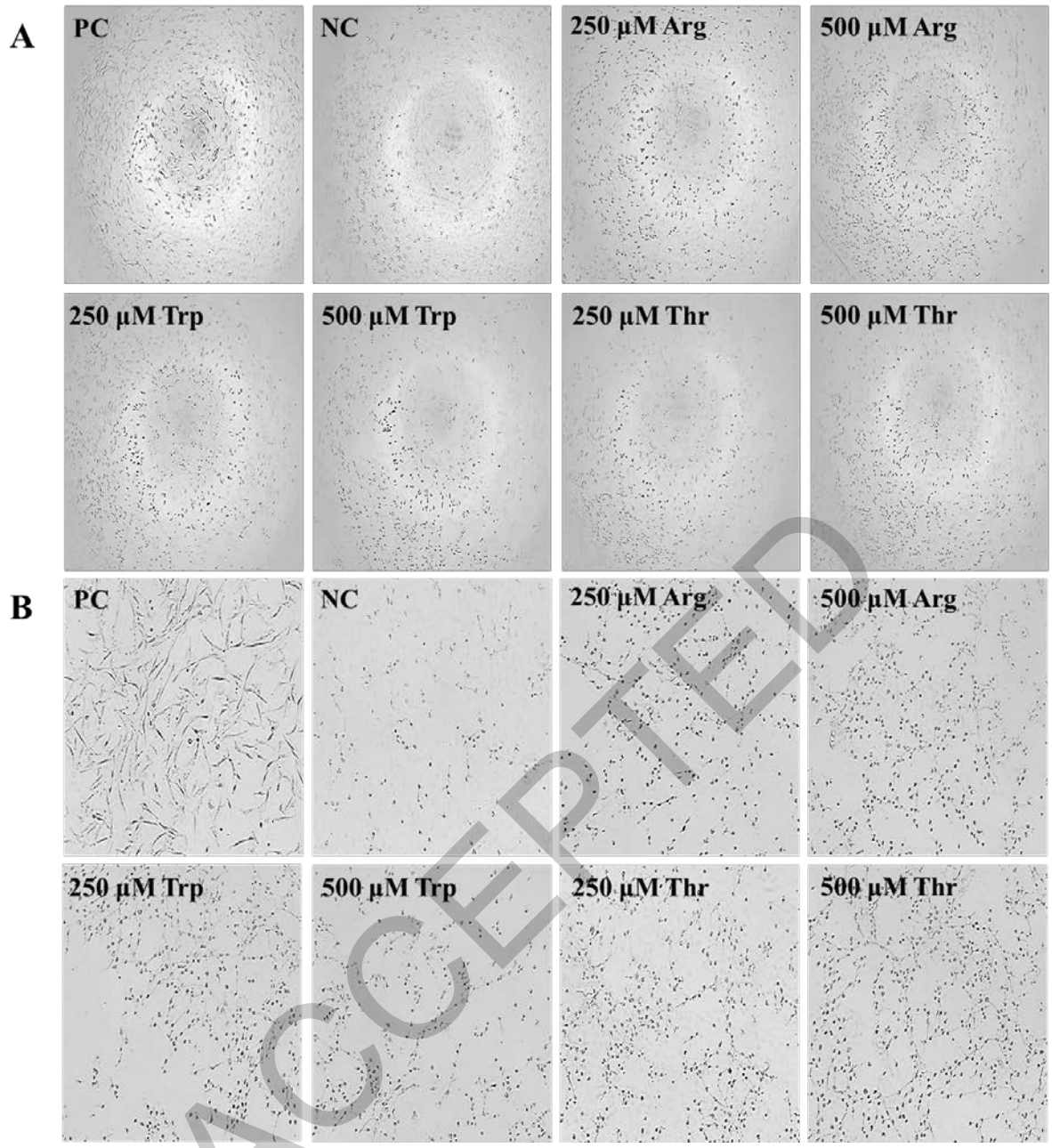


Fig. 2. Effects of arginine (Arg), tryptophan (Trp), and threonine (Thr) on cell viability induced with oxidative stress in cIECs. Cells were pretreated with Arg, Trp, or Thr at levels of 250 or 500 μM for 24 h, followed by exposure to 1 mM H_2O_2 for 6 h. All data are presented as means ± SEM (n = 6). Different letters indicate significant differences among treatments ($p < 0.05$).



677

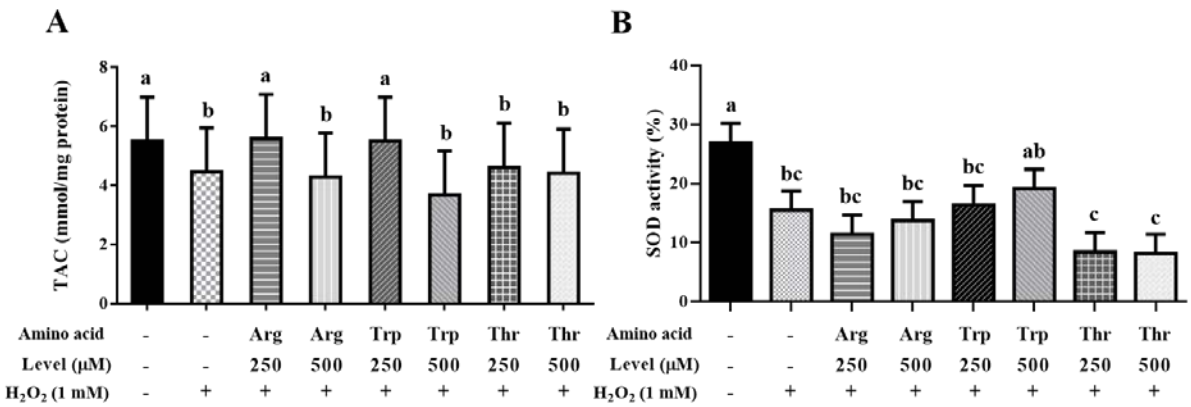
678 **Fig. 3.** Effects of arginine (Arg), tryptophan (Trp), and threonine (Thr) on morphology induced

679 with oxidative stress in cIECs. Cells were pretreated with Arg, Trp, or Thr at levels of 250 or

680 500 μM for 24 h, followed by exposure to 1 mM H_2O_2 for 6 h. (A) 2× magnification, (B) 10×

681 magnification.

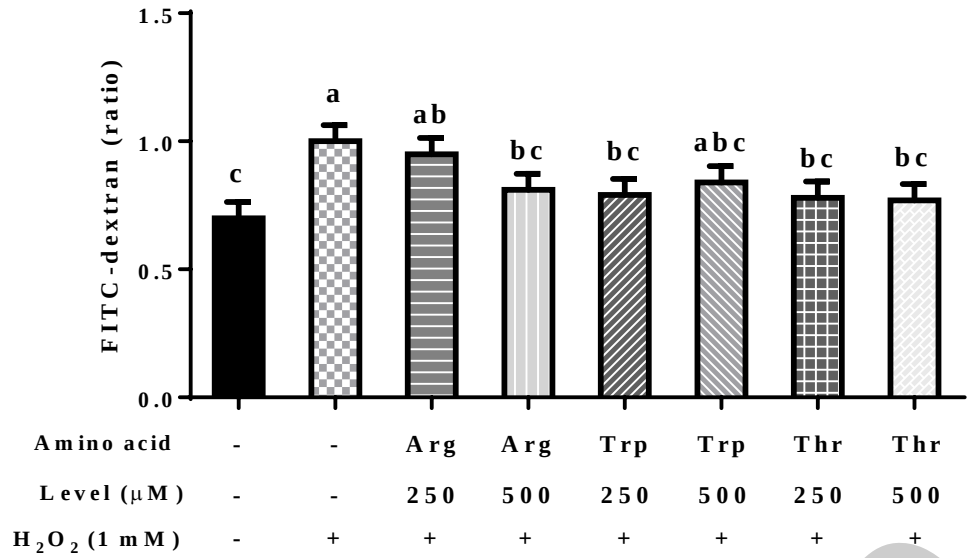
682



683

684 **Fig. 4**. Effects of arginine (Arg), tryptophan (Trp), and threonine (Thr) on antioxidant capacity
685 induced with oxidative stress in cIECs. Cells were pretreated with Arg, Trp, or Thr at levels of
686 250 or 500 μM for 24 h, followed by exposure to 1 mM H_2O_2 for 6 h. (A) TAC and (B) SOD
687 of cIECs. All data are presented as means ± SEM (n = 6). Different letters indicate significant
688 differences among treatments ($p < 0.05$).

689



690

691 **Fig. 5**. Effects of arginine (Arg), tryptophan (Trp), and threonine (Thr) on FITC-dextran
692 induced with oxidative stress in cIECs. Cells were pretreated with Arg, Trp, or Thr at levels of
693 250 or 500 μM for 24 h, followed by exposure to 1 mM H_2O_2 for 6 h. All data are presented as
694 means ± SEM (n = 6). Different letters indicate significant differences among treatments ($p <$
695 0.05).

696

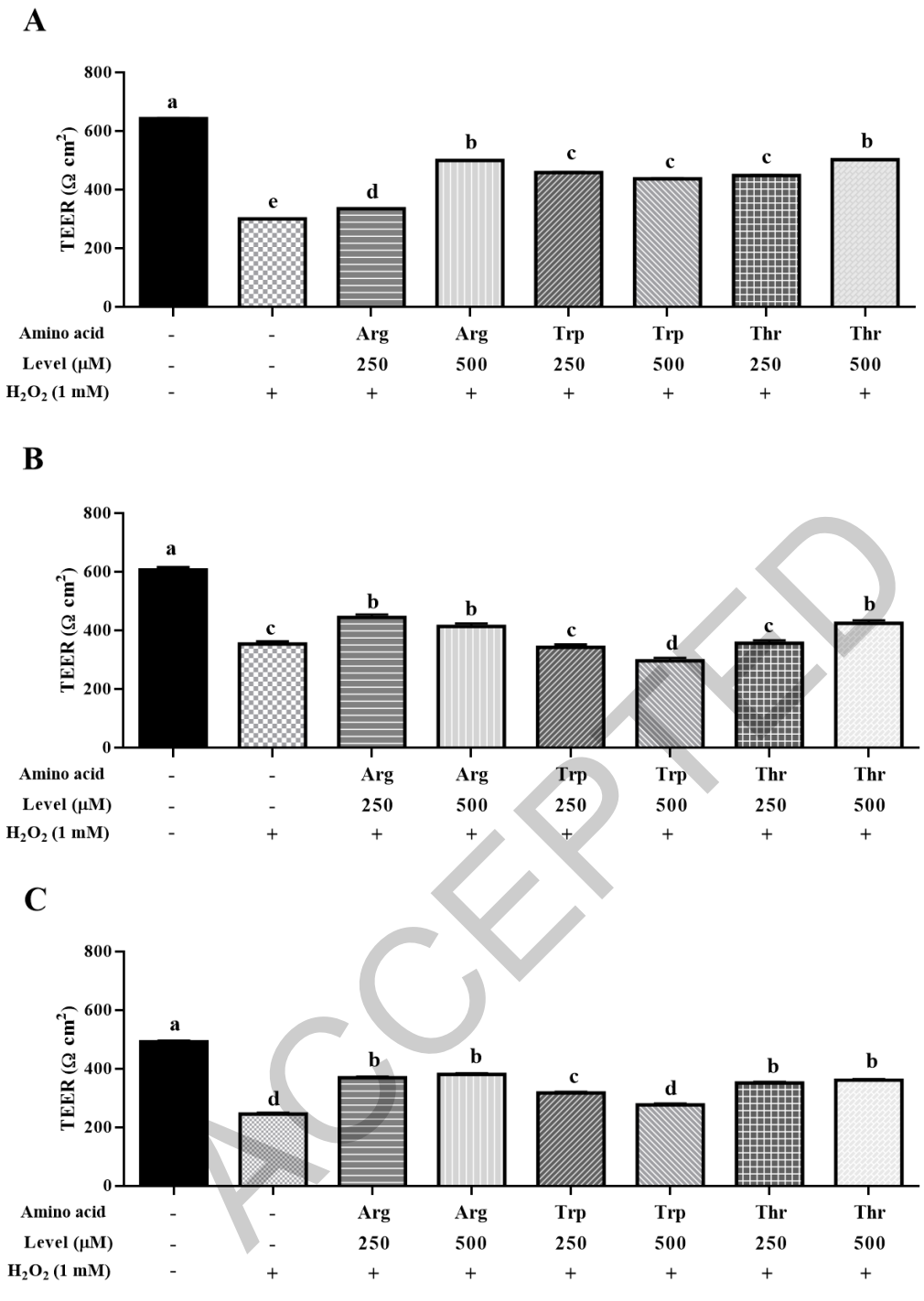


Fig. 6. Effects of arginine (Arg), tryptophan (Trp), and threonine (Thr) on transepithelial electrical resistance (TEER) induced with oxidative stress in cIECs. Cells were pretreated with Arg, Trp, or Thr at levels of 250 or 500 μM for 24 h, followed by exposure to 1 mM H_2O_2 for 6 h. (A) TEER was measured at 0 h. (B) TEER was measured at 24 h. (C) TEER was measured at 48 h. All data are presented as means ± SEM (n = 6). Different letters indicate significant differences among treatments ($p < 0.05$).

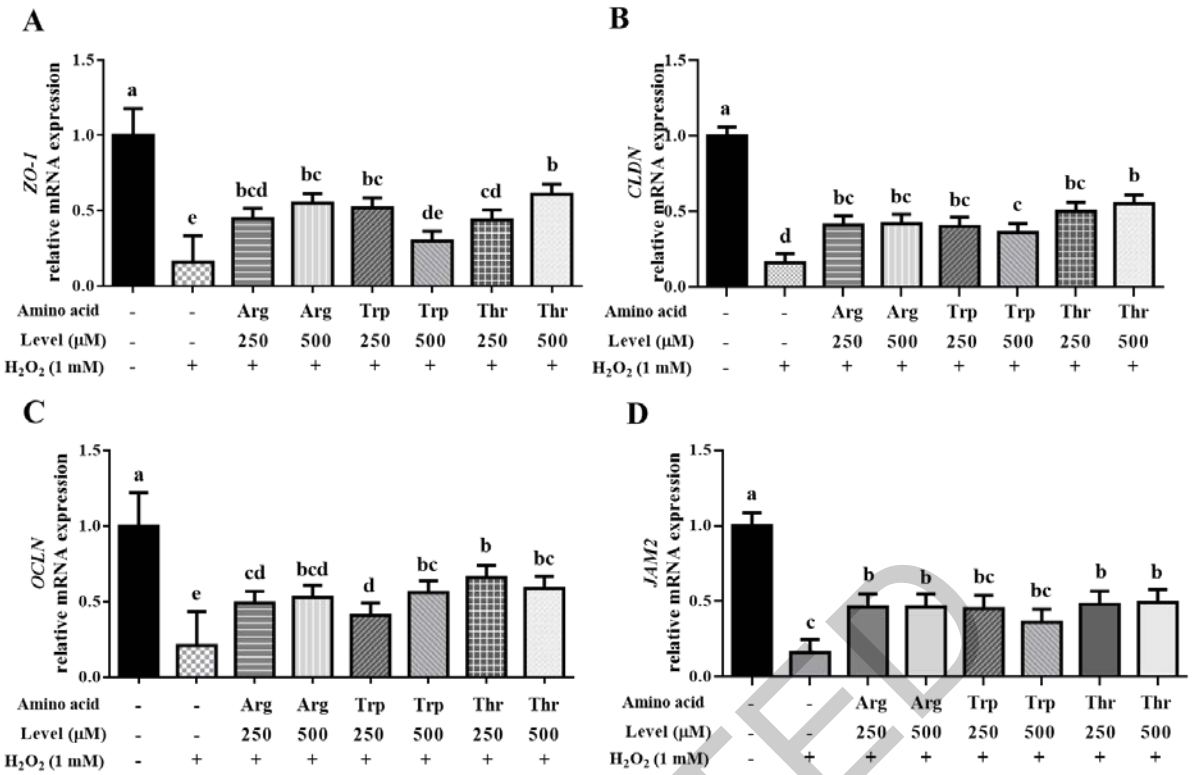


Fig. 7. Effects of arginine (Arg), tryptophan (Trp), and threonine (Thr) on mRNA expression of tight junction induced with oxidative stress in cIECs. Cells were pretreated with Arg, Trp, or Thr at levels of 250 or 500 μM for 24 h, followed by exposure to 1 mM H_2O_2 for 6 h. (A–D) The mRNA expression of *ZO-1*, *CLDN*, *OCLN*, and *JAM2*. All data are presented as means ± SEM (n = 6). Different letters indicate significant differences among treatments ($p < 0.05$).