L-Arginine inhibits hydrogen peroxide-induced oxidative damage and inflammatory response by regulating antioxidant capacity in ovine intestinal epithelial cells

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Introduction

The intestinal epithelium is constituted by intestinal epithelial cells (IECs) under proliferation and differentiation, and these IECs are arranged in the monolayer continuously, which also sets a separation between intestinal mucosa and bowel lumen (Tang et al. 2016). The IECs generate a semipermeable barrier for the efficient absorption of water and nutrients; meanwhile, they prevent the penetration of pathogens, allergens, and toxins into the blood circulation and mucosal tissues through the bowel lumen (Zhao et al. 2015). The intestinal barrier functions can be compromised by oxidative stress (OS), a status of imbalance between antioxidants and prooxidants, which results in elevated toxin and allergen permeation (Iizuka et al. 2007). Antioxidant deficiency and/or excess reactive oxygen species (ROS) production is associated with IEC injury.
species (ROS) generation can lead to endogenous OS (Nathan and Cunningham-Bussel 2013). Hydrogen peroxide ($H_2O_2$), a highly ROS, has been shown to attribute to the generation of other ROS, such as hydroxyl radicals, and the imbalanced oxidant/antioxidant status in vitro and in vivo (Yin et al. 2015; Essick et al. 2013). OS is involved in the pathogenesis of diverse gastrointestinal disorders, including inflammatory bowel disease (IBD), gastrointestinal tumours, and peptic ulcers (Bhattacharyya et al. 2014; Zou et al. 2016). Hence, we need safe and effective treatments to keep the redox balance in the intestine.

According to prior research, antioxidant or bioactive substance supplemented in diets effectively decreases OS (Talavera et al. 2017; Zhang et al. 2019a; Qiu et al. 2019). The addition of L-arginine (Arg) can be adopted to be the potential nutrition strategy to boost intestinal nutrient absorption and barrier function, thus treating endotoxin-infected neonatal lambs and infants (Zhang et al. 2019b). Arg, one of the semi-essential amino acids, exerts a vital part in some metabolic pathways, like creatine, nitric oxide (NO), polyamines and protein biosynthesis (Evoy et al. 1998). Degradation of Arg in the rumen may interfere with the use of Arg as a feed additive. Rumen-protected Arg should not be degraded by rumen microbes (Keith et al. 2018) and may increase intestinal development in ruminants. Under physiological conditions, Arg boosts the generation of NO within tissues like the small intestine, while NO exerts a vital part in the regulation of the antioxidant defense system (Rhoads and Wu 2009; Dai et al. 2013). A growing amount of evidence supports the role of the Arg – NO signalling pathway in regulating the metabolic processes for energy substrates and nutrient-sensing signals (Talavera et al. 2017; Mansouri et al. 2018). Arg stimulates hypertrophy, hyperplasia and differentiation of ovine conceptus trophoderm cells via cell signalling pathways involving nitric NO and polyamines (Bazer et al. 2015; Kim et al. 2011). Arg supplementation of whole milk powder (1% DM basis) can enhance intestinal development and integrity in the duodenum and jejunum, either through increased cell growth or proliferation or through reduced levels of cellular atrophy, but only when calves were fed the higher milk allowance (Van Keulen et al. 2020). Previous study has demonstrated that Arg supplementation was able to promote the growth of Hu suckling lambs with intra-uterine growth retardation (IUGR) and maintain their intestinal integrity, immune function, and oxidative status (Zhang et al. 2018). The IUGR suckling lambs supplemented with Arg show enhanced bowel function, which is achieved through modulating the oxidation state by a NO-dependent pathway (Zhang et al. 2019c). Nonetheless, little is known about how Arg affects the ovine intestinal epithelial cells (IOECs) redox status induced by $H_2O_2$.

In this regard, Arg was supposed to protect IOECs against $H_2O_2$-induced oxidative damage through modulating the antioxidant ability by the NO-dependent pathway. Therefore, the present work aimed to prove this hypothesis using the $H_2O_2$-induced oxidative damaged IOECs model.

**Material and methods**

**Reagents**

The antibiotics, foetal bovine serum, and the Dulbecco’s modified Eagle’s F12 Ham media (DMEM) were obtained from Invitrogen (Grand Island, NY, USA). The 2.5% trypsin solution and custom-made Arg-free DMEM (modified DMEM no.08–5009EF, Gibco) were provided by Gibco (Carlsbad, CA, USA). BD Biosciences (Bedford, MA, USA) was responsible for synthesising the epidermal growth factor (EGF), and plastic culture plates were provided by Corning Inc. (Corning, NY, USA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

**Cell cultures**

All experimental procedures were approved by the Ethics Committee of Yangzhou University (SYKK2013-0057). IOECs were obtained from the jejenum of a non-suckling neonatal lamb and cultivated according to previous description (Zhan et al. 2018). Then, IOECs (passages 20–25) were plated in the DMEM containing 5% foetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 4 mmol/L glutamine, 1% non-essential amino acids (NEAA), 1 × insulin transferrin selenium (ITS) and 15 mg/mL EGF, and incubated within the 75 cm² plastic flasks equipped with the vented capos (BD Falcon). Cell passage was conducted at an interval of 3 days according to the previous description until reaching 20–25 passages (Zhan et al. 2018). The culture media were replaced at an interval of 2 days. At confluence, cells were seeded and trypsinized in 24-well culture plates with approximately $2 \times 10^4$ cells/well. Thereafter, all cells were cultured within the humidified incubator containing 95% $O_2$ and 5% $CO_2$ at 37°C. After reaching 85–95% cell confluence, routine cell passage (at the split ratio of 1:3) was carried out. Following 16 h of incubation, cells were cultured for 6 h in the custom-made Arg-free
DMEM (modified DMEM, Gibco) to minimise the total Arg content in cells. The 5% FBS in Arg-free DMEM provided 10 μM of Arg.

The IOECs (1 × 10⁶/well) were inoculated within the 96-well plates and cultivated in complete DMEM. After incubation for 16 h, IOECs were further cultured within the Arg-free DMEM for 6 h to minimise Arg amount in cells, followed by 24 h of treatments (n = 6/group) as follows: (1) control (CON) group, in which IOECs were cultured in Arg-free DMEM containing 100 μM Arg; (2) Arg group, in which IOECs were cultured in Arg-free DMEM containing 350 μM Arg; (3) H₂O₂ group, in which IOECs were cultured in CON group plus 150 μM H₂O₂; (4) Arg + H₂O₂ group, in which IOECs were cultured in Arg group plus 150 μM H₂O₂. The Arg doses were selected according to previous studies (Xiao et al. 2017; Zhang et al. 2019a, 2019b) as well as a previous dose-response experiment performed in our laboratory (data not shown). The H₂O₂ dose was selected according to previous research (Jiang et al. 2017) and a previous dose-response experiment performed in our laboratory (data not shown).

Following incubation for 24 h, cells in 1.5 mL phosphate buffered saline (PBS) were broken through ultrasonic treatment, followed by centrifugation to collect the supernatant. One-mL cellular supernatant was harvested and preserved under the temperature of −20 °C for a subsequent experiment. Cells cultured within the 6-well plates were twice rinsed with PBS gently, followed by lysis using the radio immunoprecipitation assay (RIPA) Lysis Buffer R2220 (containing 1% PMSF) following manufacturers’ protocols. The bicinchoninic acid (BCA) reagent for protein assay was used to determine the cell protein content at the wavelength of 562 nm following the manufacturer’s protocols. Later, those collected protein samples were preserved under the temperature of −20 °C before the experiment (Tang et al. 2018). All values were standardised relative to the control and calculated according to a previous description (Zhao et al. 2014).

**Cell viability**

The viability of cells was determined using the cell counting KIT-8 (CCK-8) assay following the manufacturer’s instructions. Briefly, IOECs (6000 cells/well) were inoculated in the 96-well plates for 24 h before treatment in corresponding media. Then, cells from different treatment groups were collected and determined. The data were corrected based on the control group and calculated according to a previous description (Zhao et al. 2014).

**Content of mitochondrial reactive oxygen species (ROS)**

Isolated intestinal mitochondria was treated with 2′,7′-dichlorohydro-fluorescein diacetate (DCFH-DA) that can pass through the mitochondria membrane and is hydrolysed by intracellular esterase. ROS oxidises dichlorohydro-fluorescein (DCFH) and converts DCFH to DCFH-DA, which is highly fluorescent at 485 nm, and the emission was detected at 525 nm (Cao et al. 2019). The isolated mitochondria (0.4 mg/mL) were treated with 2 umol/L DCFH-DA and incubated at room temperature for 20 min (Cao et al. 2018). The fluorescence intensity was detected using a fluorescence microplate reader as described by Pipatpiboon et al. (2012). The fluorescence intensity of all samples was expressed as fold changes, calculated relative to the control group (Pipatpiboon et al. 2012).

**Dehydrogenase (LDH) activity and malonaldehyde (MDA) level**

Cells were cultured in corresponding media for 24 h, then 1.0 mL cell supernatant was collected and preserved in the −20 °C refrigerator before analysis. Cells in the six-well plates were washed with PBS twice, and RIPA Lysis Buffer R2220 (containing 1% PMSF) was utilised to lyse IOECs according to manufacturer protocols. The cell protein content was determined using the reagent for the bicinchoninic acid (BCA) protein assay. LDH content was determined by the LDH assay kit (Nanjing Jiangcheng Biotechnology Institute) according to a previous description (Jiao et al. 2015). MDA content was detected by the MDA assay kit (Nanjing Jiangcheng Biotechnology Institute). Every assay was repeated six times.

**Activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and total antioxidant capacity (T-AOC)**

Cells were incubated for 24 h, broken through ultrasonic treatment, and centrifuged for obtaining supernatants. Then, 1.0 mL cell supernatants were collected and preserved in the −20 °C refrigerator before analysis. The samples were analysed in two hours after sampling. Cells in the six-well plates were washed with PBS twice gently, and IOECs were lysed by the RIPA Lysis Buffer R2220 (containing 1% PMSF) following manufacturer protocols. The cell protein was determined by the BCA protein assay reagent at the wavelength of 562 nm following manufacturer protocols. The extracted protein sample was preserved in the
−20 °C refrigerator before analysis. The CAT, SOD, GSH-Px, and T-AOC contents in cells and cell supernatants were determined using the CAT, SOD, GSH-Px, and T-AOC assay kits (Nanjing Jiangcheng Biotechnology Institute) according to previous description (Xu et al. 2016). Every assay was repeated six times.

**Transepithelial electrical resistance (TEER) experiment**

TEER is a tool that applies an epithelium volt-ohmmeter (Millicell ERS-2; Millipore Corporation) in measuring tight-junction (TJ) integrity (Xia et al. 2016). Briefly, cells (5 × 10⁴/well) were cultured within cell culture Transwells (pore size, 0.4 μm; membrane area, 0.33 cm²) and incubated in 24-well plates. Thereafter, TEER was determined according to the difference in potential detected between the basolateral and apical cell layers by an epithelium volt-ohmmeter. The resistance measured across a culture insert with no seeded cells was used for background correction of all TEER measurements. Later, cells were cultivated for 24 h in different mediums. The blank resistance was then subtracted from the measured resistance to obtain the effective TEER. TEER value was calculated as: Unit Area Resistance (KΩ·cm²) = Resistance (KΩ) × Effective membrane Area (cm²). The unit area resistance was obtained by multiplying the metre reading by the effective membrane area of the insert (Mandal et al. 2020). Resistance was presented in the manner of KΩ·cm². Every assay was repeated six times.

**Paracellular permeability assay**

Cells were treated similarly to that in TEER determination. In brief, the apical cell monolayer was added with 1 g/L fluorescein isothiocyanate (FITC) –labelled dextran (20 kDa). Then, the aliquot media were discarded from the basolateral chamber; and the fluorescence intensity of FITC-dextran was determined by SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices) at the emission and excitation wavelengths of 520 nm and 490 nm, respectively. Standard curve for calculating the FITC-dextran concentration in the samples was obtained by diluting FITC-dextran in non-treated cells diluted with PBS at different rates (Park et al. 2016; Li et al. 2018). Values were converted in concentrations of FITC-dextran (ng/mL) using a standard curve. Afterward, the amount of FITC-dextran that was passed from the apical chamber to the basolateral one was defined as the monolayer cell permeability. Besides, the content of FITC-dextran was calculated by taking away the fluorescence level within the FITC-free media (He et al. 2020).

**Cytokine experiment**

The Enzyme Immunometric Assay (ELISA) kits (R&D Systems, Oxford, UK) were used to determine the tumour necrosis factor α (TNF-α), interleukin (IL)-1β and IL-6 contents in cell culture supernatants following the manufacturer’s instructions. Then, the absorbance value in every well was determined at the wavelength of 450 nm; the limits of detection for TNF-α, IL-1β, and IL-6 were 8.0, 30.0 and 9.0 pg/mL, separately. The intra- and inter-assay coefficients of variation (CV) were no more than 10% (Zhang et al. 2019).

**Content of NO content and activity of NO synthase (NOS)**

Culture supernatants were harvested to determine NO content and NOS activity within IOECs by assay kits (Nanjing Jiancheng Biological Product, Nanjing, China) following manufacturer protocols (Guo et al. 2017). Nitrate and nitrite (NOx) were measured as oxidised stable end products of NO and the total nitrite level in the sample was determined according to the method described by Zhang et al. (2008). Results of NO content were calculated as μmol/g of protein. NOS activities were measured directly by catalysing Arg method (Zhang et al. 2008). Results of NOS activities were calculated as U/g of protein (Liu et al. 2009). The absorbance value was determined at the wavelength of 450 nm. The inter-assay CV was <15%.

**Gene levels detected by Real-Time PCR assay**

The total cellular RNA was collected by the Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer protocols. The NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to determine total RNA. cDNA was synthesised by reverse transcription of 2.5 μg RNA by Prime Script RT reagent kit (Takara, Otsu, Japan) following the manufacturer’s instructions. The Primer 5.0 software was employed for designing all primers utilised in the present work following our prior protocols relative to the sheep gene sequences (Table 1). The expression of the target gene was corrected relative to β-actin (the housekeeping gene). Real-time PCR was carried out as previously depicted (Zhang et al. 2019).
Table 1. Primer sequences used in the real-time PCR.

| Gene     | Sequences (5’-3’)          | GenBank accession number |
|----------|----------------------------|--------------------------|
| CAT      | F: CACTCAGTTGGGGATTCTCT   | NM_001009784.1           |
|          | R: ATGGGGAGCGATTTCAAGG     |                         |
| GPx1     | F: GCACGCTGTTGAGCACTCAT   | AF223942.1               |
|          | R: TCCATTTGGGGATTCTCTTTCT |                         |
| SOD2     | F: GTGAAAACCTCAAGTGCTGC   | XM_013966636.1           |
|          | R: GGCTTGCTGCTGTTATAGTA   |                         |
| Nrf2     | F: ATCCAGATGTCACATGGCG    | NM_001009392             |
|          | R: TGAAGGTGGTCTGAGATGC    |                         |
| HO-1     | F: GAAGCACAAGAAGGAAAG     | XM_005674733.2           |
|          | R: CCAATCGGAGACCCTTGCT    |                         |
| MyD88    | F: ATGTTGTGTTGTTGCTCTGC   | GQ212044.1               |
|          | R: GGAACTTCTTCTTATTGGGCTT |                         |
| TLR-2    | F: CCAAGGAAAAGCCAGGAAG    | DQ890157.1               |
|          | R: TGGACAGTGGTCTCCTCA     |                         |
| TLR-4    | F: TGCTGCTGCAAATGATAGTG   | HQ343416.1               |
|          | R: CCGTCTGAAGGCGGAGAC     |                         |
| TLR-9    | F: ATGGGGGCTCATTCTGGT     | HQ263217.1               |
|          | R: CTATCCGCGCTGCTGGG      |                         |
| TRAF-6   | F: TCGAGAAGACAGGCTCCAAG  | XM_021234166.2           |
|          | R: GCTGCGGAACTGCATTTCA   |                         |
| IL-6     | F: AGCAGAGAGAGGGTGAAGCTC | NM_001009392             |
|          | R: GACACAGCTGTTTGTTGTACAA|                         |
| IL-1β    | F: CGTCCTCTGCGAAGGCTTTAG | NM_001009465             |
|          | R: CTGGATGCTTCTTTAGGG     |                         |
| NF-kB    | F: ATAGCTGCGCCGGTGTCTAT  | XM_005226864.2           |
|          | R: GGAAATCTGTGCCTGGTATAG |                         |
| TNF-α    | F: ACACCATGAGACCAAAAGC   | NM_001002840.1           |
|          | R: AGGCAAGAGCAATCCCTGGGA |                         |
| ZO-1     | F: AGAAGATAGGCTTCGAGCCA  | AJ313188                 |
|          | R: CCT CTC CTT TGT TAA AAC AAT GTC |                |
| Occludin | F: CTGTTACGAAATGCCCCCA   | AJ313191                 |
| Claudin-1| F: ACCTCCTACCTTCTCCTCTATG| HM117762.1               |
|          | R: AGCAATGGAAGGACCTCTGA  |                         |
| iNOS     | F: TGGAGAAGCAGCTTGCTTC   | AF223942.1               |
|          | R: GGTGATCTGCAAGAATGGGTA |                         |
| eNOS     | F: AGCTACACCTGACACACTCTG | NM_001129901.1           |
| β-actin  | F: GCCATTCACCTCGCACTTTT  |                         |
|          | R: TGCAAGTGCTGCTGAGATGC  |                         |

CAT: catalase; GPx1: glutathione peroxidase 1; SOD2: superoxide dismutase 2; Nrf2: nuclear factor erythroid 2-related factor 2; HO-1: haem oxygenase-1; NQO1: quinone oxidoreductase 1; MyD88: myeloid differentiation factor 88; TRAF-6: TNF receptor-associated factor 6; TLR: toll-like receptor; IL-1: interleukin; TNF-α: tumour necrosis factor α; NF-kB: nuclear factor kappa B (p65); ZO-1: zona occludens-1; eNOS: endothelial NO synthase; iNOS: inducible NO synthase; F: forward; R: reverse.

Relative gene expression levels in the treatment groups were corrected based on the control group.

Western blotting assay

The total cellular protein was collected by the commercial kit (Beyotime Biotechnology, Jiangsu, China) following manufacturer protocols. The protein assay kit (Pierce, Rockford, IL, USA) was used to detect protein level. In this study, the primary antibodies include CAT (66765-1-lg, 1: 2000, Protein Tech, Chicago, USA), SOD2 (NB100-1969, 1:5000, Novus, CO, USA), glutathione peroxidase 1 (GPx1) (ab22604, 1:2000, Abcam, Cambridge, UK), haem oxygenase-1 (HO-1) (ab13248, 1:1000, Abcam, Cambridge, UK), Nrf2 (16396-1-AP, 1:2000, Protein Tech, Chicago, USA), quinone oxidoreductase 1 (NQO1) (ab110340, 1: 1000, Abcam, Cambridge, UK), NF-κB (p65) (3572S, 1:300, Cell Signalling, BSN, USA), phospho-NF-κB (p65) (pp65) (6923S, 1: 300, Cell Signalling, BSN, USA), TNF-α (sc-12737, 1:300, Santa Cruz Biotechnology, CA, USA), IL-1β (sc-12657, 1:300, Santa Cruz Biotechnology, CA, USA), zonula occludens-1 (ZO-1) (sc-14456, 1:1000, Santa Cruz Biotechnology, CA, USA). Claudin-1 (sc-12689, 1:1000, Santa Cruz Biotechnology, CA, USA), zonula occludens-1 (ZO-1) (sc-12689, 1:1000, Santa Cruz Biotechnology, CA, USA), inducible NOS (iNOS) (6132S, 1:1000, Cell Signalling, BSN, USA), epidermal NO synthase (eNOS) (6074S, 1:1000, Cell Signalling, BSN, USA), actin (sc-12657, 1:3000, Cell Signalling, BSN, USA), actin (sc-12657, 1:3000, Cell Signalling, BSN, USA), actin (sc-12657, 1:3000, Cell Signalling, BSN, USA).

Later, proteins were incubated with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (A13253, 1:5000, Antigene Biotech, HK, CHN) for 1 h. Then, the enhanced chemiluminescence kit (ECL-Plus, Thermo, Waltham, MA, USA) was used to detect signals. Later, the BioRad gel detection system was used to detect fluorescence intensity to scan signals. Each densitometric value was corrected relative to β-actin and was expressed as the level ± pooled SEM. The statistical significance was declared at p ≤ .05.

Statistical analysis

Data were evaluated using the ANOVA according to the SAS general linear model procedures of 2 × 2 factorial design (SAS Institute). The model included Arg, H2O2 and their interaction. All values were expressed as means ± pooled SEM. Bonferroni’s multiple comparison test was used to detect differences among treatment means. The statistical significance was declared at p ≤ .05.

Results

Effects of arg and H2O2 administration on cell viability and ROS content in IOECs during 24 h incubation period

There were no Arg x H2O2 treatment interactions (p > .05) on ROS content and cell viability (Figure 1). Compared to CON, adding H2O2 reduced cell viability and increased the ROS content, respectively (p < .05). Compared to both CON and H2O2 treatments, the addition of Arg reduced ROS content, but enhanced cell viability and ROS content, respectively (p < .05) on ROS content and cell viability (Figure 1).
viability, respectively \( (p < .05) \). The co-administration of Arg and H\(_2\)O\(_2\) normalised the cell viability ROS content.

**Effects of Arg and H\(_2\)O\(_2\) administration on LDH and MDA production in IOECs and culture medium during 24 h incubation period**

There were no Arg \( \times \) H\(_2\)O\(_2\) treatment interactions \( (p > .05) \) on the cell viability (Table 2). Compared with CON, the LDH and MDA production within cells and culture medium were increased by H\(_2\)O\(_2\) treatment \( (p < .05) \) but were reversed by Arg administration. The co-addition of Arg and H\(_2\)O\(_2\) normalised the production levels of LDH and MDA within IOECs and culture medium.

**Effects of Arg and H\(_2\)O\(_2\) administration on antioxidant capacity in IOECs and the cell supernatant during 24 h incubation period**

The activity of CAT was affected in cells and cell supernatant by an Arg \( \times \) L-NAME interaction \( (p < .05) \) (Table 3). Relative to the CON, the concentration of CAT, GSH-Px and SOD in both cells and supernatants were increased by Arg addition, decreased by H\(_2\)O\(_2\) addition but were normalised to the CON levels by the co-administration of Arg and H\(_2\)O\(_2\), respectively \( (p < .05) \). The T-AOC contents in both cells and supernatants followed the same trend i.e., increased with Arg, but decreased by H\(_2\)O\(_2\) addition, except with co-administration of Arg and H\(_2\)O\(_2\) where T-AOC contents were higher than those observed for the CON \( (p < .05) \).

**Effects of Arg and H\(_2\)O\(_2\) administration on intestinal barrier function, cytokine concentrations, NO content and NOS activity in IOECs during 24 h incubation period**

There were no Arg \( \times \) H\(_2\)O\(_2\) treatment interactions \( (p > .05) \) on the intestinal barrier function, cytokine concentrations, the level of NO and the activity of NOS in IOECs (Table 4). Arg addition increased TEER but decreased that of FITC-dextran and the reverse was observed after H\(_2\)O\(_2\) addition, relative to CON, respectively \( (p < .05) \). The co-administration of Arg and H\(_2\)O\(_2\) normalised the levels of the intestinal barrier function markers (TEER and FITC-dextran) to the
baseline state ($p < .05$). Relative to the CON, the cytokines (TNF-$\alpha$, IL-1$\beta$ and IL-6) levels were decreased by Arg addition and increased by H$_2$O$_2$ addition $p < .05$. Except for IL-6 level, the co-administration of Arg and H$_2$O$_2$ did not decrease the cytokine levels to values observed with CON nor the Arg treatments but decreased their values to those observed with H$_2$O$_2$ treatment alone ($p < .05$). Relative to the CON, the NO production was increased by Arg addition and decreased by H$_2$O$_2$ whereas the co-administration of Arg and H$_2$O$_2$ alleviated the decline in NO production compared to the H$_2$O$_2$ treatment only but did not recover its production to the CON state ($p < .05$). The NOS activity was increased by Arg, decreased by H$_2$O$_2$ and normalised by the co-administration of Arg and H$_2$O$_2$ relative to the CON, respectively ($p < .05$).

**Effects of Arg and H$_2$O$_2$ administration on mRNA and protein expression of intestinal integrity, immune function, antioxidant capacity and nitric oxide-dependent pathway in IOECs during 24 h incubation period**

The mRNA expressions of intestinal integrity-related genes (ZO-1, occludin and claudin-1) were up-regulated by Arg, down-regulated by H$_2$O$_2$, but were normalised by the co-administration of Arg and H$_2$O$_2$ relative to the CON, respectively ($p < .05$) (Table 5). Protein expressions of intestinal integrity-related genes (ZO-1, occludin and claudin-1) followed the same trend of mRNA expression except for occludin where its protein expression was not normalised by the co-administration of Arg and H$_2$O$_2$ relative to the CON ($p < .05$) (Figure 2).

The mRNA expressions of immune function-related genes (MyD88, TLR-2, TLR-4, TLR-9, TRAF-6, IL-6, IL-1$\beta$, NF-$k$B and TNF-$\alpha$) were down-regulated by Arg addition and up-regulated by H$_2$O$_2$ addition relative to the CON treatment, respectively ($p < .05$) (Table 5). Relative to the CON, the co-administration of Arg and H$_2$O$_2$ normalised the mRNA expressions of immune function-related genes except for TLR-4, TRAF-6 and TNF-$\alpha$ which did not return to the baseline values but were still lower than those observed for H$_2$O$_2$ treatment ($p < .05$). The protein expressions of immune function-related genes (TNF-$\alpha$, IL-6, IL-1$\beta$, p65 and pp65) followed the same trend of mRNA expression (down-regulated by Arg addition and up-regulated by H$_2$O$_2$ addition relative to the CON treatment, respectively) ($p < .05$) (Figure 3). Relative to the CON, the co-administration of Arg and H$_2$O$_2$ normalized the protein expressions of TNF-$\alpha$, IL-1$\beta$ and p65, but further up-regulated ($p < .05$) those of IL-6 and pp65, respectively.

The mRNA expression of antioxidant capacity-related genes (CAT, GPx1, SOD2, Nrf2, HO-1 and NQO1) were upregulated by Arg, downregulated by H$_2$O$_2$, but were normalised by the co-administration of Arg and H$_2$O$_2$ except for Nrf2 and NQO1 which were greater than those observed for H$_2$O$_2$ alone but did not return to the normal state, relative to the CON treatment, respectively ($p < .05$) (Table 5). The protein expression of antioxidant capacity-related genes (CAT, GPx1, SOD2, Nrf2, HO-1 and NQO1) followed the same trend of mRNA expression (were upregulated by Arg and downregulated by H$_2$O$_2$, relative to the CON) (Figure 4). The co-administration of Arg and H$_2$O$_2$ normalised the protein expressions of these genes except for CAT and NQO1 which were their protein expressions were greater than those observed for H$_2$O$_2$ alone but did not return to the normal state, relative to the CON treatment, respectively.

The mRNA and protein expressions of NO-dependent pathway-related genes (iNOS and eNOS) were up-

| Items              | Treatments         | $p$ Value |
|--------------------|-------------------|-----------|
|                   | CON | Arg | H$_2$O$_2$ | Arg + H$_2$O$_2$ | SEM | Arg | H$_2$O$_2$ | Arg + H$_2$O$_2$ |
| Cell supernatant   |     |     |            |                  |     |     |            |                  |     |
| T-AOC, U/mL        | 1.67$^{c}$ | 2.56$^{p}$ | 1.13$^{d}$ | 1.99$^{b}$ | 0.09 | 0.07 | 0.12 | 0.108 |
| CAT, U/mL          | 1.92$^{b}$ | 2.89$^{p}$ | 1.36$^{c}$ | 2.02$^{b}$ | 0.11 | 0.09 | 0.21 | 0.37  |
| GSH-Px, U/mL       | 41.2$^{b}$ | 56.7$^{p}$ | 32.1$^{c}$ | 42.9$^{b}$ | 3.7  | 0.14 | 0.09 | 0.97  |
| SOD, U/mL          | 34.9$^{b}$ | 53.8$^{p}$ | 25.9$^{c}$ | 31.2$^{b}$ | 2.6  | 0.15 | 0.24 | 0.209 |
| Cells              |     |     |            |                  |     |     |            |                  |     |
| T-AOC, U/mg protein| 3.08$^{c}$ | 5.97$^{p}$ | 1.69$^{d}$ | 4.61$^{b}$ | 0.07 | 0.09 | 0.24 | 0.192 |
| CAT, U/mg protein  | 7.11$^{b}$ | 10.24$^{p}$ | 5.32$^{c}$ | 7.02$^{b}$ | 0.15 | 0.11 | 0.14 | 0.25  |
| GSH-Px, U/mg protein| 45.8$^{b}$ | 61.9$^{p}$ | 30.7$^{c}$ | 48.8$^{b}$ | 3.5  | 0.21 | 0.08 | 0.114 |
| SOD, U/mg protein  | 39.2$^{b}$ | 52.9$^{p}$ | 28.3$^{c}$ | 37.9$^{b}$ | 1.8  | 0.09 | 0.22 | 0.228 |

Table 3. Effects of Arg and H$_2$O$_2$ administration on antioxidant capacity in IOECs and cell supernatant during 24 h incubation period$^{1}$.

$^{1}$Data are means and pooled SEMs, $n = 6$. $^{a,b}$Means in a row with superscripts without a common letter differ, $p < .05$. IOECs: ovine intestinal epithelial cells; CAT: catalase; GSH-Px: glutathione peroxidase; SOD: superoxide dismutase; T-AOC: total antioxidant capacity; CON: Arg-free DMEM supplemented with 100 $\mu$M Arg; Arg treatment: Arg-free DMEM supplemented with 350 $\mu$M Arg; H$_2$O$_2$ treatment: CON supplemented with 150 $\mu$M H$_2$O$_2$; Arg + H$_2$O$_2$ treatment: Arg treatment supplemented with 150 $\mu$M H$_2$O$_2$. DMEM: Dulbecco’s modified Eagle’s F12 Ham media.
Table 4. Effects of Arg and H2O2 administration on intestinal barrier function, cytokine concentrations, NO content and NOS activity in IOECs during 24 h incubation period.

| Treatments                      | CON  | Arg  | H2O2 | Arg + H2O2 | SEM  | Arg  | H2O2 | Arg × H2O2 |
|---------------------------------|------|------|------|------------|------|------|------|------------|
| TEER, KΩ cm⁻²                  | 1.13b| 1.76c| 0.53c| 1.06b      | 0.08 | 0.09 | 0.07 | 0.213      |
| FITC-dextran, ng/mL            | 321  | 230  | 457  | 317b       | 8.3  | 0.11 | 0.03 | 0.107      |
| IL-1β, pg/mL                   | 89.21c| 64.12d| 156.1 | 112.3b     | 4.8  | 0.09 | 0.01 | 0.214      |
| IL-6, pg/mL                    | 36.5b | 25.6c| 58.4c| 32.2b      | 2.4  | 0.017| 0.009| 0.112      |
| TNF-α, pg/mL                   | 33.8  | 23.5d| 55.8b| 41.9b      | 3.1  | 0.016| 0.011| 0.087      |
| NO, μmol/g of protein          | 4.91b| 6.69a| 2.68c| 3.74c      | 0.61 | 0.009| 0.008| 0.103      |
| NOS, U/g of protein            | 501  | 579  | 411  | 499b       | 21   | 0.013| 0.025| 0.226      |

1Data are means and pooled SEMs, n = 6. a,b,cMeans in a row with superscripts without a common letter differ, p < .05. IOECs: ovine intestinal epithelial cells; TEER: transepithelial electrical resistance; FITC-dextran: fluorescein isothiocyanate (FITC)-conjugated dextran; TNF-α: tumour necrosis factor α; IL: interleukin; NO: nitric oxide; NOS: NO synthase; CON: Arg-free DMEM supplemented with 150 μM Arg; Arg treatment: Arg-free DMEM supplemented with 350 μM Arg; H2O2 treatment: CON supplemented with 150 μM H2O2; Arg + H2O2 treatment: Arg treatment supplemented with 150 μM H2O2; DMEM: Dulbecco’s modified Eagle’s F12 Ham media.

Table 5. Effects of Arg and H2O2 on the mRNA abundance of genes in IOECs for 24 h.

| Treatments                      | CON  | Arg  | H2O2 | Arg + H2O2 | SEM  | Arg  | H2O2 | Arg × H2O2 |
|---------------------------------|------|------|------|------------|------|------|------|------------|
| Intestinal integrity-related genes |      |      |      |            |      |      |      |            |
| ZO-1                            | 1.00b| 1.56a| 0.49c| 0.97b      | 0.08 | 0.09 | 0.012| 0.192 |
| Occludin                        | 1.00c| 1.62a| 0.57d| 1.39b      | 0.07 | 0.03 | 0.007| 0.086 |
| Claudin-1                       | 1.00b| 1.41a| 0.62b| 1.08b      | 0.10 | 0.007| 0.010| 0.203 |
| Immune function-related genes    |      |      |      |            |      |      |      |            |
| MyD88                           | 1.00b| 0.54a| 1.47a| 0.91b      | 0.06 | 0.24 | 0.008| 0.115 |
| TLR-2                           | 1.00b| 0.68a| 1.37a| 1.12b      | 0.09 | 0.07 | 0.031| 0.079 |
| TLR-4                           | 1.00b| 0.61a| 1.65a| 1.34b      | 0.05 | 0.35 | 0.04 | 0.278 |
| TLR-9                           | 1.00b| 0.46a| 1.35a| 1.04b      | 0.04 | 0.12 | 0.009| 0.174 |
| TRAF-6                          | 1.00b| 0.59b| 1.71a| 1.36b      | 0.06 | 0.07 | 0.005| 0.094 |
| IL-6                            | 1.00b| 0.46c| 1.41b| 1.06b      | 0.11 | 0.25 | 0.006| 0.099 |
| IL-1β                           | 1.00b| 0.65a| 1.58a| 0.93b      | 0.08 | 0.08 | 0.05 | 0.126 |
| NF-kB                           | 1.00b| 0.44a| 1.43a| 0.97b      | 0.09 | 0.03 | 0.021| 0.868 |
| TNF-α                           | 1.00b| 0.59d| 1.57a| 1.36b      | 0.13 | 0.09 | 0.012| 0.104 |
| Antioxidant-related genes        |      |      |      |            |      |      |      |            |
| CAT                             | 1.00b| 1.37a| 0.63a| 0.95b      | 0.06 | 0.02 | 0.026| 0.093 |
| GPx1                            | 1.00b| 1.54a| 0.52a| 1.02b      | 0.08 | 0.09 | 0.010| 0.119 |
| SOD2                            | 1.00b| 1.45a| 0.69a| 0.89b      | 0.07 | 0.07 | 0.008| 0.301 |
| Nrf2                            | 1.00b| 1.41a| 0.43a| 0.78b      | 0.08 | 0.09 | 0.012| 0.245 |
| HO-1                            | 1.00b| 1.31a| 0.68a| 1.06b      | 0.08 | 0.09 | 0.008| 0.129 |
| NQO1                            | 1.00b| 1.40a| 0.42a| 0.73b      | 0.07 | 0.02 | 0.029| 0.085 |
| NO dependent pathway-related genes |      |      |      |            |      |      |      |            |
| iNOS                            | 1.00c| 1.57a| 0.53a| 1.29b      | 0.09 | 0.09 | 0.006| 0.118 |
| eNOS                            | 1.00b| 1.46a| 0.60a| 0.89b      | 0.07 | 0.02 | 0.009| 0.104 |

1Data are means and pooled SEMs, n = 6. a,b,cMeans in a row with superscripts without a common letter differ, p < .05. CON: Arg-free DMEM supplemented with 100 μM Arg; Arg treatment: Arg-free DMEM supplemented with 350 μM Arg; H2O2 treatment: CON supplemented with 150 μM H2O2; Arg + H2O2 treatment: Arg treatment supplemented with 150 μM H2O2; DMEM: Dulbecco’s modified Eagle’s F12 Ham media.

Discussion

Arg has been shown to protect against heat stress-induced intestinal epithelial barrier injury by enhancing the tight junction proteins through activation of AMPK signalling (Xia et al. 2019) and by promoting the synthesis of NO, the potent protector against oxidative stress (Varasteh et al. 2018), thus maintain gut health and integrity. In ovine, Arg has been shown to protect IECs from lipopolysaccharide-induced intestinal barrier injury and apoptosis (Zhang et al. 2019a, 2019b). Markers are applied in assessing OS in the cultivated cells, including MDA content and LDH production (Pirincicgilo et al. 2010; Todd et al. 2016). According to our findings, the LDH and MDA contents increased following H2O2 treatment, which suggested the presence of OS in IOECs. Arg can reduce H2O2-induced OS in IOECs, which was verified by the reduced ROS content and the enhanced cell viability in the current work. GSH-Px, SOD, and T-AOC were recognised as the vital components in the antioxidant system to scavenge ROS (Circu and Aw 2010). The lipid peroxide metabolic product, MDA, is used as a biomarker of ROS-induced OS (Zhan et al. 2007). Thus, the markedly reduced T-AOC content and antioxidant enzyme activities, as well as the remarkably increased MDA content within IOECs induced by H2O2 verified that oxidative damage occurred. Such alterations may result in gut-related disorders through inducing oxidative damage to lipids, proteins and DNA, and by increasing the membrane permeability (Murphy 2009). The elevated TNF-α, IL-1β and IL-6 contents within H2O2-injured IOECs compared with those within CON IOECs verify that OS was accompanied by increased production of pro-inflammatory cytokines (Rada et al. 2011).

regulated by Arg, but down-regulated by H2O2, relative to CON, respectively (p < .05) (Table 5; Figure 5).

The co-administration of Arg and H2O2 normalised both eNOS mRNA expression and iNOS protein expression, but further upregulated that of iNOS and downregulated the eNOS protein expression, relative to the CON, respectively (p < .05).
Within H$_2$O$_2$-injured IOECs, inflammatory cytokines and free radicals were greatly produced and released; these markers are indicators of cell and organ dysfunctions (Zhang et al. 2016; Jia et al. 2019). Based on this study, the Arg addition to H$_2$O$_2$-injured IOECs reduced the contents of inflammatory cytokines (such as TNF-$\alpha$, IL-6, and IL-1$\beta$), together with their mRNA abundances within H$_2$O$_2$-injured IOECs. Besides, Arg also down-regulated the protein expression levels of TNF-$\alpha$ and pNF-$\kappa$B within H$_2$O$_2$-injured IOECs. Based on the above-mentioned results, Arg could prevent inflammatory responses in the intestine. Similarly, dietary Arg supplementation attenuated OS in the intestinal tissues of weaned piglets (Zheng et al. 2017). In conclusion, these findings emphasised the role of Arg in alleviating intestinal dysfunction and maintaining intestinal integrity and health.

The intestinal epithelial barrier represents the first-line defense to resist the hostile environment in the
intestinal cavity (Wijtten et al. 2011). TEER stands for a tool for measuring intestinal epithelial permeability and integrity (Sun et al. 2017), which reflects the paracellular permeability of the intestinal mucosa and the opening of tight junctions (TJs) between epithelial cells (Wijtten et al. 2011). The paracellular pathways are mainly involved in FITC-dextran flux crossing intestinal epithelium (Jiao et al. 2015). The reduced TEER and elevated FITC-dextran flux indicate intestinal barrier impairment (Wijtten et al. 2011). According to our results, the Arg-treated IOECs had increased TEER but decreased FITC-dextran flux, which suggested the beneficial effect of Arg on the intestinal barrier function. Based on our results, Arg addition modulated the claudin-1, occludin, and ZO-1 contents within the H2O2-induced damaged IOECs. The above findings indicated that Arg administration could be used as an effective nutritional strategy to alleviate intestinal mucosal barrier dysfunction.

Nuclear factor erythroid 2-related factor (Nrf2) plays an important role in maintaining the homeostasis of intestinal barrier function. Figure 4. Effects of Arg and H2O2 administration on the protein expression of antioxidant capacity in IOECs during 24 h incubation period. Representative charts of western blot results (A), CAT, GPx1, SOD2, Nrf2, HO-1 and NQO1 (B) were determined. Data are means and pooled SEMs, n = 6. Labelled means without a common letter differ, p < .05. CAT: catalase; GPx1: glutathione peroxidase 1; SOD2: superoxide dismutase 2; Nrf2: nuclear factor erythroid 2-related factor 2; HO-1: haem oxygenase-1; NQO1: quinone oxidoreductase 1; IOECs: ovine intestinal epithelial cells; CON: Arg-free DMEM supplemented with 100 μM Arg; Arg treatment: Arg-free DMEM supplemented with 350 μM Arg; H2O2 treatment: CON supplemented with 150 μM H2O2; Arg + H2O2 treatment: Arg treatment supplemented with 150 μM H2O2. DMEM: Dulbecco’s modified Eagle’s F12 Ham media.

Figure 5. Effects of Arg and H2O2 administration on the protein expression of genes involved in the NO-dependent pathway in IOECs during 24 h incubation period. Representative charts of western blot results (A), iNOS and eNOS (B) were determined. Data are means and pooled SEMs, n = 6. Labelled means without a common letter differ, p < .05. NO: nitric oxide; NOS: inducible NO synthase; eNOS: epithelial NO synthase; IOECs: ovine intestinal epithelial cells; CON: Arg-free DMEM supplemented with 100 μM Arg; Arg treatment: Arg-free DMEM supplemented with 350 μM Arg; H2O2 treatment: CON supplemented with 150 μM H2O2; Arg + H2O2 treatment: Arg treatment supplemented with 150 μM H2O2. DMEM: Dulbecco’s modified Eagle’s F12 Ham media.
cells subjected to chemical oxidative stress, which is achieved through modulating diverse genes related to detoxification and antioxidation (Ryoo et al. 2015). The Nrf2 signalling pathway exerts a vital part in OS and initiates massive antioxidant genes that follow nuclear translocation (Yin et al. 2013). At resting conditions, Nrf2 is kept in the cytosol and bound onto Keap1, which represents the ubiquitin-dependent proteasomal degradation (Kuhn et al. 2011). In the case of OS, Nrf2 is dissociated from nuclear translocates and Keap1, and Nrf2 is bound onto the antioxidant response elements (AREs) in target genes, including antioxidant enzymes (such as CAT, SOD, GSH-Px) and phase II metabolic enzymes (such as quinine oxidoreductase NQO1, as well as haem oxygenase HO-1) (Park et al. 2016). HO-1 is the rate-limiting enzyme that catalyses haem into biliverdin, carbon monoxide (CO), and free iron (You et al. 2013). HO-1, together with metabolites such as CO and bilirubin, possesses anti-oxidative and anti-inflammatory activities. In addition, HO-1 is also the remarkable target gene of Nrf2 triggered depending on Nrf2 (Lv et al. 2016). NQO1 is also a phase II metabolising enzyme-mediated by Nrf2, which alleviates oxidative damage through decreasing the activity of NADPH and the production of ROS (Park et al. 2011). Antioxidant enzymes, such as CAT, SOD and GSH-Px, exert vital parts in maintaining the redox balance in cells (Gou et al. 2015). Based on our results, Arg activated Nrf2 in IOECs injured with H$_2$O$_2$, which thereby triggered the antioxidant enzymes (such as CAT, SOD and GSH-Px), as well as the phase II metabolising enzymes (HO-1 and NQO1). Arg was identified to up-regulate the protein expression levels of phase II metabolic enzymes, Nrf2 and antioxidant enzymes to reduce the H$_2$O$_2$-induced oxidative injury in IOECs.

The eNOS-mediated Arg metabolism produces NO, the critical molecule that damages blood vessels (Jobgen et al. 2006). NO plays a key role in Arg’s effect on the proliferation and growth of intestinal epithelial cells, such as cell migration stimulation (Rhoads et al. 2008). Based on our findings, Arg prevented the H$_2$O$_2$-induced injury to the intestinal barrier in IOECs, and this was associated with the Arg role as a precursor of NO. For exploring the NO pathway’s role in the function of intestinal barrier enhanced by Arg addition, iNOS and eNOS mRNA and protein expression, NO level, and NOS activity within IOECs were examined. Based on our findings, Arg up-regulated iNOS and eNOS mRNA and protein expression, NO level, and NOS activity within IOECs. Such beneficial effects indicated that Arg was associated with the superior IOECs antioxidant state in IOECs, which was partly because of the increased flux via the NO pathway.

**Conclusions**

This study indicates the potential protective effect of Arg on IOECs to resist the H$_2$O$_2$-induced cell injury, which is achieved through up-regulating the expression of Nrf2-mediated phase II metabolising enzymes and antioxidative enzymes to alleviate oxidative damage. Arg improves the intestinal epithelial barrier function, as suggested by the increased TEER and decreased paracellular permeability. Notably, the beneficial effects of Arg are related to the suppression of OS, attenuation of inflammatory response and increase in TJ integrity within H$_2$O$_2$-induced IOECs through modulating antioxidant capacity via the NO-dependent pathway.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Ethical approval**

All procedures were approved by the Animal Care and Use Committee of Yangzhou University, Yangzhou, China.

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**Data availability statement**

All data included in this study are available upon request by contact with the corresponding author.

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