Dietary Daidzein Supplementation Improved Growth Performance and Antioxidant Properties in Weaned and Growing Pigs

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Abstract

Background: In previous study, we found that soybean isoflavones in soybean meal play important roles in improving growth performance and antioxidant properties in pigs. However, it is still not known whether long-term supplementation with daidzein, an active molecule deglycosylated from daidzin, in a corn-soybean meal diet can enhance growth performance in pigs. Thus, in the present study, an animal trial was carried out to investigate the effects of dietary supplementation with daidzein on the growth performance and antioxidant capacity of pigs. Porcine intestinal epithelial cells (IPEC-J2) were also used as an *in vitro* model to explore the underlying antioxidant mechanisms of daidzein. Weanling pigs were fed a diet supplemented with 0, 25, 50, and 100 mg/kg daidzein, and IPEC-J2 cells were treated with 0.6 mM hydrogen peroxide (H_2O_2) in the presence or absence of 40 μM daidzein.

Results: Adding 50 mg/kg daidzein to the diet significantly improved body weight on day 72, average daily gain during days 0-72 and plasma superoxide dismutase activity on day 42 ($P < 0.05$). Treatment with 0.6 mM H_2O_2 for 1 h significantly decreased cell viability and catalase (CAT) activities and increased intracellular reactive oxygen species (ROS) levels and malondialdehyde (MDA) content ($P < 0.05$), while pretreatment with 40 μM daidzein prevented the decrease in cell viability and CAT activities and the increase in intracellular ROS levels and MDA content caused by H_2O_2 ($P < 0.05$). In addition, H_2O_2 stimulation significantly suppressed the expression of nuclear factor erythoid-2-related factor 2 (Nrf2), CAT, occludin and zonula occludens-1 (ZO-1), while pretreatment with daidzein preserved the expression of Nrf2, CAT, occludin and ZO-1 in H_2O_2-stimulated IPEC-J2 cells ($P < 0.05$).

Conclusions: Long-term dietary supplementation with 50 mg/kg daidzein improved growth performance and antioxidative properties in pigs. Daidzein exerted protective effects against H_2O_2-induced oxidative stress in IPEC-J2 cells, and the underlying mechanism may be related to the activation of the Kelch-like ECH-associated protein 1-Nrf2/antioxidant response element signaling pathway.

Introduction

The antioxidant system of the body maintains a balance between the generation and elimination of reactive oxygen species (ROS) [1]. Oxidative stress occurs when the production of ROS exceeds the antioxidant capacity of the body [2]. It results in damage to DNA, proteins and lipids [3], eventually leading to diseases such as aging, cardiovascular diseases, and Alzheimer's disease [4-6]. Oxidative stress in the intestinal tract can injure the intestinal structure, increase the permeability of epithelial cells, and influence the absorption function, eventually inducing gastrointestinal diseases, such as intestinal mucosal infection, colon cancer, and Crohn's disease [7]. Protecting intestinal cells from the damage caused by oxidative stress will improve intestinal function, thereby increasing growth performance in pigs.

Our previous study showed that eliminating soybean isoflavones from the diet decreased antioxidative properties, while replenishing soybean isoflavones prevented a decrease in antioxidative properties [8], indicating that soybean isoflavones play an essential role in antioxidation. Daidzin and genistin are two major components of soybean isoflavones. Soybean isoflavones are deglycosylated to aglycones by intestinal bacteria and absorbed by the intestine [9, 10]. Daidzein (4,7-dihydroxyisoflavone) is deglycosylated from daidzin. The antioxidative property of daidzein has been demonstrated in several studies. On the basis of *in vivo* studies, Xiao et al. (2015) reported that adding daidzein to a diet without any soy source significantly improved the antioxidant capacity of weaning

piglets [11]. Zhao et al. (2017) observed that dietary supplementation with daidzein increased the antioxidant capacity of bull calves [12]. In *in vitro* studies, Xu et al. (2009) evaluated the protective effects of daidzein against hydrogen peroxide (H_2O_2)-induced oxidative stress in HUVECs [13]. Wijeratne and Cuppett (2007) assessed the protective effects of daidzein against oxidative damage in Caco-2 cells [14]. Nevertheless, there is limited literature concerning whether long-term supplementation with daidzein in corn-soybean meal diets affects the growth performance and antioxidant capacity of pigs.

Therefore, the present study evaluated the effect of long-term supplementation with daidzein on the growth performance and antioxidant capacity of pigs. The antioxidant mechanism of daidzein was investigated with porcine intestinal epithelial cells (IPEC-J2), a non-transformed porcine intestinal epithelial cell line isolated from the jejunal epithelia of neonatal unsuckled piglets. The results will have implications for the application of daidzein in pig production.

Methods

Animals and experimental design

This study was approved by the Animal Care and Use Committee of the Feed Research Institute of the Chinese Academy of Agricultural Sciences. A total of 80 Large White × Landrace F1 crossbred piglets (40 barrows and 40 females), with similar initial body weights (BWs, 7.35 ± 0.14 kg) and the same age (23 days), were randomly assigned to 4 treatments, with 5 pens per treatment and 4 piglets per pen according to BW and sex (half male and half female), for a 72-day trial. The dietary treatments included a corn-soybean meal basal diet supplemented with 0 (control group), 25, 50 or 100 mg/kg daidzein. The daidzein (purity ≥ 98%) used in this experiment was purchased from Guanghan Biochemical Products Co., Ltd. (Guanghan, China). The diets were formulated according to National Research Council (2012) nutrient requirements [15], and the composition and nutrient levels in the basal diets are shown in Table 1. The barn was maintained at a temperature between 25 °C and 28 °C with a 12-h light/dark cycle. Throughout the experiment, the pigs were allowed *ad libitum* access to water and feed.

Sample collection

On days 14, 28 and 42 of the trial, one piglet from each pen was selected randomly to collect blood samples via jugular veins. Then, blood samples were centrifuged at 3,000×*g* for 10 min at 4 °C to obtain plasma; subsequently, the plasma was stored at −20 °C until analysis.

Growth performance measurement

Pigs were individually weighed on day 0 of the trial. However, BW by pen was measured on days 14, 28, 42 and 72 of the trial. Feed intake was recorded daily, and the residual feed was measured when pigs were weighed. Growth performance was evaluated by calculating the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion rate (FCR) for each pen.

Assay of plasma antioxidant indices

The activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and the content of malondialdehyde (MDA) in the plasma were determined using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Cell culture

IPEC-J2 cells were obtained from Dr. Guoyao Wu's laboratory at Texas A&M University and cultured in Dulbeco's modified Eagle's medium/F12 (DMEM/F12, Thermo Fisher Scientific, MA, USA) supplemented with 5% fetal bovine serum (FBS, Thermo Fisher Scientific, MA, USA), 0.1% ITS (5 μg/L insulin, 5 μg/L transferrin and 5 ng/L selenious acid, Corning Inc., NY, USA), 0.01% epidermal growth factor (5 μg/L, Corning Inc., NY, USA) and 1% pen-strep (Thermo Fisher Scientific, MA, USA) at 37 °C in a humidified atmosphere with 5% CO_2. Passage 13-15 cells were used in our experiment.

Establishment of cell oxidative stress model

To select the optimal H_2O_2 concentration, IPEC-J2 cells were seeded at 1×10^5 cells/mL (100 μL per well) in 96-well plates (Corning Inc., NY, USA) with 6 replications (wells) per treatment. After 48 h of incubation, oxidation was induced by exposing IPEC-J2 cells to 0, 0.2, 0.4, 0.6, and 0.8 mM H_2O_2 for another 1 h. Subsequently, the supernatant was removed, the cells were washed twice with PBS (pH 7.4, Thermo Fisher Scientific, MA, USA), and cell viability was determined using a cell counting kit (CCK-8) (MedChemExpression, NJ, USA) according to the manufacturer's instructions. Briefly, 110 μL of FBS-free DMEM/F-12 (containing 10 μL of CCK-8 reagent) was added to each well, and after 3 h of incubation at 37 °C, the absorbance was measured at 450 nm using an Epoch microplate spectrophotometer (BioTek Instruments, Inc., VT, USA). Cell viability was calculated using the following equation: Cell viability = (As - Ab)/(Ac - Ab) ×100%. As represents the absorbance of the H_2O_2-treated group, Ac represents the absorbance of the H_2O_2 untreated group, and Ab represents the absorbance of the blank group which contained culture medium and CCK-8 without cells and H_2O_2. The cell viability of the H_2O_2 untreated group was considered 100%.

Selection of daidzein concentration

Daidzein was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) at 10 mg/mL and diluted to the final concentration in medium before use. To select the optimal daidzein concentration, IPEC-J2 cells were seeded at 1×10^5 cells/mL (100 μL per well) in 96-well plates (Corning Inc., NY, USA) with 6 replications (wells) per treatment. After 24 h of incubation, daidzein at different concentrations (0, 20, 40, 60, 80, 100 μM) was added to the wells and incubated for another 24 h. In addition, the daidzein untreated group contained 0.2% DMSO. Then, 0.6 mM H_2O_2 was added to daidzein treated or untreated wells and incubated for 1 h. Cell viability was tested with the CCK-8 assay as described above.

Measurement of intracellular reactive oxygen species (ROS)

IPEC-J2 cells were seeded at 1×10^5 cells/mL (100 μL per well) in 96-well plates (Corning Inc., NY, USA) with 6 replications (wells) per treatment, pretreated with or without 40 μM daidzein for 24 h, and then treated with or without 0.6 mM H_2O_2 for 1 h. At the end of the experiment, cells were incubated with DCFH-DA probes (Beyotime Biotechnology, Shanghai, China) for 30 min and then washed twice with PBS (pH 7.4). The fluorescence was read at 488 nm for excitation and 525 nm for emission with a fluorescence microplate reader (Infinite M Plex, Tecan, Männedorf).

Measurements of SOD, CAT and GSH-Px activity and MDA content

IPEC-J2 cells were seeded at 1.5×10^5 cells/mL (2 mL per well) in 6-well plates (Corning Inc., NY, USA) with 6 replications (wells) per treatment, pretreated with or without 40 μM daidzein for 24 h, and then treated with or without 0.6 mM H_2O_2 for 1 h. The supernatant was removed, and the cells were washed twice with ice-cold PBS (pH 7.4) and lysed using RIPA buffer (Thermo Fisher Scientific, MA, USA) containing 1% protease inhibitors and a phosphatase inhibitor cocktail (Thermo Fisher Scientific, MA, USA) for 30 min at 4 °C. The supernatant was collected after centrifugation at 13,000×*g* for 30 min at 4 °C and stored at −20 °C. The SOD, CAT and GSH-Px activities and MDA content were determined using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

RNA isolation, reverse transcription and quantitative real-time PCR (qRT-PCR)

IPEC-J2 cells were seeded at 1.5×10^5 cells/mL (1 mL per well) in 12-well plates (Corning Inc., NY, USA) with 6 replications (wells) per treatment, pretreated with or without 40 μM daidzein for 24 h, and then treated with or without 0.6 mM H_2O_2 for 1 h. At the end of the experiment, cells were washed twice with ice-cold PBS (pH 7.4); subsequently, total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. The concentration and quality of total RNA were determined using an Epoch microplate spectrophotometer (BioTek Instruments, Inc., VT, USA). Reverse transcription was performed using the TransScript First-Strand cDNA Synthesis Super Mix Reagent Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. qRT-PCR was performed using SYBR Green as a reagent (Thermo Fisher Scientific, MA, USA) on a QuantStudio™ Real-Time PCR System (Thermo Fisher Scientific, MA, USA). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference. The gene expression of superoxide dismutase 1 (SOD1), CAT, glutathione peroxidase 1 (GPX1), nuclear factor-erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO1), zonula occludens-1 (ZO-1), occludin and claudin 1 was measured. The primer sequences used for qRT-PCR are shown in Table 2. The comparative CT method was used [16], determining fold changes in gene expression, calculated as $2^{-\Delta\Delta CT}$. The relative expression of each target gene was normalized to the mRNA level of the GAPDH gene.

Western blotting

IPEC-J2 cells were seeded at 1.5×10^5 cells/mL (2 mL per well) in 6-well plates (Corning Inc., NY, USA) with 4 replications (wells) per treatment, pretreated with or without 40 μM daidzein for 24 h, and then treated with or without 0.6 mM H_2O_2 for 1 h. At the end of the experiment, cells were washed twice with ice-cold PBS (pH 7.4) and lysed using RIPA buffer (Thermo Fisher Scientific, MA, USA) containing 1% protease inhibitors and a phosphatase inhibitor cocktail (Thermo Fisher Scientific, MA, USA) for 30 min at 4 °C. The supernatant was collected after centrifugation at 13,000×*g* for 30 min at 4 °C, and the protein concentration was determined using a BCA protein assay kit (Applygen, Beijing, China). For denaturation, 25 μg of protein and 4× loading buffer (Bio-Rad Laboratories Inc., CA, USA) were boiled at 95 °C for 10 min. The denatured proteins were separated by SDS-PAGE (12%) and subsequently transferred to PVDF membranes (Bio-Rad Laboratories Inc., CA, USA) for 2 h at 200 mA using the Bio-Rad Mini-PROTEAN Tetra electrophoresis system (Bio-Rad Laboratories Inc., CA, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) for 3 h at room temperature and then incubated with primary antibodies (Nrf2, ab92946, Abcam, Cambridge, UK, diluted 1:1000; ZO-1: 61-7300, Thermo Fisher Scientific, MA, USA, diluted 1:1000; Occludin: ab31721, Abcam, Cambridge, UK, diluted 1:1000; GAPDH: #2118, CST, Boston, USA, diluted 1:2000) at 4 °C overnight. After washing 3 times with TBST to remove residual primary antibodies, the membranes were incubated with secondary antibodies for 1 h at

room temperature. The membranes were washed 3 times with TBST to remove residual secondary antibodies, and an ECL agent was added for chemiluminescence imaging. The images were detected by a ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc., CA, USA). GAPDH was used as an internal reference.

Statistical analysis

Data related to growth performance were analyzed by ANOVA using a completely randomized block design with SPSS 20.0. The remaining data were analyzed using the one-way ANOVA procedure of SPSS 20.0. The pen represents the experimental unit for growth performance, and the individual piglet is the experimental unit for antioxidant parameters. Treatment comparisons were performed using Tukey's honest significant difference test for multiple testing. Significant differences among the treatments were determined at $P < 0.05$, whereas a treatment effect trend was noted for $0.05 < P < 0.10$.

Results

Growth performance

The effect of daidzein on the growth performance of pigs is shown in Table 3. Compared with the control diet, supplementation with daidzein at 50 mg/kg increased BW on day 72 and ADG during days 0-72 ($P < 0.05$) and tended to increase ADFI during days 42-72 ($P = 0.094$). In addition, compared with pigs fed dietary daidzein at 25 mg/kg, those fed dietary daidzein at 50 mg/kg tended to increase BW on day 72 ($P = 0.088$) and ADG during days 0-72 ($P = 0.085$).

Antioxidative properties

Table 4 presents the effect of daidzein on the plasma antioxidative properties of pigs. Compared with the control group, dietary supplementation with 50 mg/kg daidzein enhanced plasma SOD activity on day 42 ($P < 0.05$) and tended to decrease plasma MDA content on day 14 ($P = 0.062$). In addition, compared with pigs fed dietary daidzein at 25 mg/kg, those fed dietary daidzein at 50 mg/kg tended to decrease plasma MDA content on day 14 ($P = 0.062$).

The concentration of H_2O_2 in the model

As displayed in Figure 1, 0.2, 0.4, 0.6, and 0.8 mM H_2O_2 significantly inhibited cell viability compared to the untreated group ($P < 0.05$), reducing cell viability from 100 ± 0.85% to 91.2 ± 0.85%, 78.9 ± 0.61%, 68.6 ± 0.54%, and 58.8 ± 0.76%, respectively. H_2O_2 (0.6 mM) led to an approximately 31.4% loss in cell viability. Therefore, a concentration of 0.6 mM was selected in our study to conduct the following experiments.

The concentration of daidzein in the model

As shown in Figure 2, pretreatment with 20 and 40 μM daidzein effectively prevented H_2O_2-induced cell damage ($P < 0.05$), restoring cell viability from 71.2 ± 1.84% to 83.1 ± 1.75% and 84.5 ± 1.28%, respectively. Because 40 μM daidzein led to higher cell viability, the concentration of 40 μM was selected in our study to carry out the following experiments.

Intracellular ROS

As presented in Figure 3, the H_2O_2-treated group had significantly increased intracellular ROS levels compared to the control group ($P < 0.05$). However, pretreatment with 40 μM daidzein prior to H_2O_2 exposure significantly decreased intracellular ROS levels compared to the H_2O_2-treated group ($P < 0.05$).

SOD, CAT and GSH-Px activity and MDA content in H_2O_2-treated IPEC-J2 cells

As demonstrated in Figure 4, the H_2O_2-treated group had significantly decreased CAT activity and increased MDA content compared to the control group ($P < 0.05$). However, pretreatment with 40 μM daidzein prior to H_2O_2 exposure significantly increased CAT activity and decreased MDA content compared to the H_2O_2-treated group ($P < 0.05$).

Gene expression of antioxidant enzymes in H_2O_2-treated IPEC-J2 cells

As illustrated in Figure 5, the H_2O_2-treated group had significantly decreased gene expression of CAT compared to the control group ($P < 0.05$). However, pretreatment with 40 μM daidzein prior to H_2O_2 exposure significantly increased the gene expression of SOD1 and CAT compared to that in the H_2O_2-treated group ($P < 0.05$). In addition, daidzein treatment alone significantly enhanced the gene expression of SOD1 and CAT compared to the control group ($P < 0.05$).

Gene expression of Nrf2 and phase II detoxifying enzymes in H_2O_2-treated IPEC-J2 cells

As summarized in Figure 6, the H_2O_2-treated group had significantly decreased gene expression of Nrf2 compared to the control group ($P < 0.05$). However, pretreatment with 40 μM daidzein prior to H_2O_2 exposure significantly increased the gene expression of Nrf2, HO-1 and NQO1 compared to that in the H_2O_2-treated group ($P < 0.05$). In addition, daidzein treatment alone significantly enhanced the gene expression of Nrf2, HO-1 and NQO1 compared to that in the control group ($P < 0.05$).

Gene expression of tight junctions in H_2O_2-treated IPEC-J2 cells

As shown in Figure 7, the H_2O_2-treated group had significantly decreased gene expression of ZO-1 and occludin compared to the control group ($P < 0.05$). However, pretreatment with 40 μM daidzein prior to H_2O_2 exposure significantly increased the gene expression of occludin compared to that in the H_2O_2-treated group ($P < 0.05$). In addition, daidzein treatment alone significantly enhanced the gene expression of occludin compared to the control group ($P < 0.05$).

Protein expression of Nrf2 in H_2O_2-treated IPEC-J2 cells

As displayed in Figure 8, the H_2O_2-treated group had significantly decreased protein expression of Nrf2 compared to the control group ($P < 0.05$). However, pretreatment with 40 μM daidzein prior to H_2O_2 exposure significantly increased the protein expression of Nrf2 compared to that in the H_2O_2-treated group ($P < 0.05$).

Protein expression of tight junctions in H_2O_2-injured IPEC-J2 cells

As presented in Figure 9, the H_2O_2-treated group exhibited significantly lower protein expression of occludin than the control group ($P < 0.05$). However, pretreatment with 40 μM daidzein prior to H_2O_2 exposure significantly

increased the protein expression of occludin compared to that in the H_2O_2-treated group ($P < 0.05$).

Discussion

In the present study, dietary supplementation with 50 mg/kg daidzein significantly improved the growth performance of pigs during days 0-72 of the trial, indicating that long-term addition of daidzein to a corn-soybean diet benefits pig growth. This result corroborates our previous study in which soybean isoflavones in soybean meal were proven to play important roles in enhancing growth performance in pigs [8]. Our results were also in agreement with other studies on the beneficial effects of daidzein on growth performance. Greiner et al. (2001) found that 200 or 400 ppm daidzein could enhance body growth in porcine reproductive and respiratory syndrome virus-infected pigs [17]. Zhao et al. (2017) observed that dietary supplementation with 100, 200 and 400 mg/kg daidzein significantly increased the ADG of bull calves [12]. However, other studies showed no growth-promoting effect with daidzein addition. Xiao et al. (2015) reported that adding different concentrations of daidzein to the diet without any soy source did not significantly affect the growth performance of pigs [11]. Payne et al. (2001) observed that isoflavone supplementation two or five times as high as regular corn-soybean did not significantly affect the growth performance of growing-finishing pigs [18]. Rochell et al. (2015) reported that increasing the dietary soybean meal level from 17.5% to 29% did not significantly affect the growth of porcine reproductive and respiratory syndrome virus-infected pigs [19]. The discrepancy between our results and those of others may be caused by the level of soybean isoflavones in the diet, specific composition of the diet, initiation of the feeding phase of pigs, or the exposure time to soybean isoflavones.

Daidzein is a polyphenol compound, and the hydrogen atoms in the phenolic hydroxyl group can react with free radicals, thereby scavenging free radicals. Numerous studies have revealed the reactivity of daidzein and active oxygen species [20, 14]. The antioxidant activity of daidzein has been demonstrated by many previous studies. Xiao et al. (2015) demonstrated that pigs fed diets supplemented with 200 mg/kg daidzein had significantly higher serum SOD activity and lower MDA content [11]. Zhang et al. (2018) found that supplementation with 50 mg/kg daidzein increased the serum total antioxidant capacity and SOD activity and tended to decrease the MDA content in rats [21]. Zhao et al. (2017) observed that supplementation with 100, 200 and 400 mg/kg daidzein increased serum SOD activity in bull calves [12]. Liu et al. (2013) indicated that the serum SOD and GSH-Px activities of late lactation cows under heat stress were enhanced after adding 200, 300, 400 mg/kg daidzein [22]. These results were consistent with our study in which pigs fed a diet supplemented with 50 mg/kg daidzein had increased SOD activity and decreased MDA content in the plasma. However, the antioxidative mechanism of daidzein remains unclear.

To explore the mechanism underlying antioxidation by daidzein, we employed an *in vitro* model with the IPEC-J2 cell line, a non-transformed porcine intestinal epithelial cell line, with H_2O_2 stimulation mimicking oxidative stress [23-26].

Under normal physiological conditions, the antioxidant system of the body maintains a balance between the generation and elimination of ROS [1]. However, ROS levels dramatically increase under oxidative stress [27], and it has been reported that ROS production is related to cell damage and death [28]; thus, ROS production is a vital indicator of oxidative stress [29]. In the present study, exposure of IPEC-J2 cells to H_2O_2 significantly enhanced ROS levels. In addition, CAT activities significantly decreased, while MDA content significantly increased after H_2O_2 treatment. These results indicate that the oxidative stress model was successfully established. Daidzein

pretreatment followed by H_2O_2 exposure remarkably decreased ROS levels, increased CAT activity, and decreased MDA content. This corroborates our *in vivo* results in which daidzein could act as a potent antioxidant to protect IPEC-J2 cells against oxidative stress. This observation is in accordance with previous studies. Gao et al. (2016) reported that kudzu root extract (containing daidzein) possessed antioxidant properties and protected human umbilical vein endothelial cells against rotenone-induced oxidative stress [30]. Wijeratne et al. (2007) found that daidzein supplementation of Caco-2 cells could reduce oleic acid hydroperoxide- mediated cell injury [14].

The Kelch-like ECH-associated protein 1 (Keap1)-Nrf2/antioxidant response element (ARE) signaling pathway plays important roles in preventing oxidative stress in cells [31, 32]. Under normal physiological conditions, Nrf2 is mainly located in the cytoplasm and binds to Keap1. Due to proteasomal degradation mediated by Keap1, Nrf2 is inactive. Under oxidative stress, the cysteine residues of Keap1 can be modified, and its conformational changes result in a decrease in its binding affinity to Nrf2. Subsequently, activated Nrf2 translates from the cytoplasm to the nucleus, specifically binds to the ARE, promotes the expression of downstream antioxidant enzymes and phase II detoxifying enzyme genes, and enhances the antioxidant capacity of the body to resist the injury caused by oxidative stress [33, 34]. In the present study, compared to H_2O_2 treatment, daidzein pretreatment followed by H_2O_2 exposure dramatically enhanced the gene expression of CAT and Nrf2 and the protein expression of Nrf2. In addition, daidzein treatment alone significantly increased the gene expression of SOD1, CAT, Nrf2, HO-1 and NQO1. These results suggested that daidzein could upregulate the expression of antioxidant enzymes and phase II detoxifying genes at the transcriptional level through activation of the Keap1- Nrf2/ARE signaling pathway.

Tight junctions are important part of the intestinal mucosal epithelial barrier [35]. Disruption of tight junctions increases intestinal permeability, which results in infectious and inflammatory factors in the systemic circulation, eventually leading to tissue damage [36, 37]. Occludin, claudin1 and ZO-1 are 3 crucial proteins of tight junctions [38]. Previous studies reported that increased expression of occludin and ZO-1 can reduce the intestinal permeability of weaned piglets [39, 40]. In the present study, the exposure of IPEC-J2 cells to H_2O_2 significantly decreased the gene expression of occludin and ZO-1 and the protein expression of occludin, while daidzein pretreatment followed by H_2O_2 exposure significantly increased the gene and protein expression of occludin. Our results indicated that daidzein exhibited a protective effect on intestinal barrier function.

Conclusions

In conclusion, adding 50 mg/kg daidzein to a corn-soybean basal diet can effectively improve the growth performance and antioxidant capacity of pigs. Daidzein has a protective effect on IPEC-J2 cells against H_2O_2-induced oxidative stress. The mechanism by which daidzein exerts antioxidant capacity may be related to activation of the Keap1-Nrf2/ARE signaling pathway in IPEC-J2 cells.

Abbreviations

ADFI: Average daily feed intake; ADG: Average daily gain; ARE: Antioxidant response element; BW: Body weight; CAT: Catalase; CCK-8: Cell counting kit-8; DMEM/F12: Dulbeco's modified eagle medium/F12; DMSO: Dimethyl sulfoxide; FBS: Fetal bovine serum; FCR: Feed conversion rate; GSH-Px: Glutathione peroxidase; H2O2: hydrogen peroxide; HO-1: Heme oxygenase-1; IPEC-J2: Porcine intestinal epithelial cells; Keap1: Kelch-like ECH-associated protein 1; MDA: Malondialdehyde; Nrf2: Nuclear factor erythoid-2-related factor 2; NQO1: NAD(P)H: quinone

oxidoreductase 1; ROS: Reactive oxygen species; SOD: Superoxide dismutase; TBST: Tris-buffered saline with Tween 20; ZO-1: Zonula occludens-1.

Declarations

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Authors' contributions

Yanpin Li, Xianren Jiang, Jingdong Yin and Xilong Li designed the research. Yanpin Li, Long Cai, Yanli Zhang conducted the research. Yanpin Li, Xianren Jiang, Hongbiao Ding and Xilong Li analyzed the data and wrote the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

This study was approved by the Animal Care and Use Committee of the Feed Research Institute of the Chinese Academy of Agricultural Sciences.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Ingredient and nutrient composition of corn-soybean basal diets (as fed basis)

	Prestarter	Starter	Growing
Ingredients, %			
Corn	22.00	41.12	64.25
Extruded corn	23.58	21.00	-
Soybean meal	15.00	21.00	20.00
Extruded soybean	14.50	4.00	4.00
Solvent rice bran meal	-	-	8.00
Fish meal	5.50	3.00	-
Whey	15.00	5.00	-
Soybean oil	0.50	0.70	-
Dicalcium phosphate	0.55	0.60	0.45
Limestone ($CaCO_3$)	0.65	0.92	1.30
L-Lysine HCl	0.55	0.53	0.50
DL-Methionine	0.09	0.07	0.04
Threonine	0.08	0.06	0.08
Tryptophan	-	-	0.01
Salt	0.25	0.30	0.40
Choline chloride (60%)	0.08	0.08	-
Premix[1]	1.37	1.42	0.97
Zinc oxide	0.30	0.20	-
Analyzed nutrient content			
Dry matter, %	94.42	93.79	92.62
Crude protein, %	19.57	18.14	16.42
Calcium, %	0.91	0.83	0.75
Phosphorus, %	0.65	0.50	0.47
Calculated nutrient content			
ME, MJ/kg	14.35	13.93	13.67
Crude fat, %	5.20	3.84	3.43
Crude fiber, %	2.13	2.23	2.82
Lysine, %	1.31	1.15	0.98

Methionine, %	0.40	0.35	0.28
Threonine, %	0.73	0.65	0.59
Tryptophan, %	0.21	0.19	0.18

[1]Premix supplied per kg of diet: vitamin A, 35.2 mg; vitamin D_3, 7.68 mg; vitamin E, 128 mg; vitamin K_3, 8.16 mg; vitamin B_1, 4 mg; vitamin B_2, 12 mg; vitamin B_6, 8.32 mg; vitamin B_{12}, 4.8 mg; niacin, 38.4 mg; calcium pantothenate, 25 mg; folic acid, 1.68 mg; biotin, 0.16 mg; iron ($FeSO_4 \cdot H_2O$), 171 mg; manganese ($MnSO_4 \cdot H_2O$), 42.31 mg; copper ($CuSO_4 \cdot 5H_2O$), 125 mg; selenium (Na_2SeO_3), 0.19 mg; cobalt ($CoCl_2$), 0.19 mg; iodine ($Ca(IO_3)_2$), 0.54 mg.

Table 2 Primer sequences used for quantitative real-time PCR

Gene[1]	Forward (5´- 3´)	Reverse (5´- 3´)	Product Length, bp	Accession No.
GAPDH	GCTTGTCATCAATGGAAAGG	CATACGTAGCACCAGCATCA	86	NM_001206359.1
SOD1	GAAGACAGTGTTAGTAACGG	CAGCCTTGTGTATTATCTCC	93	NM_001190422.1
CAT	CCTGCAACGTTCTGTAAGGC	GCTTCATCTGGTCACTGGCT	72	NM_214301.2
GPX1	TCTCCAGTGTGTCGCAATGA	TCGATGGTCAGAAAGCGACG	104	NM_214201.1
Nrf2	GACCTTGGAGTAAGTCGAGA	GGAGTTGTTCTTGTCTTTCC	103	XM_005671981.3
HO-1	GAGAAGGCTTTAAGCTGGTG	GTTGTGCTCAATCTCCTCCT	74	NM_001004027.1
NQO1	GGACATCACAGGTAAACTGA	TATAAGCCAGAGCAGTCTCG	68	NM_001159613.1
Occludin	TCAGGTGCACCCTCCAGATT	TGGACTTTCAAGAGGCCTGG	112	NM_001163647.2
ZO-1	CGATCACTCCAGCATACAAT	CACTTGGCAGAAGATTGTGA	111	CV870309
Claudin1	CCTCAATACAGGAGGGAAGC	CTCTCCCCACATTCGAGATGATT	76	NM_001244539.1

[1]GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SOD1, superoxide dismutase 1; CAT, catalase; GPX1, glutathione peroxidase 1; Nrf2, nuclear factor-erythroid2-related factor 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H: quinone oxidoreductase 1; ZO-1, zonula occludens-1.

Table 3 Effect of daidzein on growth performance of pigs[1]

Items	daidzein, mg/kg				SEM	*P*-value		
	0	25	50	100		ANOVA	Linear	Quadratic
BW, kg								
Day 0	7.41	7.34	7.33	7.33	0.555	0.366	0.923	0.952
Day 14	10.26	10.12	10.33	10.07	0.696	0.778	0.910	0.934
Day 28	16.22	16.06	16.96	16.36	1.098	0.493	0.790	0.845
Day 42	22.67	23.12	24.70	23.90	1.536	0.320	0.465	0.697
Day 72	41.78^{b}	$42.53^{ab,y}$	$45.98^{a,x}$	43.45^{ab}	1.920	0.037	0.349	0.414
ADG, g								
Day 0-14	204	199	214	196	14.6	0.797	0.904	0.656
Day 14-28	426	424	474	450	31.7	0.311	0.425	0.734
Day 28-42	461	504	553	538	42.9	0.410	0.185	0.531
Day 42-72	637	647	709	652	19.8	0.109	0.319	0.159
Day 0-72	477^{b}	$489^{ab,y}$	$537^{a,x}$	502^{ab}	20.0	0.033	0.211	0.277
ADFI, g								
Day 0-14	328	321	334	308	23.5	0.756	0.664	0.693
Day 14-28	753	745	769	730	50.8	0.753	0.847	0.760
Day 28-42	920	960	1026	983	90.6	0.716	0.558	0.673
Day 42-72	1391^{y}	1423^{xy}	1556^{x}	1436^{xy}	61.7	0.102	0.368	0.253
Day 0-72	968	987	1062	991	52.1	0.152	0.557	0.414
FCR								
Day 0-14	1.62	1.61	1.56	1.58	0.040	0.712	0.396	0.815
Day 14-28	1.81	1.76	1.63	1.62	0.066	0.264	0.056	0.836
Day 28-42	2.06	1.88	1.88	1.83	0.114	0.667	0.233	0.585
Day 42-72	2.18	2.20	2.19	2.21	0.063	0.995	0.847	0.957
Day 0-72	2.03	2.01	1.98	1.98	0.036	0.649	0.296	0.877

SEM, standard error of the mean; BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion rate.

[a, b] Values within a row without common letters differ significantly ($P < 0.05$).

[x, y] Values listed in the same row with different superscripts are tended to be different ($0.05 < P < 0.10$).

[1] n = 5.

Table 4 Effect of daidzein on plasma antioxidative properties of pigs[1]

Items	daidzein, mg/kg				SEM	*P*-value		
	0	25	50	100		ANOVA	Linear	Quadratic
Day14								
CAT (U/ml)	12.56	12.76	12.99	12.93	0.348	0.833	0.419	0.719
SOD (U/ml)	19.61	19.33	19.21	19.19	0.748	0.979	0.690	0.868
MDA (nmol/ml)	2.52^{x}	2.54^{x}	2.31^{y}	2.50^{xy}	0.055	0.040	0.250	0.109
GSH-Px (U/ml)	493	479	499	490	21.8	0.930	0.918	0.924
Day28								
CAT (U/ml)	12.51	12.82	12.99	12.71	0.447	0.892	0.711	0.514
SOD (U/ml)	22.06	21.87	22.04	21.29	0.527	0.742	0.404	0.621
MDA (nmol/ml)	2.58	2.47	2.56	2.23	0.143	0.341	0.180	0.430
GSH-Px (U/ml)	499	492	517	509	15.7	0.741	0.474	0.985
Day42								
CAT (U/ml)	12.31	12.43	13.17	12.67	0.544	0.702	0.469	0.586
SOD (U/ml)	18.19^{b}	18.58^{ab}	20.54^{a}	19.01^{ab}	0.544	0.042	0.094	0.098
MDA (nmol/ml)	2.07	2.05	1.98	2.02	0.155	0.986	0.765	0.866
GSH-Px (U/ml)	510	588	566	559	29.6	0.332	0.368	0.169

SEM, standard error of the mean; CAT, catalase; SOD, superoxide dismutase; MDA, malondialdehyde; GSH-Px, glutathione peroxidase.

[a, b] Values within a row without common letters differ significantly (P < 0.05).

[x, y] Values listed in the same row with different superscripts are tended to be different (0.05 < P < 0.10).

[1] n = 5.

Figures

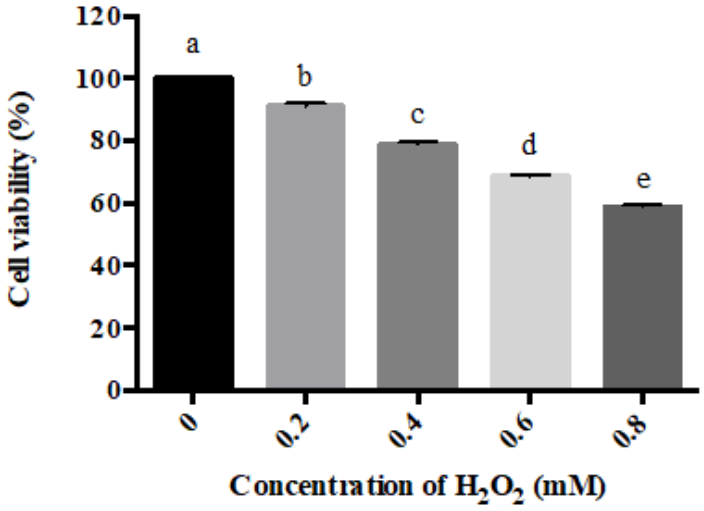

Figure 1

The viability of H2O2-treated IPEC-J2 cells. IPEC-J2 cells were seeded in 96-well plates and treated with 0-0.8 mM H2O2 for 1 h after 48 h of incubation. The results are presented as the mean ± SE, n = 6. The value is expressed as a percentage of the H2O2 untreated group. Values without common letters (a, b, c, d, e) differ significantly (P < 0.05). IPEC-J2, porcine intestinal epithelial cells; H2O2, hydrogen peroxide.

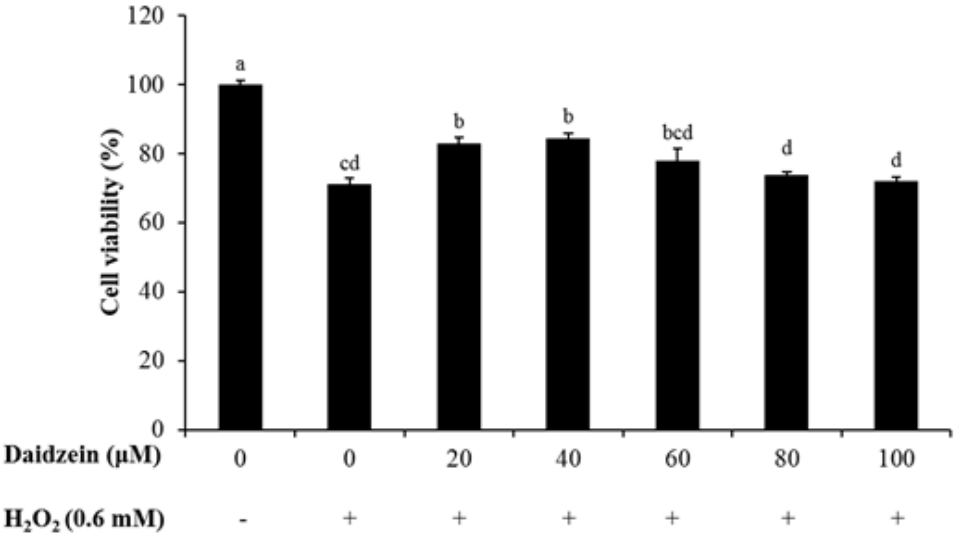

Figure 2

Effect of daidzein on the viability of H2O2-treated IPEC-J2 cells. IPEC-J2 cells were seeded in 96-well plates, pretreated with different daidzein concentrations (0-100 μM) for 24 h, and then treated with or without 0.6 mM H2O2 for 1 h. The results are presented as the mean ± SE, n = 6. The value is expressed as a percentage of the group not treated with H2O2 or daidzein. Values without common letters (a, b, c, d) differ significantly (P < 0.05). IPEC-J2, porcine intestinal epithelial cells; H2O2, hydrogen peroxide.

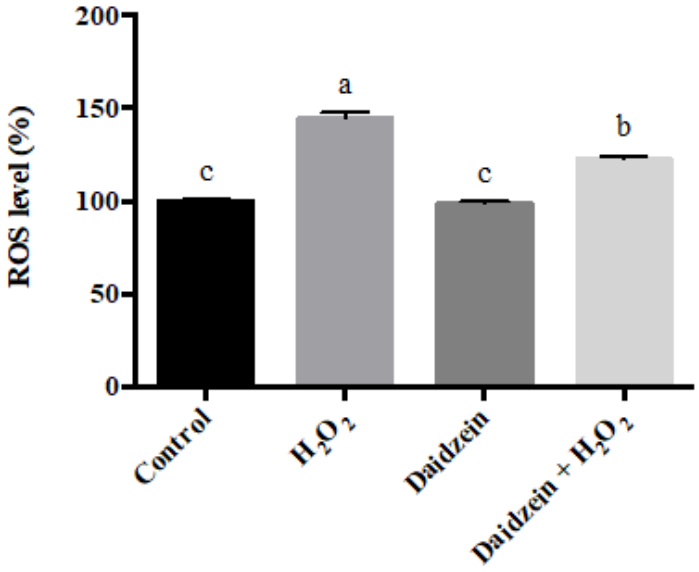

Figure 3

Effect of daidzein on ROS levels in H2O2-treated IPEC-J2 cells. IPEC-J2 cells were seeded in 96-well plates, pretreated with or without 40 μM daidzein for 24 h, treated with or without 0.6 mM H2O2 for 1 h, and subsequently incubated with DCFH-DA probes for 30 min. The results are presented as the mean ± SE, n = 6. Values without common letters (a, b, c) differ significantly ($P < 0.05$). IPEC-J2, porcine intestinal epithelial cells; H2O2, hydrogen peroxide; ROS, reactive oxygen species.

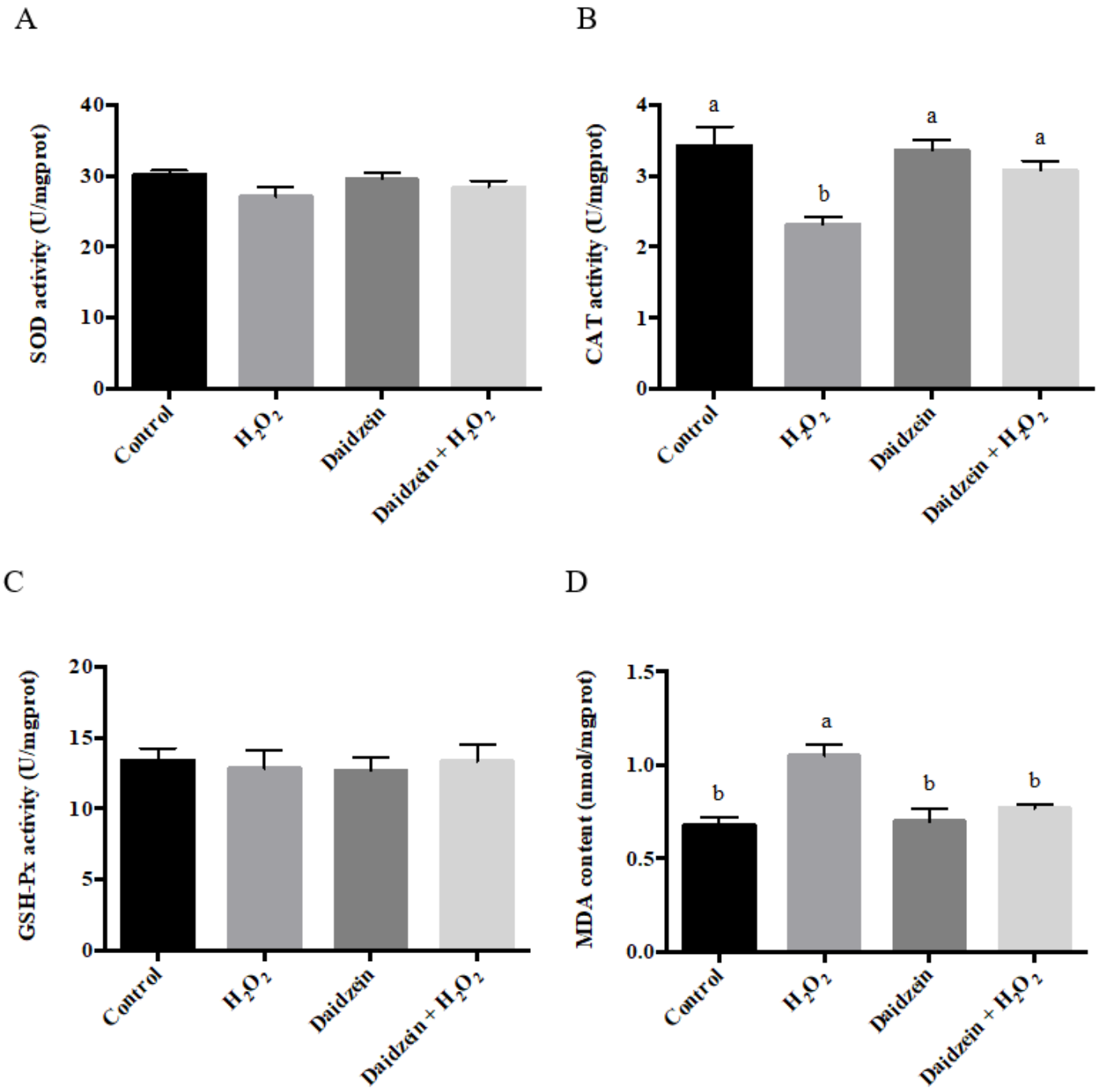

Figure 4

Effects of daidzein on antioxidant enzyme activity and MDA content in H2O2-treated IPEC-J2 cells. A: SOD activity; B: CAT activity; C: GSH-Px activity; D: MDA content. IPEC-J2 cells were seeded in 6-well plates, pretreated with or without 40 μM daidzein for 24 h, and then treated with or without 0.6 mM H2O2 for 1 h. The results are presented as the mean ± SE, n = 6. Values without common letters (a, b) differ significantly ($P < 0.05$). IPEC-J2, porcine intestinal epithelial cells; H2O2, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde.

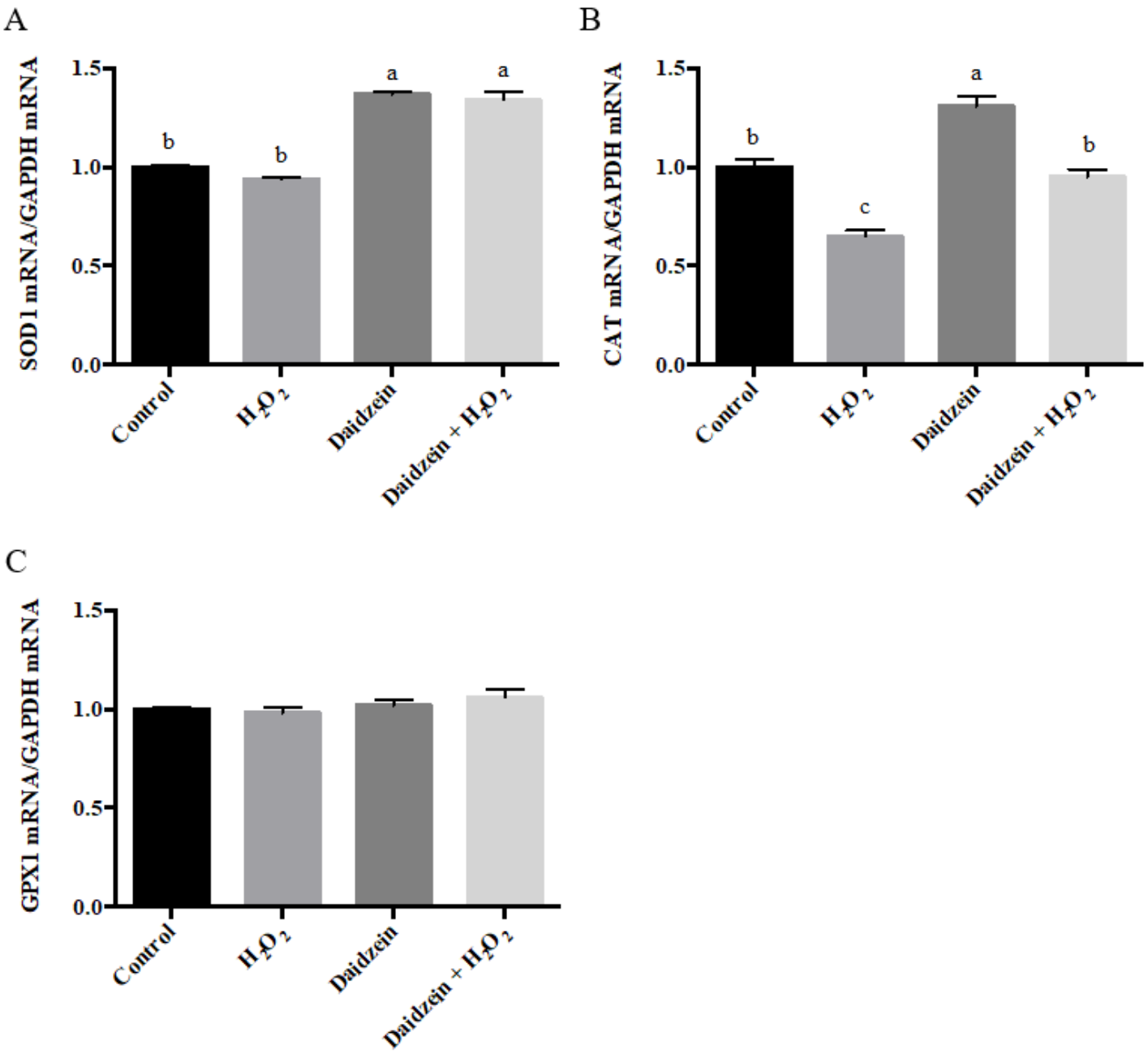

Figure 5

Effect of daidzein on the gene expression of antioxidant enzymes in H2O2-treated IPEC-J2 cells. A: Gene expression of SOD1; B: Gene expression of CAT; C: Gene expression of GPX1. IPEC-J2 cells were seeded in 12-well plates, pretreated with or without 40 μM daidzein for 24 h, and then treated with or without 0.6 mM H2O2 for 1 h. The results are presented as the mean ± SE, n = 6. Values without common letters (a, b, c) differ significantly ($P < 0.05$). IPEC-J2, porcine intestinal epithelial cells; H2O2, hydrogen peroxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SOD1, superoxide dismutase 1; CAT, catalase; GPX1, glutathione peroxidase 1.

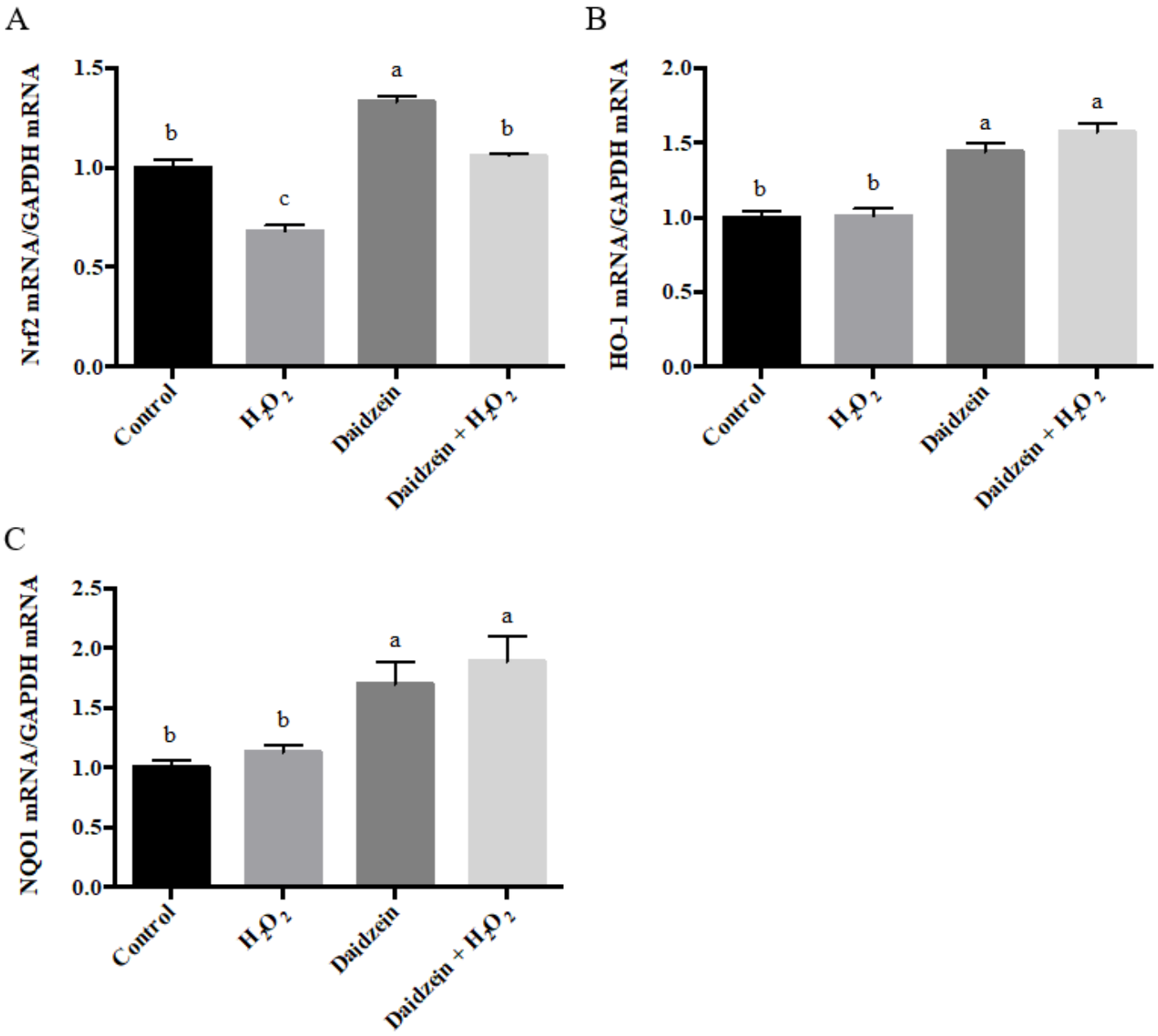

Figure 6

Effects of daidzein on the gene expression of Nrf2 and phase II detoxifying enzymes in H2O2-treated IPEC-J2 cells. A: Gene expression of Nrf2; B: Gene expression of HO-1; C: Gene expression of NQO1. IPEC-J2 cells were seeded in 12-well plates, pretreated with or without 40 μM daidzein for 24 h, and then treated with or without 0.6 mM H2O2 for 1 h. The results are presented as the mean ± SE, n = 6. Values without common letters (a, b, c) differ significantly ($P < 0.05$). IPEC-J2, porcine intestinal epithelial cells; H2O2, hydrogen peroxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Nrf2, nuclear factor-erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H: quinone oxidoreductase 1.

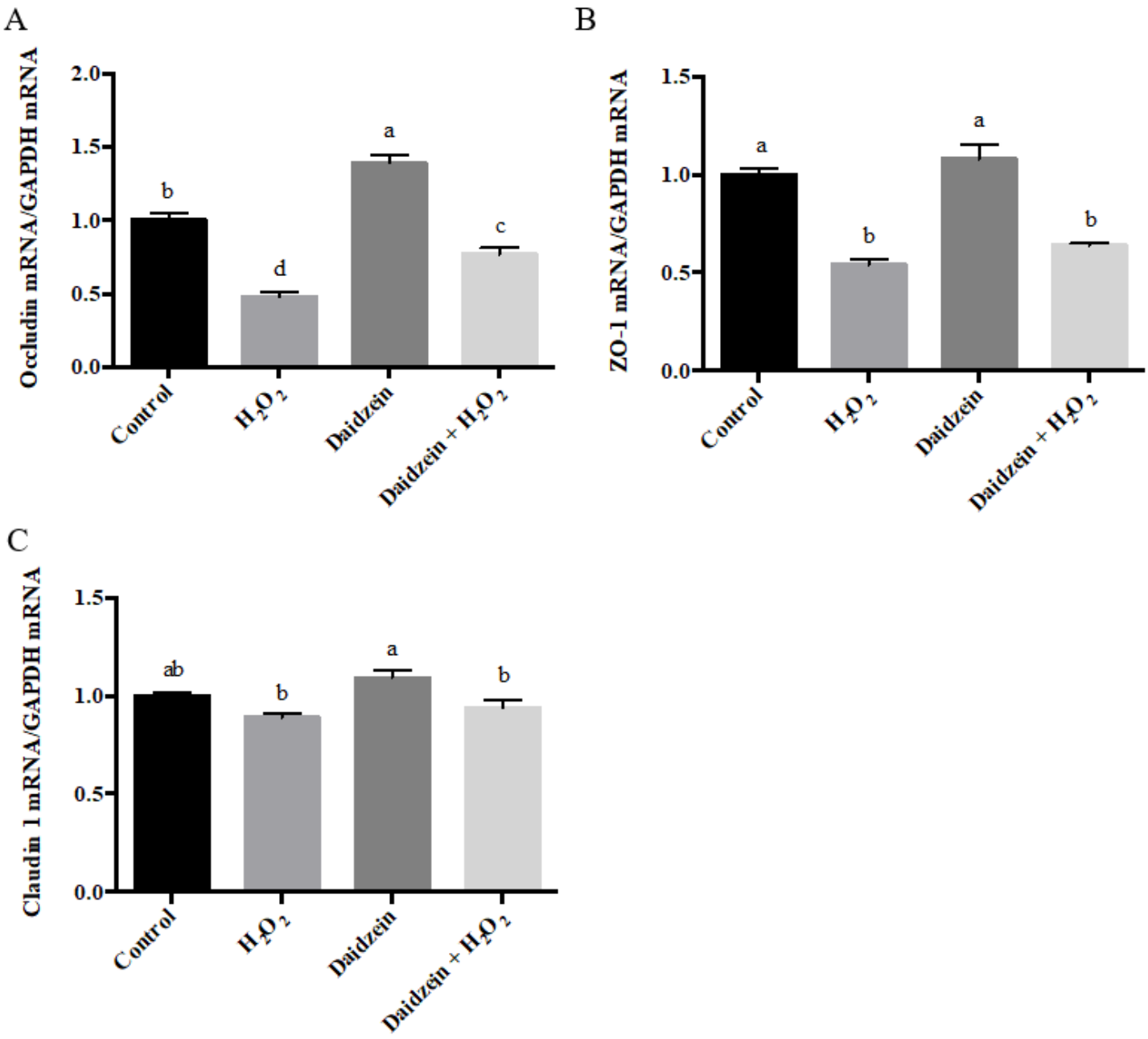

Figure 7

Effect of daidzein on the gene expression of tight junctions in H2O2-treated IPEC-J2 cells. A: Gene expression of occludin; B: Gene expression of ZO-1; C: Gene expression of claudin 1. IPEC-J2 cells were seeded in 12-well plates, pretreated with or without 40 μM daidzein for 24 h, and then treated with or without 0.6 mM H2O2 for 1 h. The results are presented as the mean ± SE, n = 6. Values without common letters (a, b, c, d) differ significantly ($P < 0.05$). IPEC-J2, porcine intestinal epithelial cells; H2O2, hydrogen peroxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ZO-1, zonula occludens-1.

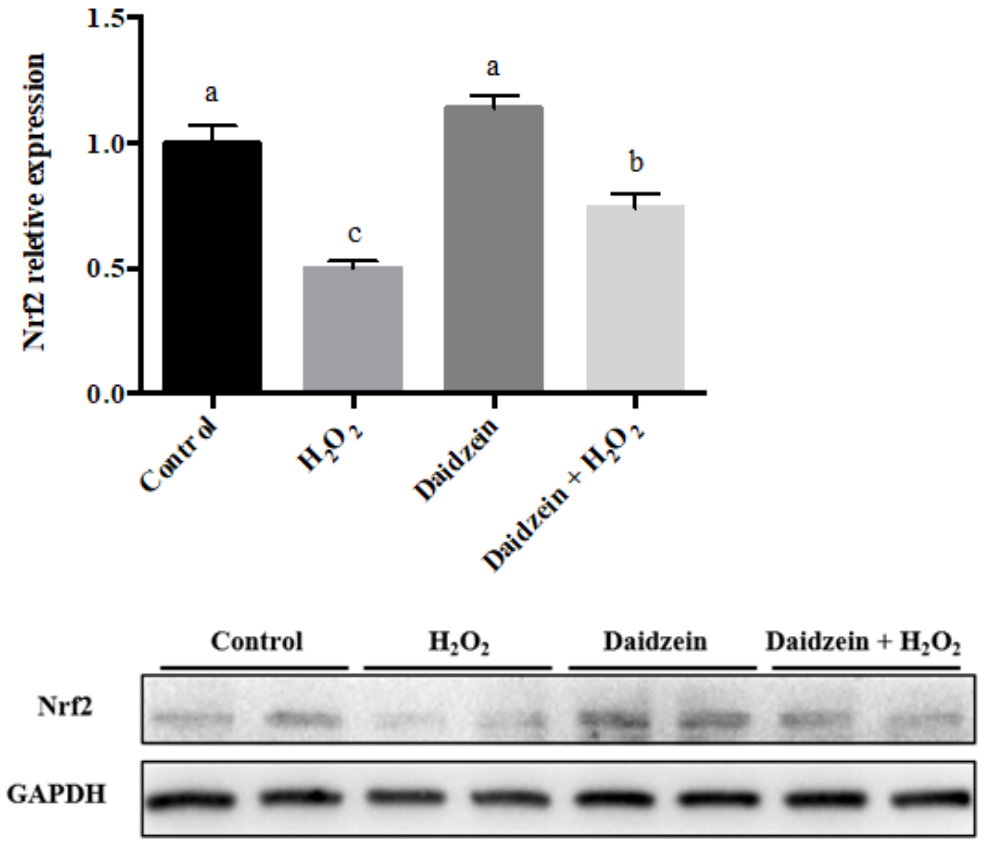

Figure 8

Effect of daidzein on the protein expression of Nrf2 in H2O2-treated IPEC-J2 cells. IPEC-J2 cells were seeded in 6-well plates, pretreated with or without 40 μM daidzein for 24 h, and then treated with or without 0.6 mM H2O2 for 1 h. The results are presented as the mean ± SE, n = 4. Values without common letters (a, b, c) differ significantly ($P < 0.05$). IPEC-J2, porcine intestinal epithelial cells; H2O2, hydrogen peroxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Nrf2, nuclear factor-erythroid 2-related factor 2.

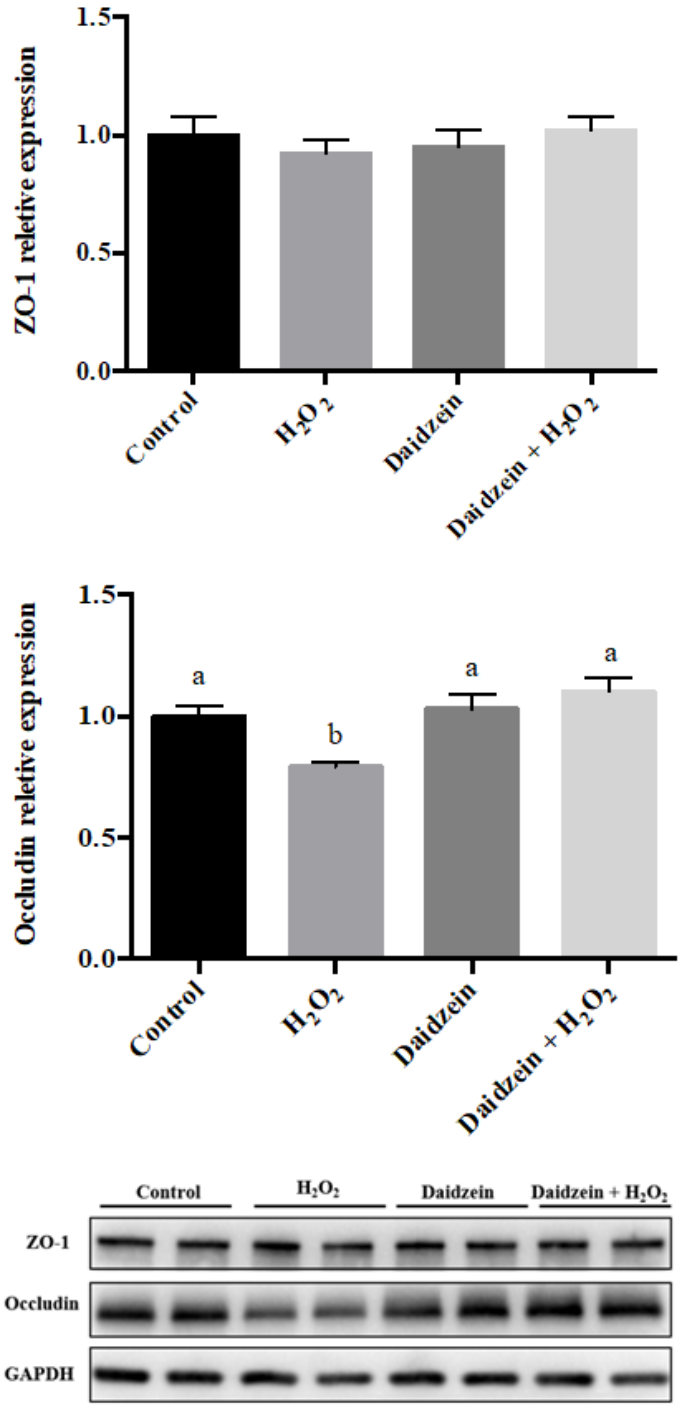

Figure 9

Effect of daidzein on the protein expression of tight junctions in H2O2-treated IPEC-J2 cells. IPEC-J2 cells were seeded in 6-well plates, pretreated with or without 40 μM daidzein for 24 h, and then treated with or without 0.6 mM H2O2 for 1 h. The results are presented as the mean ± SE, n = 4. Values without common letters (a, b) differ significantly ($P < 0.05$). IPEC-J2, porcine intestinal epithelial cells; H2O2, hydrogen peroxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ZO-1, zonula occludens-1.