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Research Article

Protective Effects of Feruloyl Oligosaccharides from Fermented Wheat Bran against Oxidative Stress in IPEC-J2 Cells In Vitro and in a Zebrafish Model In Vivo

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This study aims to evaluate the protective effects of feruloyl oligosaccharides from fermented wheat bran (FOs-FWB) against oxidative stress in IPEC-J2 cells in vitro and in a zebrafish model in vivo. Results showed that FOs-FWB effectively reduces reactive oxygen species (ROS) and malondialdehyde (MDA) content in lipopolysaccharide (LPS)-stimulated IPEC-J2 cells while elevating glutathione (GSH) content and superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activities. The real-time PCR data illustrated that FOs-FWB upregulated the mRNA expression levels of glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM), NAD (P) H: quinone oxidoreductase-1 (NQO-1), and heme oxygenase-1 (HO-1). In the model of AAPH-stimulated zebrafish embryos, we observed that FOs-FWB suppressed cell death, ROS generation, and lipid peroxidation, along with improvements in SOD, CAT, and GSH-Px activities. Therefore, FOs-FWB exerted protective effects against oxidative stress in IPEC-J2 cells and zebrafish.

1. Introduction

The excessive generation of reactive oxygen species (ROS), which cannot be counteracted by the antioxidative defense system of living organisms, induces oxidative stress (OS) [1]. Oxidative stress leads to DNA, cellular protein, and membrane lipid damage [2] and ultimately causes oxidative damage in the organs. A previous study has found that the gastrointestinal tract is more susceptible to OS than other organs due to the continuous exposure to exogenous oxidant species [3]. The increasing amount of evidence confirms the correlation between OS and various gastrointestinal diseases, such as necrotizing enterocolitis [4], Crohn's disease [5], and gastrointestinal cancers [6]. Thus, extra antioxidants as direct scavengers of free radicals and/or as regulators of the antioxidative defense system are critical to prevent gastrointestinal tract from OS.

Recently, a great deal of attention in functional food and pharmaceutical science has focused on finding cheap, abundant, and renewable natural antioxidants from plants, animals, and microorganisms [7, 8]. Feruloyl oligosaccharides (FOs), a group of oligosaccharides acylated with ferulic acid (FA) released from cereal bran, possess valuable antioxidative properties. It has been reported that FOs could effectively scavenge free radicals [9] and eliminate OS in lymphocytes [10], pheochromocytoma cells (PC 12 cells) [11], and hepatocellular carcinoma cells (HepG2) [12]. Yet, the protective effects of FOs on intestinal epithelial cells against OS are limited.

In our previous study, we fermented WB with mixed bacteria to release FOs and confirmed its strong antioxidative activity in vitro [13, 14]. However, the antioxidative activity of FOs from fermented wheat bran (FOs-FWB) has not been fully investigated. The intestinal porcine epithelial cell line (IPEC-J2) is a nontransformed, nontumorigenic small intestinal epithelial cell line for evaluation and mechanism study of the antioxidative activity of natural antioxidants [15, 16]. Additionally, zebrafish have become the third-largest model organism due to their high physiological similarity to mammals [17]. Therefore, we aimed to figure out the effects of FOs-FWB on the cell viability, ROS and MDA content, activities, and mRNA expression of antioxidative/phase II detoxifying enzymes in IPEC-J2 cells under OS. Based on this, we preliminarily confirm the protective effects of FOs-FWB against OS in the zebrafish embryo model.

2. Materials and Methods

2.1. Materials. DMEM/F12 medium, fetal bovine serum (FBS), insulin transferrin selenium (ITS), penicillin, and streptomycin were purchased from GIBCO (Grand Island, USA). Cell Counting Kit-8 (CCK-8) was purchased from Solarbio (Beijing, China). ROS, catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), glutathione (GSH), and malondialdehyde (MDA) assay kits were purchased from Jiancheng (Nanjing, China). The TRNzol Universal reagent was purchased from Tiangen (Beijing, China). A bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, USA). PrimeScript™ RT reagent Kit with gDNA Eraser and SYBR® Premix Ex Taq™ II kit were purchased from Takara Bio (Dalian, China). Lipopolysaccharide (LPS), 2, 2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2, 7-dichlorofluorescein diacetate (DCF-DA), diphenyl-1-pyrenylphosphine (DPPP), and acridine orange (AO) were purchased from Sigma (St. Louis, USA). Other chemicals were purchased from China National Medicines Corporation Ltd. (Shanghai, China).

2.2. Preparation of FOs-FWB. The preparation of FOs-FWB was conducted under optimal conditions as described in previous studies [13, 14]. In brief, wheat bran was fermented by *Bacillus subtilis* CGMCC 1.892, *Bacillus licheniformis* CGMCC 1.813, and *Saccharomyces cerevisiae* CGMCC 2.119 (1 : 1 : 1) at 42.5°C for 58.5 h. For hot water extraction, 1 g of dried fermented wheat bran was mixed with 10 mL of water and then incubated at 70°C for 24 h. The crude FOs-FWB was obtained after ethanol precipitation (4-fold volumes of 80% ethanol at 4°C for a night) and deproteinization (3-fold volumes of Sevag reagent). The crude FOs were separated and purified by the Amberlite XAD-2 and Sephadex LH-20 columns. After lyophilization, the FOs from fermented WB were obtained and named FOs-FWB. Preliminary structure analysis revealed that the FOs-FWB was hetero-oligosaccharides with a weight-average molecular weight of 11.81 kDa and mainly consisted of glucose (29.79%), arabinose (29.81%), and xylose (33.42%). The esterified FA content in FOs-FWB was 37.34 mmol/g.

2.3. IPEC-J2 Cell Culture and Oxidative Stress Induction. The IPEC-J2 cells were maintained in DMEM/F12 medium containing 10% (v/v) FBS, 1% ITS, and 1% penicillin-streptomycin at 37°C in an incubator with 5% CO_2 (v/v). Prior to oxidative induction, the IPEC-J2 cells were seeded in 96-well plates at an intensity of 1×10^5 cells/well and incubated to 80% confluence. The supernatant was replaced with serum-free DMEM/F12 medium for 20 h to keep fasting. Then, cells were incubated with FOs-FWB at the final concentrations of 0 (control), 25, 50, 100, and 200 μg/mL for 24 h and then washed twice with phosphate-buffered saline (PBS). After that, the cells were incubated with 10 μg/mL LPS for 24 h to induce OS.

2.4. Cell Viability. Protective effects of FOs-FWB on cell viability of IPEC-J2 were analyzed by the CCK8 kit according to the manufacturer's instructions. After 24 h of fasting, cells were cultured with FOs-FWB for 24 h, then treated with LPS for another 24 h. Then, 10 μL of CCK8 solution was added and further incubated at 37°C for 2 h. Finally, the absorbance at 450 nm was measured.

2.5. Determination of Intracellular ROS. The intracellular ROS contents inside the IPEC-J2 cells were determined in accordance with the instructions of the ROS assay kit. Cells were cultured as per the method of 2.3. After treatment with FOs and/or LPS, the cells were stained with DCFH-DA for 20 min, rinsed in serum-free culture medium three times, and air-dried. The fluorescence intensity was examined under an optical microscope (TS2R, Nikon, Japan) equipped with a digital camera [18].

2.6. Determination of MDA, GSH, and Antioxidative Enzymes for Cells. The MDA and GSH contents and antioxidative enzyme activities (GSH-Px, CAT, and SOD) of IPEC-J2 cells were measured by commercial assay kits, respectively. After incubation as the method of 2.3, cells were harvested by scrapers, lysed in cell lysis buffer via sonication, and centrifuged at 10,000 × g for 10 min, and then the supernatant was collected for further determination. The protein concentration of the supernatant was detected by the BCA protein assay kit.

2.7. Real-Time PCR for Cells. The RNA of IPEC-J2 cells was extracted using a TRIzol plus RNA purification kit and quantified with a Nanodrop 2000 (Thermo Scientific, Shanghai, China). The reverse transcription was performed using the PrimeScript™ RT reagent Kit with gDNA Eraser. cDNA was amplified and quantified with the SYBR® Premix Ex Taq™ II kit using the ABI7500 system (Applied Biosystems, CA, USA). Table 1 indicates the primer sequences for NAD (P) H: quinone oxidoreductase-1 (NQO-1), heme oxygenase-1 (HO-1), glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM), and the reference gene GAPDH. Relative mRNA expression levels were calculated as $2^{-\Delta\Delta Ct}$ [11].

Table 1: Primers sequences used for real-time PCR.

Gene	Product length (bp)		Sequence (5′-3′)
GCLC	188	Forward	CAAATTGGCAGACGATGAGAT
		Reverse	AACCTTCGACAGAGGGATGA
GCLM	125	Forward	AAGATGGGGTTCATCTGTCCT
		Reverse	CTGCTCCAACTGGGTTTTGT
NQO1	242	Forward	AACTTCAATCCCGTCATCTCC
		Reverse	GCAAACTCCCCTATGAGCACA
HO-1	124	Forward	ACGCCTACACCCGCTACA
		Reverse	GCGACATTGGGGAAAGTGA
GAPDH	117	Forward	GGCTACACTGAGGACCAGGTTG
		Reverse	CCAGGAAATGAGCTTGACGAA

2.8. Zebrafish Maintenance and Oxidative Stress Induction. The maintenance conditions of zebrafish in their adult stage and embryo collection were performed according to Chen et al. [19]. Prior to oxidative induction, embryos approximately 7–9 h post fertilization (hpf) were transferred to 24-well plates (10 embryos/well) and maintained in 2 mL of embryo media containing FOs-FWB (0, control; 50, 100, 200, and 400 μg/mL) for 1 h. Then AAPH solution (15 mmol/mL) was added to the coculture for up to 24 hpf to induce OS.

2.9. Determination of Antioxidative Enzymes Activities for Zebrafish Embryos. Zebrafish embryos were treated as the method of 2.8. Then, embryos were washed with fresh embryo media, lysed in PBS, and analyzed for CAT, SOD, and GSH-Px activities with the assay kits. The 240 embryos (6 pools of 40 eggs per treatment) were collected.

2.10. Determination of Cell Death, ROS Production, and Lipid Peroxidation for Zebrafish Embryos. Cell death, ROS production, and lipid peroxidation of zebrafish embryos were detected using oxidation-sensitive fluorescent probe dyes (AO, DCFH-DA, and DPPP) [20]. Zebrafish embryos were treated as the method of 2.8. Then, the supernatant was replaced with fresh embryo media and cultured up to 72 hpf. The 40 embryos per treatment were stained with AO solution (7 μg/mL), DCF-DA solution (20 μg/mL), and DPPP solution (25 μg/mL) for 0.5 h, 1 h and 1 h, respectively. After staining, zebrafish were observed under an optical microscope and their fluorescence intensity was quantified.

2.11. Statistical Analysis. The data are presented as the mean ± standard error (SE) from three independent experiments. Statistical analysis was conducted by one-way ANOVA and Tukey multiple comparison tests. The significance differences were established as $p < 0.05$.

3. Results and Discussion

3.1. The Protective Effects of FOs-FWB in LPS-Stimulated IPEC-J2 Cells. The gastrointestinal tract is the main site of nutrient digestion and absorption and acts as the body's first barrier against external stressors [21]. LPS, as the major bacterial endotoxin, displays harmful influences on the intestinal epithelium including mitochondria dysfunction, redox homeostasis disruption, and morphological damage [22]. Therefore, LPS was used in this study to establish a model of OS in IPEC-J2 cells. With the LPS induction, the survival rate of IPEC-J2 cells (76.42%) was significantly lower ($p < 0.05$) than that in the control group (Figure 1), suggesting that the IPEC-J2 cell OS model was successfully established. After pretreatment with 100 and 200 μg/mL FOs-FWB for 24 h, the survival rates of stimulated cells were significantly increased to 92.88% and 103.35% ($p < 0.05$). Pretreatment of FOs-FWB at 50–200 μg/mL exhibited no toxicity to IPEC-J2 cells (Figure S1). In accordance with our study, it was found that FOs could inhibit hemolysis induced by AAPH in rat and human erythrocytes [23, 24]. Furthermore, previous studies have reported increases in the viability of PC 12 cells and HepG2 cells under OS [11, 12].

ROS are produced during normal metabolism in the mitochondria of intestinal epithelium cells, while excessive ROS production results in redox homeostasis disruption [25]. As shown in Figure 2(a), the ROS content presented as fluorescence intensity in LPS-stimulated cells was significantly higher in comparison with the control ($p < 0.05$). It is suggested that ROS production was accelerated by LPS, which is consistent with Zhao et al. [26]. However, FOs-FWB at concentrations of 50–200 μg/mL significantly reduced the ROS content compared with the LPS group ($p < 0.05$). In HepG2 cells, it was reported that the ROS generation triggered by AAPH could be suppressed by FOs [12]. MDA is a product of lipid peroxidation induced by ROS and reflects the degree of oxidative damage in living organisms [27]. Figure 2(b) shows that the MDA content in the LPS-stimulated cells was significantly increased, while pretreatment of FOs-FWB (50–200 μg/mL) significantly relieved the increase ($p < 0.05$). In line with Yao's study, exposure of FOs prohibited the formation of MDA in PC 12 cells injured by H_2O_2 [11]. The results of our study indicated that FOs-FWB could inhibit ROS and MDA generation and alleviate oxidative damage in IPEC-J2 cells under OS. It could be attributed the in vitro antioxidative activity of FOs-FWB, such as reducing power and scavenging activities against DPPH and hydroxyl radicals [13]. Furthermore, the inhibitory effects of FOs-FWB may be partly associated with the activation of the

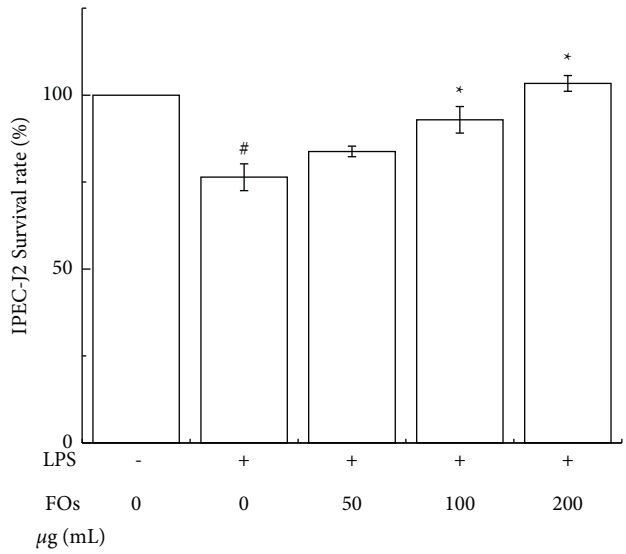


Figure 1: The effects of various concentrations of FOs-FWB on survival rate of LPS-stimulated IPEC-J2 cells. The data are expressed as the mean ± SE ($n = 3$). #Indicates significant differences from control. *Indicates significant differences from LPS. FOs-FWB: feruloyl oligosaccharides from fermented wheat bran.

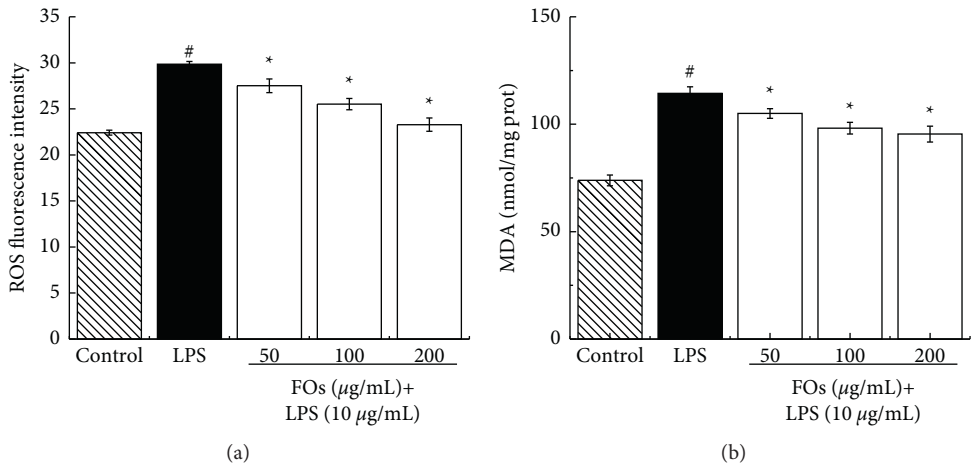


Figure 2: The effects of various concentrations of FOs-FWB on the level of ROS (a) and MDA (b) of LPS-stimulated IPEC-J2 cells. The data are expressed as the mean ± SE ($n = 3$). #Indicates significant differences from control. *Indicates significant differences from LPS. FOs-FWB: feruloyl oligosaccharides from fermented wheat bran.

antioxidative defense system of cells, because higher activities and mRNA expressions of phase II detoxifying/antioxidative enzymes were also observed in our study.

The regulating effects of FOs-FWB on GSH content and antioxidative enzymes (SOD, CAT, and GSH-Px) activities were investigated in LPS-stimulated IPEC-J2 cells. SOD catalyzed dismutase reaction of superoxide anion radicals into H_2O_2, and then CAT converts H_2O_2 into H_2O and oxygen. In addition, GSH-Px works on GSH to reduce H_2O_2 and lipid hydroperoxides to H_2O and the corresponding alcohols [28, 29]. These antioxidative enzymes could effectively alleviate oxidative stress by minimizing ROS generation and removing cytotoxic peroxides in cells [30]. As shown in Figure 3, compared with the control group, LPS induction significantly decreased SOD, CAT, and GSH-Px activities ($p < 0.05$), which suggested that LPS could induce OS in IPEC-J2 cells. The cells were pretreated with FOs-FWB at concentrations of 50–200 μg/mL prior to LPS induction, and significantly enhancements in the activities of SOD, CAT, and GSH-Px were observed ($p < 0.05$). Yao et al. showed that FOs significantly improved SOD activity in H_2O_2-stimulated PC 12 cells [11]. Moreover, Zhang et al. discovered that activities of SOD, CAT, and GPx in AAPH-treated HepG2 cells were increased FOs, and further confirmed the negative correlation between the activities of antioxidative enzymes and ROS content [12].

As the most abundant nonenzymatic thiol, GSH defends against oxidative stress in mammalian cells [31]. The synthesis of GSH is restricted by glutamate cysteine ligase, which is composed of a catalytic (GCLC) and a modifier

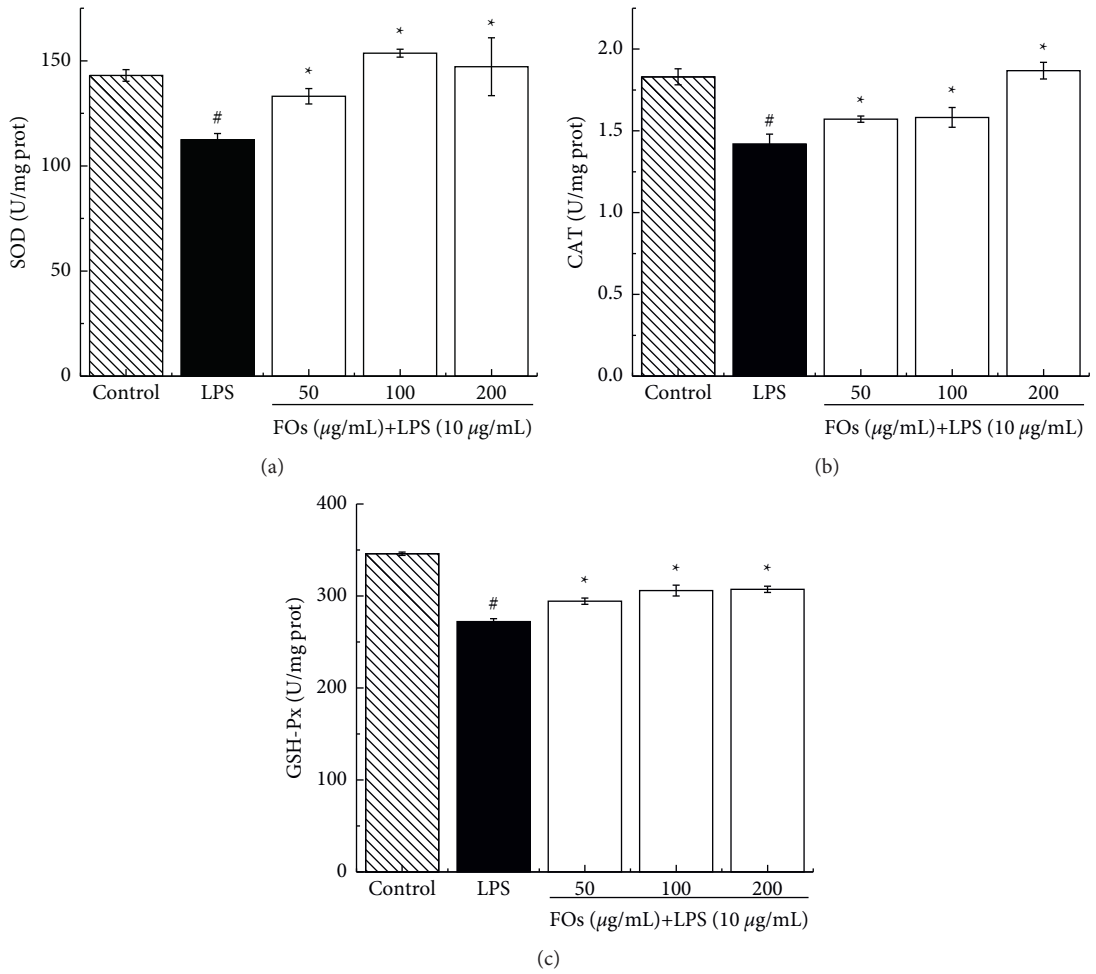
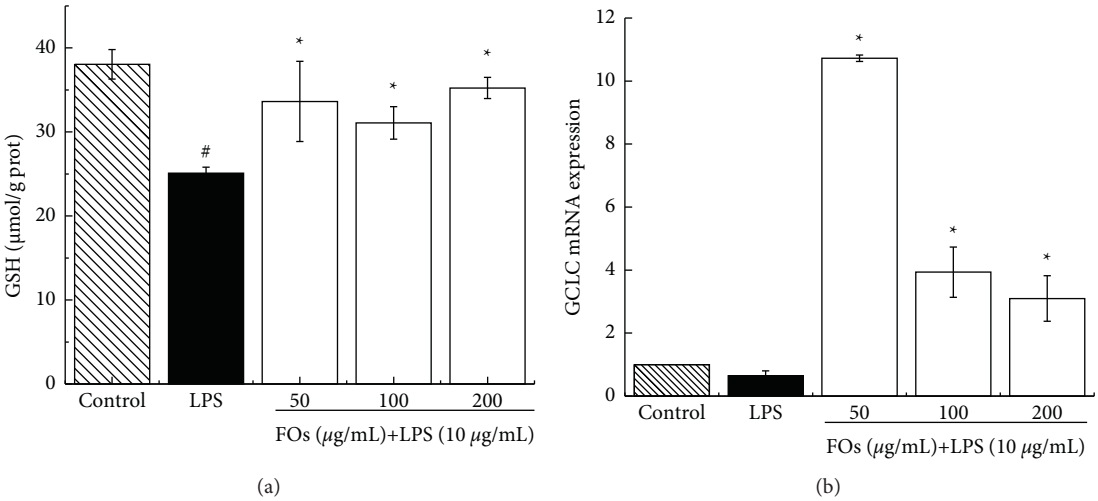


Figure 3: The effects of various concentrations of FOs-FWB on the activity of SOD (a), CAT (b), and GSH-Px (c) of LPS-stimulated IPEC-J2 cells. The data are expressed as the mean ± SE ($n = 3$). #Indicates significant differences from control. *Indicates significant differences from LPS. FOs-FWB: feruloyl oligosaccharides from fermented wheat bran.



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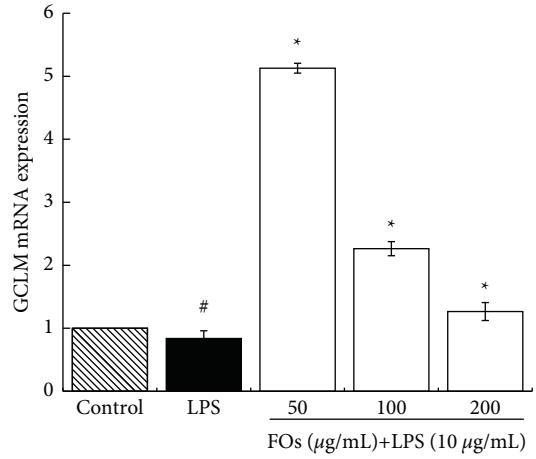
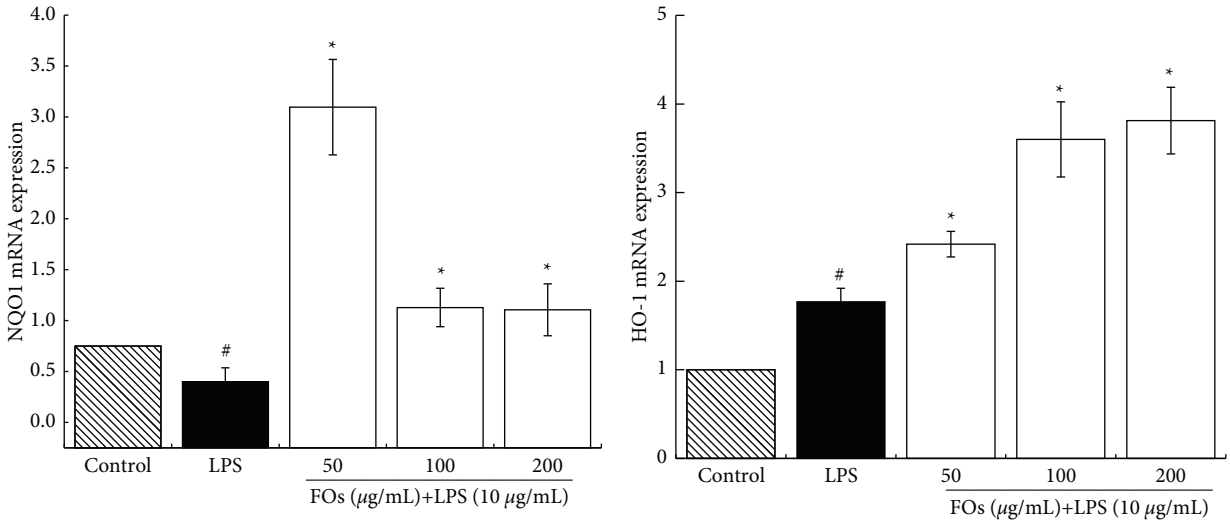


Figure 4: The effects of various concentrations of FOs-FWB on GSH content (a) and GCLC mRNA expression (b) and GCLM mRNA expression (c) of LPS-stimulated IPEC-J2 cells. The data are expressed as the mean ± SE ($n = 3$). #Indicates significant differences from control. *Indicates significant differences from LPS. FOs-FWB: feruloyl oligosaccharides from fermented wheat bran.

(GCLM) subunit [32]. Our study in IPEC-J2 cells presented as Figure 4(a) revealed that GSH level was significantly decreased by LPS induction, while pretreatment of FOs-FWB remarkably normalized the GSH level ($p < 0.05$). Additionally, as shown in Figure 4(b), significantly reduced GCLM mRNA expression was observed in IPEC-J2 cells with LPS treatment ($p < 0.05$). However, the nondiffered GCLC mRNA expression was unexpected in LPS-stimulated IPEC-J2 cells, as shown in Figure 4(c). FOs-FWB at 50–200 μg/mL significantly increased the relative mRNA expression of GCLM and GCLC ($p < 0.05$). The upregulated mRNA expression of GCLC and GCLM consisted of the increase of GSH content in IPEC-J2 cells. These results were consistent with the observed improvement of GSH level with FOs in human erythrocytes exposed to AAPH [24].

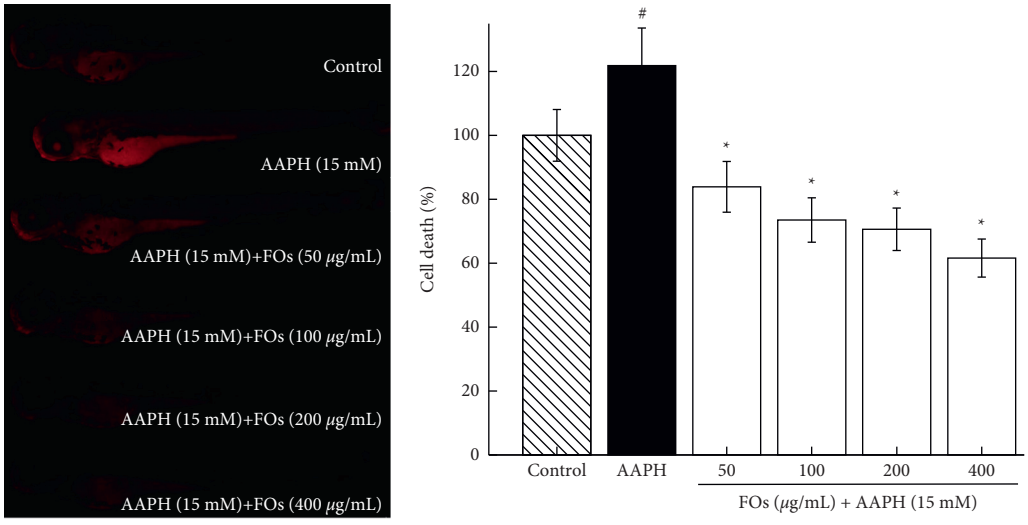
Key phase II detoxifying enzymes including HO-1 and NQO-1 play an important role in protecting cells against oxidative damage [22, 33]. Expressions of HO-1 and NQO-1 have been widely studied as biomarker of OS in cells [34]. Thus, the effects of FOs-FWB on mRNA expression levels of NQO1 and HO-1 are shown in Figure 5. The mRNA expression level of NQO1 was significantly reduced in IPEC-J2 cells after LPS stimulation, whereas the mRNA levels of HO-1 were significantly increased ($p < 0.05$). However, cells pretreated with FOs-FWB for 24 h had increased mRNA expressions of NQO1 and HO-1 ($p < 0.05$). However, there is no previous report on the regulating ability of FOs-FWB in phase II detoxifying enzymes, it is difficult to make any direct comparison. Based on all these results, we suggested that the protective effects of FOs-FWB against OS in IPEC-J2 cells are involved in regulation of antioxidative/phase II detoxifying enzyme expression and GDH synthesis.

3.2. The Protective Effects of FOs-FWB in AAPH-Stimulated Zebrafish Embryos. Zebrafish are vertebrates with special advantages over other experimental animal models, such as small and transparent larvae, a short lifespan, rapid embryogenesis, and genomic similarity to humans [35]. The zebrafish embryo OS model induced with AAPH has been widely used to search and evaluate natural antioxidants [20, 36]. To confirm the protective effects of FOs-FWB against OS in vitro, AAPH-induced OS zebrafish embryos were chosen as the in vivo model. No toxicity of FOs-FWB (50–400 μg/mL) treatment was observed based on mortality rate of zebrafish embryos (Figure S2). Cell death, ROS production and lipid peroxidation of AAPH-treated zebrafish embryos are displayed in Figure 6. Significant increases in the cell death (121.87%), ROS production (128.26%), and lipid peroxidation (131.75%) were detected in AAPH treated zebrafish embryos compared to untreated embryos ($p < 0.05$). However, pretreatment with FOs-FWB at concentrations of 100–400 μg/mL before AAPH induction significantly decreased ROS production to 96.79%, 96.32%, and 87.89%, respectively ($p < 0.05$). The cell death and lipid peroxidation in zebrafish embryos were significantly reduced by FOs-FWB (50–400 μg/mL) pretreatment ($p < 0.05$), and the lowest levels of cell death and lipid peroxidation were 61.65% and 73.45% at 400 μg/mL. This indicated that FOs-FWB effectively suppressed AAPH-induced cell death, ROS generation, and lipid peroxidation in zebrafish embryos. To further investigate the protective effects of FOs-FWB, we tested the antioxidative enzymes (SOD, CAT, and GSH-Px) activities in zebrafish embryos. As shown in Figure 7, CAT, GSH-Px, and SOD activities were significantly decreased in zebrafish stimulated with AAPH as compared to the control group ($p < 0.05$). Conversely, as compared to the AAPH-stimulated group, the CAT activity of zebrafish embryos was significantly improved by pretreatment of FOs-FWB at a concentration of 50–400 μg/mL ($p < 0.05$). Zebrafish embryos pretreated with 100 μg/mL FOs-FWB showed a significant increase in GSH-Px activity ($p < 0.05$). Pretreatment with 100 and 200 μg/mL FOs-FWB significantly enhanced the SOD activity in zebrafish embryos ($p < 0.05$). These results indicated that



(a) (b)

Figure 5: The effects of various concentrations of FOs-FWB on NQO-1 mRNA expression (a) and HO-1 mRNA expression (b) of LPS-stimulated IPEC-J2 cells. The data are expressed as the mean ± SE ($n = 3$). #Indicates significant differences from control. *Indicates significant differences from LPS. FOs-FWB: feruloyl oligosaccharides from fermented wheat bran.



(a) (b)

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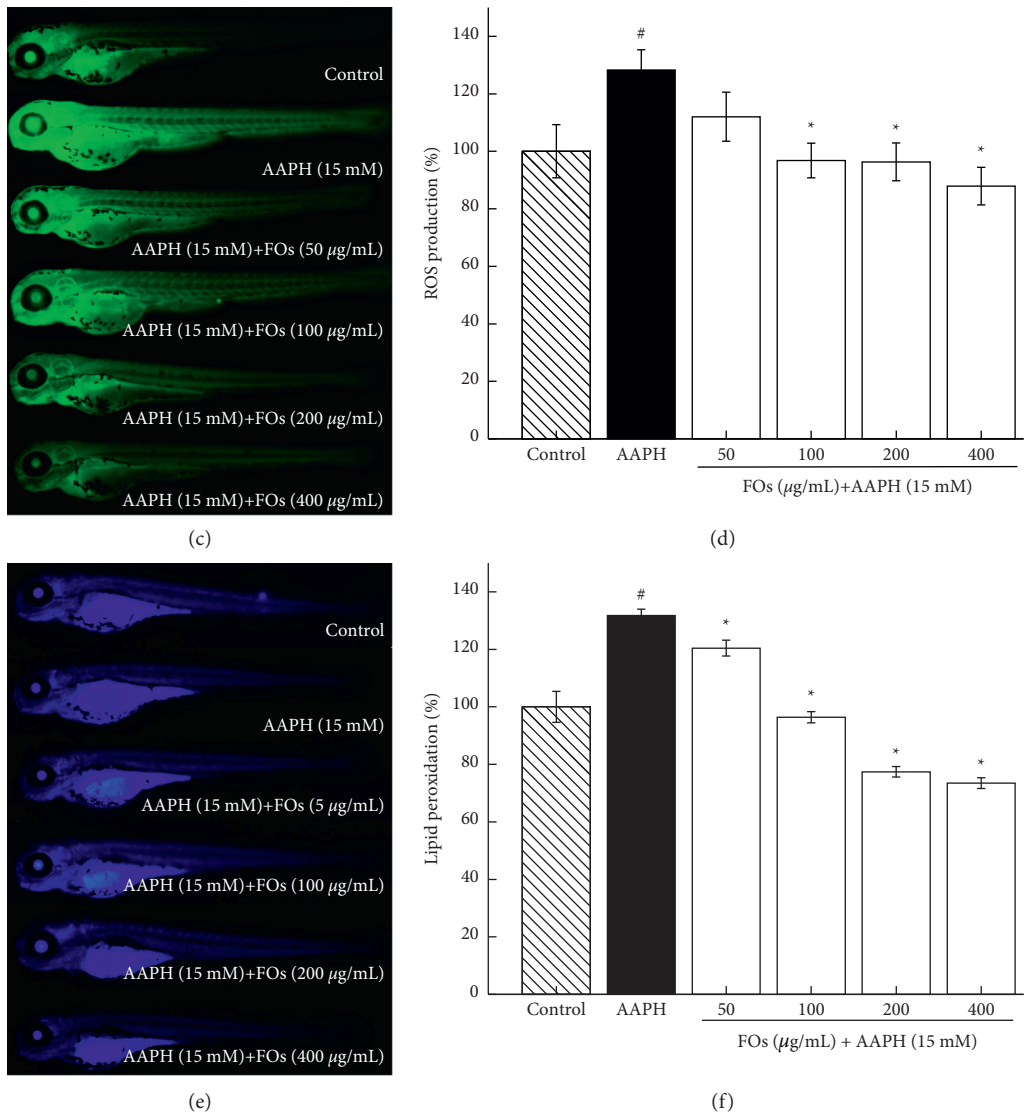
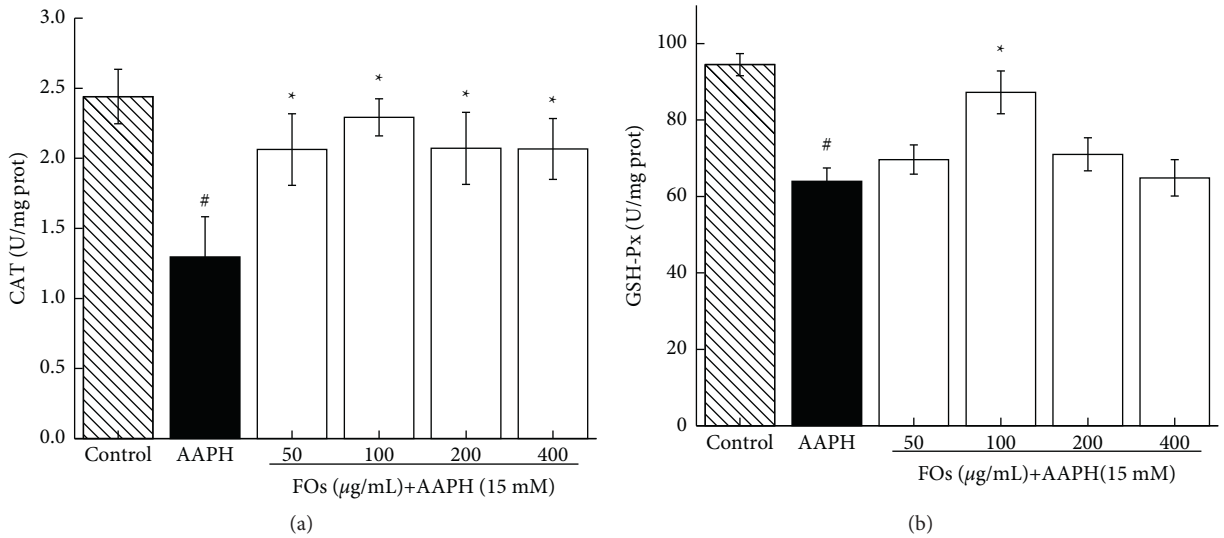


Figure 6: The effects of various concentrations of FOs-FWB on cell death (a, b), ROS generation (c, d), and lipid peroxidation (e, f) in AAPH-stimulated zebrafish embryos measured by fluorescence microscopy and image J software. The data are expressed as the mean ± SE ($n = 3$). #Indicates significant differences from control. *Indicates significant differences from AAPH. FOs-FWB: feruloyl oligosaccharides from fermented wheat bran.



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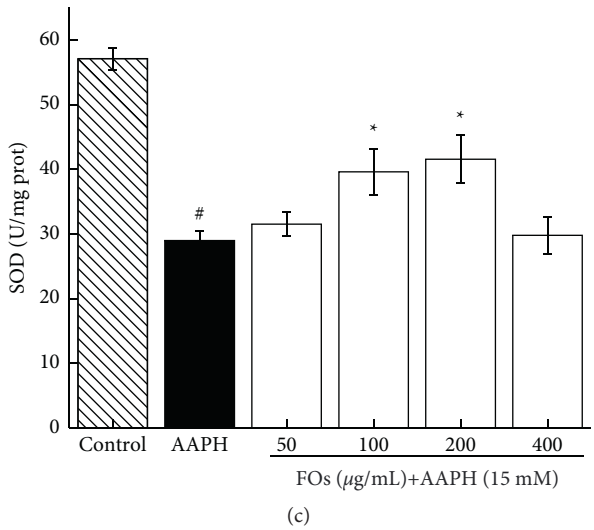

Figure 7: The effects of various concentrations of FOs-FWB on the activity of CAT (a), GSH-Px (b), and SOD (c), in AAPH-stimulated zebrafish embryos. The data are expressed as the mean ± SE ($n = 3$). #Indicates significant differences from control. *Indicates significant differences from AAPH. FOs-FWB: feruloyl oligosaccharides from fermented wheat bran.

FOs-FWB effectively suppressed AAPH-induced cell death, ROS generation, and lipid peroxidation in zebrafish embryos by improving antioxidative enzyme activities, which is consistent with our findings in vitro. In a similar study, it was reported that the MDA formation induced by H_2O_2 in the liver of mice was dramatically inhibited by FOs [37]. Additionally, Zhang et al. clarified that FOs exhibited positive impacts on MDA accumulation and antioxidative enzymes activities reduction in the heart, liver, and kidney of AAPH-treated rats [38].

4. Conclusion

In summary, FOs-FWB exhibited protective effects on IPCE-J2 cells against OS induced by LPS, evidenced by suppressing ROS and MDA formation through enhancing antioxidative/phase II detoxifying enzyme expression and GDH synthesis. Furthermore, FOs-FWB effectively suppressed AAPH-induced cell death, ROS generation, and lipid peroxidation in zebrafish embryos by improving antioxidative enzyme activities. Prospectively, FOs isolated from mixed bacterial fermented wheat bran might be a potential natural antioxidant to prevent the gastrointestinal tract from oxidative stress.

Data Availability

All the data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

The authors Jia Zhang and Qiuyan Chen contributed equally to this work.

Acknowledgments

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Supplementary Materials

Figure S1. The effects of various concentrations of FOs-FWB survival rate of IPEC-J2 cells. The data are expressed as the mean ± SE ($n = 3$). *Indicates significant differences from control. △indicates significant differences between the two feet compared. FOs-FWB: feruloyl oligosaccharides from fermented wheat bran. Figure S2. The effects of various concentrations of FOs-FWB on the viability of zebrafish. The data are expressed as the mean ± SE ($n = 3$). *Indicates significant differences from control. FOs-FWB: feruloyl oligosaccharides from fermented wheat bran. (*Supplementary Materials*)

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