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Hydrolyzed wheat gluten alleviates deoxynivalenol-induced intestinal injury by promoting intestinal stem cell proliferation and differentiation via upregulation of Wnt/β-catenin signaling in mice

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ABSTRACT

Disintegration of the intestine caused by deoxynivalenol (DON), which is a fungal metabolite found in cereal grain-based human and animal diets, triggers severe intestinal inflammatory disease. Hydrolyzed wheat gluten (HWG) can promote the development of intestine. Therefore, HWG was administered orally to male mice on 1–14 days, and DON was administered to them on 4–11 days. Feed, water intake and body weight were recorded all over the experimental period. Blood samples were collected then the mice were sacrificed to collect the jejunum for crypt isolation and culture. The intestinal morphology was observed by electron microscopy, and Western blotting was used to investigate intestinal stem cell (ISC) proliferation and differentiation, as well as the primary regulatory mechanism of the Wnt/β-catenin signaling. The results showed that HWG increased the average daily gain and average daily water intake of mice under DON-induced injury conditions, and increased the jejunum weight, villous height in the jejunum, and promoted jejunal crypt cell expansion. The DON-induced decrease in Wnt/β-catenin activity, the expression of Ki67, PCNA and KRT20 were rescued by HWG in the jejunum, crypt and enteroid, as well as the number of goblet cells and Paneth cells. Furthermore, HWG increased jejunum diamine oxidase (DAO) activity. In conclusion, HWG alleviates DON-induced intestinal injury by enhancing ISC proliferation and differentiation in a Wnt/β-catenin-dependent manner.

1. Introduction

The integrity of intestinal epithelium is a crucial defense against bacteria and viruses living in the environment, as well as natural toxins occurring in food and feed (Sartor and Wu, 2017; Vignal et al., 2018). The maintenance of intestinal homeostasis depends on the continuous proliferation and differentiation of Lgr5-positive intestinal stem cells (ISCs), which are interspersed between terminally differentiated Paneth cells and located at the lower third of the crypts with a highly dynamic niche (Snippert et al., 2010). The Paneth cell-derived niche supplies ISCs with essential pro-proliferative and differentiative factors that include Wnts; thus, the activity of ISCs is tightly controlled by the Wnt/β-catenin pathway (Krausova and Korinek, 2012; Sato et al., 2011). Additionally, β-catenin accumulates in the cell cytoplasm and enters the nucleus, where it displaces the Groucho from TCF/LEF transcription factors, and TCF/LEF-β-catenin complexes thus act as bipartite transcriptional activators of specific target genes such as Lgr5 (Kretzschmar and Clevers, 2017).

Deoxynivalenol (DON), a *Fusarium* metabolite, mainly contaminates cereal-based food and feed worldwide (Rodríguez-Carrasco et al., 2014). DON is considered an important food safety issue since it is an extremely prevalent mycotoxin (Streit et al., 2013). Since DON mainly enters the body via the oral route, the intestinal epithelium is one of the primary targets following dietary DON exposure, which can cause the destruction of intestinal architecture, a decrease in trans-epithelial electrical resistance, modulation of the opening of tight junctions, an increase in intestinal permeability and bacterial translocation, reduced goblet cell density and β-defensin production, and alteration in the intestinal cell proliferative and apoptotic index (Akbari et al., 2014; Cheat et al., 2016; Gerez et al., 2015; Ghareeb et al., 2015; Li et al., 2019; Park et al., 2017; Springler et al., 2016; Wang et al., 2014). In addition, DON exposure may be associated with allergies (Akbari et al., 2017). However, whether DON exposure causes disintegration of intestinal epithelial integrity by changing the fate of ISC proliferation and

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differentiation in a Wnt/β-catenin-dependent manner remains unclear. Therefore, it will be of great significance to conduct further investigations into the mechanism of DON-induced intestinal toxicity.

Nutritional intervention for intestinal injury is emphasized by the enormous harm of DON to human and animal health. Hydrolyzed wheat gluten (HWG), obtained by enzymatic hydrolysis of wheat gluten and purified by molecular size or electric charge, exhibits immunity-strengthening properties in healthy human subjects, prevents diarrhea and promotes growth (Han et al., 2017; Horiguchi et al., 2005; Wang et al., 2011). Consequently, HWG is widely used as a practical, natural, functional food to improve the health of humans due to its glutamate, glutamine and a variety of biologically active-wheat peptides (Wang et al., 2007).

Considering the beneficial effects of HWG as a nutritional fortifier and its role in the protection of the anti-diarrhea function, we hypothesized that HWG could prevent DON-induced disruption of intestinal integrity. This is the first study to find the that the preventive effects of HWG against DON-induced intestinal epithelial injury in mice depend on promoting the proliferation and differentiation of ISCs by activating Wnt/β-catenin signaling and enhancing intestinal barrier function.

2. Materials and methods

2.1. Chemicals and reagents

HWG was provided by Zhengzhou Newwill Nutrition Technology Co., Ltd. (China) and the ingredient composition of the HWG (Table 1) was described by Wang et al. (2011). Physiological saline was used to dissolve the HWG, and the final concentration used was equivalent to a gavage administration of 1000 and 2000 mg/kg body weight (BW).

The purified DON (MW: 296.32) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in physiological saline, the final concentration was 2 mg/kg BW. In addition, Glutamine, N-acetylcysteine, nicotinamide and SB202190 were purchased from Sigma-Aldrich (St. Louis, MO, USA); N2 supplement and B27 supplement were purchased from Invitrogen (Carlsbad, CA, USA); Matrigel was purchased from BD Biosciences (San Jose, CA, USA); recombinant murine epidermal growth factor was purchased from PeproTech (Rocky Hill, NJ, USA); Y27632 and CHIR99021 were purchased from Stemgent (Cambridge, MA, USA); LY2157299 was purchased from Selleck (Houston, TX, USA); DAO kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); lipopolysaccharide (LPS) ELISA kit was purchased from Shanghai Enzyme Linked Biotechnology Co., Ltd. (Shanghai, China).

The primary antibodies, LYZ (A0099) antibody was purchased from Dako (Denmark); Claudin-1 (#374900) antibody was purchased from Thermo Fisher (Waltham, MA, USA); Lgr5 (TA503316) antibody was purchased from OriGene Technologies (Rockwell, MD, USA); β-actin (#600149), β-catenin (#201328) and PCNA (#200947) antibodies were obtained from Zen BioScience (Chengdu, Sichuan, China); TCF4 (ab130014) and KRT20 (ab238034) antibodies were obtained from Abcam (Cambridge, MA, USA); and Ki67 (NB500-170) was purchased from Novus Biologicals (Littleton, CO, USA). MUC2 (sc-15334) antibody, anti-rabbit IgG (#7074) and anti-mouse IgG (#7056) second antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Table 1
Ingredients of HWG.[a]

Amino acid		Peptides	
Ingredients	Content (%)	Molecular weight (Da)	Content (%)
Glutamic acid	33.93	< 180	4.59
Asparticacid	2.78	180–500	33.03
Serine	3.93	500–1000	17.92
Histidine	1.76	1000–2000	10.22
Glycine	2.84	2000–3000	4.03
Threonine	2.18	3000–5000	7.78
Arginine	2.79	5000–10000	16.31
Alanine	2.21	10000–20000	5.14
Tyrosine	2.98	> 20000	0.96
Valine	3.41		
Methionine	2.59		
Phenylalanine	3.25		
Isoleucine	3.89		
Leucine	5.84		
Lysine	1.22		
Proline	11.54		
Crude protein	75		
Σ AA	87.66		
Σ EAA[b]	26.92		

[a] Values are expressed on dry matter basis.
[b] EAA, essential amino acid.

2.2. HWG and DON administration for mice

Healthy male C57BL/6 mice (4 weeks of age) of clean grade were provided by the Medical Experimental Animal Center of Guangdong Province (Foshan, China). The mice were housed in a mouse colony (temperature, 23 ± 1 °C; relative humidity, 45–60%; lighting cycle, 12 h/day; 08:00–20:00 for light) and had free access to food and drinking water. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of South China Agricultural University (Guangzhou, China) and experiments were approved by the Animal Ethics Committee of South China Agricultural University (Guangzhou, China).

Seventy-two mice were randomly divided into six groups (n = 12): control (CON) , low-dose HWG (LD, 1000 mg/kg BW), high-dose HWG (HD, 2000 mg/kg BW), DON (D, 2 mg/kg BW), low-dose HWG + DON (LD + D) and high-dose HWG + DON (HD + D). The mice in the LD, HD, LD + D and HD + D groups were treated orally with HWG 3 days before and 3 days after 7 days of the combined treatment with DON plus HWG, the mice in group of DON were treated orally physiological saline 3 days and 3 days after 7 days of the treatment with DON, and the mice in the CON group were treated with physiological saline of the same volume. Feed, water intake and body weight were recorded all over the experimental period.

2.3. Serum and intestine collection, crypt isolation and culture

Mice were bled retro-orbitally to collect blood samples, and then to collect the jejunum after they were euthanized with CO_2 inhalation followed by cervical dislocation to ensure death. The jejunum was collected after phosphate buffered saline (PBS) washing, and samples were fixed in fresh 4% paraformaldehyde or 2.5% glutaraldehyde, or snap frozen in liquid nitrogen for protein analysis, while another sample was used to isolate crypts. Specifically, the mucosal surface of the jejunum was scraped with a glass slide to remove the villi, cut into pieces and incubated in soaking buffer. Fresh Dulbecco's phosphate buffered saline (DPBS) was added until crypts were obtained; finally, they were suspended in Matrigel (BD Biosciences, San Jose, CA, USA), and expanded in culture medium, which consisted of 90% WRN conditioned medium, 10% FBS, 1× N2 supplement, 1× B27 supplement, 1× glutamine, 1 mmol/L N-acetylcysteine, 50 ng/mL recombinant murine epidermal growth factor, 10 mmol/L nicotinamide, 10 μmol/L Y27632, 0.5 μmol/L LY2157299, 10 μmol/L SB202190and 10 μmol/L CHIR99021. Enteroid forming efficiency, was calculated as percentage of colony number to the number of crypts seeded, and enteroid area, was measured by Image-Pro Plus software, used to assess enteroid activity.

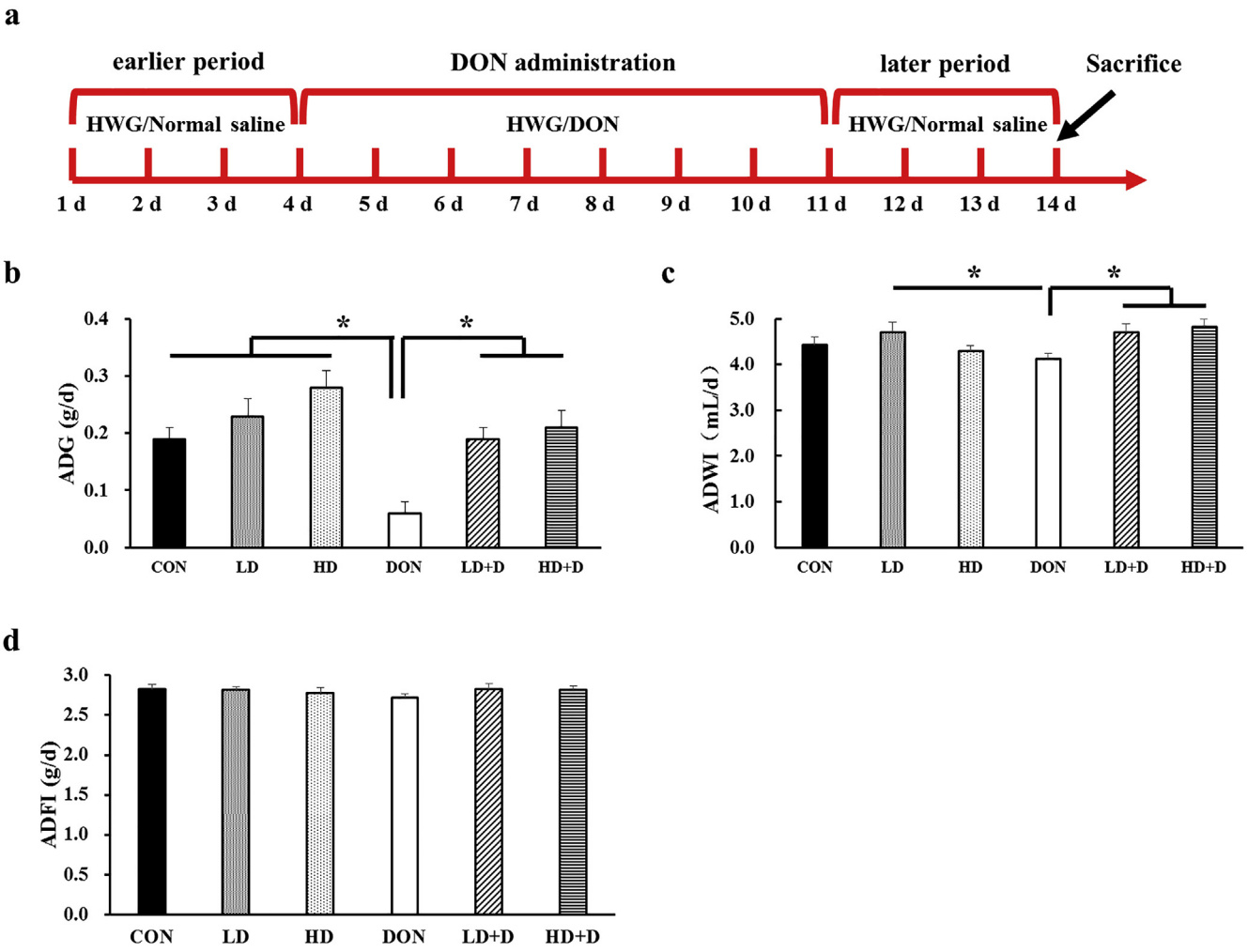

Fig. 1. Effect of HWG on body weight gain, feed and water intake in mice treated with DON. (a) Experimental procedure, (b) average daily gain (ADG), (c) average daily feed intake (ADFI), (d) average daily water intake (ADWI).

2.4. Histological examination of tissues

This was performed using hematoxylin and eosin (H&E) staining. Briefly, jejunum samples were fixed with 4% paraformaldehyde overnight and then washed with PBS, dehydrated with alcohol and embedded in paraffin blocks. Sections of 5 μm were cut for histological analysis. The sections were deparaffinized and hydrated, and then stained with H&E. Villus height and crypt depth were measured using Image-Pro Plus software. Immunohistochemistry against MUC2 and LYZ were used for goblet cell and Paneth cell staining, respectively. Images were taken by confocal microscopy (Ti2, Nikon, Tokyo, Japan). Quantification of villus height, crypt depth, number of goblet cells and Paneth cells were performed in at least six villi or crypts per slide. To determine the villus height, the height from the tip of the villus to the crypt opening was measured, and the associated crypt depth was measured from the base of the crypt to the level of the crypt opening. Then, the villus/crypt ratio was calculated with the ratio of villus height to relative crypt depth. Six mice were studied in each group. The data collectors were unaware of the treatment status of the examined slides.

2.5. Scanning electron microscopy evaluation

The jejuna were fixed with 2.5% glutaraldehyde overnight and then washed with PBS, treated with 1% osmium tetroxide in sodium cacodylate buffer for 1 h, dehydrated with alcohol and dried to the critical point (CPD 300 Critical Point BALTIC Dryer, Leica Microsystems, Liechtenstein). After drying, the jejuna were glued on stubs using carbon tape and coated with gold (Sputter Coater BALTIC ACE 600, Leica Microsystems, Liechtenstein). The jejuna were analyzed using an EVO MA 15 scanning electron microscope.

2.6. Detection of diamine oxidase (DAO) activity

The activity of diamine oxidase (DAO) was determined in the jejunum and serum using a commercial DAO kit. The procedure was performed according to the manufacturer's protocols. The color optical density absorbance was measured by visible spectrophotometer at a wavelength of 340 nm.

2.7. Enzyme-linked immunosorbent assay

The concentration of lipopolysaccharide (LPS) was determined in the jejunum and serum using a commercial ELISA kit. The procedure was performed according to the manufacturer's protocols. The color optical density absorbance was measured at a wavelength of 450 nm. The sample concentrations were calculated using a microtiter plate reader (Thermo, USA) according to the relevant standard curves.

2.8. Isolation of total protein and Western blotting

The total protein in the jejunum and crypt were extracted using a radio immunoprecipitation assay (RIPA) lysis buffer containing 0.1%

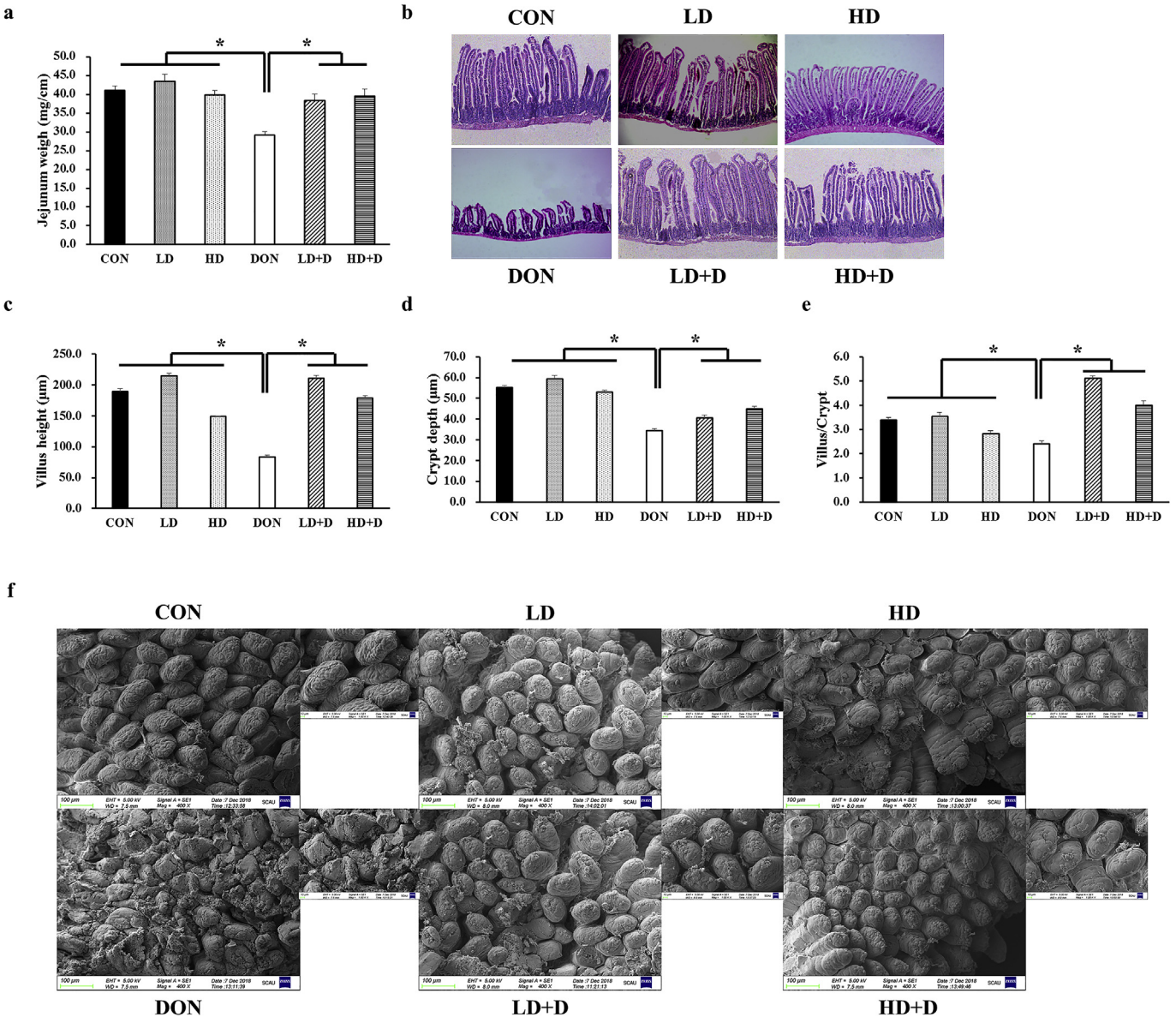

Fig. 2. Effect of HWG on the restitution of intestinal epithelial injury in mice treated with DON. (a) Jejunum weight, (b) H&E staining in the jejunum (×100), (c) villus height, (d) crypt depth, (e) the ratio of villus to crypt and (f) images of scanning electron micrographs in the jejunum (×400 and ×1000).

phenylmethylsulfonyl fluoride (PMSF). The protein concentrations in the homogenates were determined using the BCA protein assay kit. The proteins (10 μg) were separated on 8–10% SDS/PAGE gels and then were transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking, the membranes were incubated with the primary antibody and secondary antibody. The proteins were visualized using the Beyo ECL Plus chemiluminescence detection kit (Beyotime Institute of Biotechnology, Shanghai, China). The proteins were visualized using the Beyo ECL Plus chemiluminescence detection kit (Beyotime Institute of Biotechnology, Shanghai, China). Enhanced chemiluminescence (ECL) signals were scanned using a FluorChem M apparatus (Protein Simple, Inc., Santa Clara, CA, USA), and the band densities were analyzed using image analysis software (Tanon, Shanghai, China).

2.9. Automated capillary WES

Enteroids were lysed in RIPA buffer for 30 min and then were centrifuged at 12,000 × *g* and 4 °C for 15 min. The protein concentration of the supernatant was determined using the BCA Protein Assay Reagent kit. The diluted protein lysate was mixed with 5× fluorescent master mix and was heated at 95 °C for 5 min. The samples, blocking reagent, wash buffer, primary antibodies, secondary antibodies, and chemiluminescent substrate were dispensed into designated wells in a manufacturer manufacturer-provided microplate. The plate was loaded into the instrument, and protein was drawn into individual capillaries on a 25-capillary cassette provided by the manufacturer. Protein separation was performed automatically on the individual capillaries using default settings. The data were analyzed using Compass software (Protein Simple, San Jose, CA, USA).

2.10. Statistical analysis

The results were analyzed by SAS (Version 9.2; SAS Inst. Inc., Cary, NC) software. All data are represented by the mean ± SEM. The Duncan's multiple-range test was used to evaluate the differences in six groups or four groups following a standard one-way ANOVA. P-values < 0.05 were considered statistically significant: *P < 0.05.

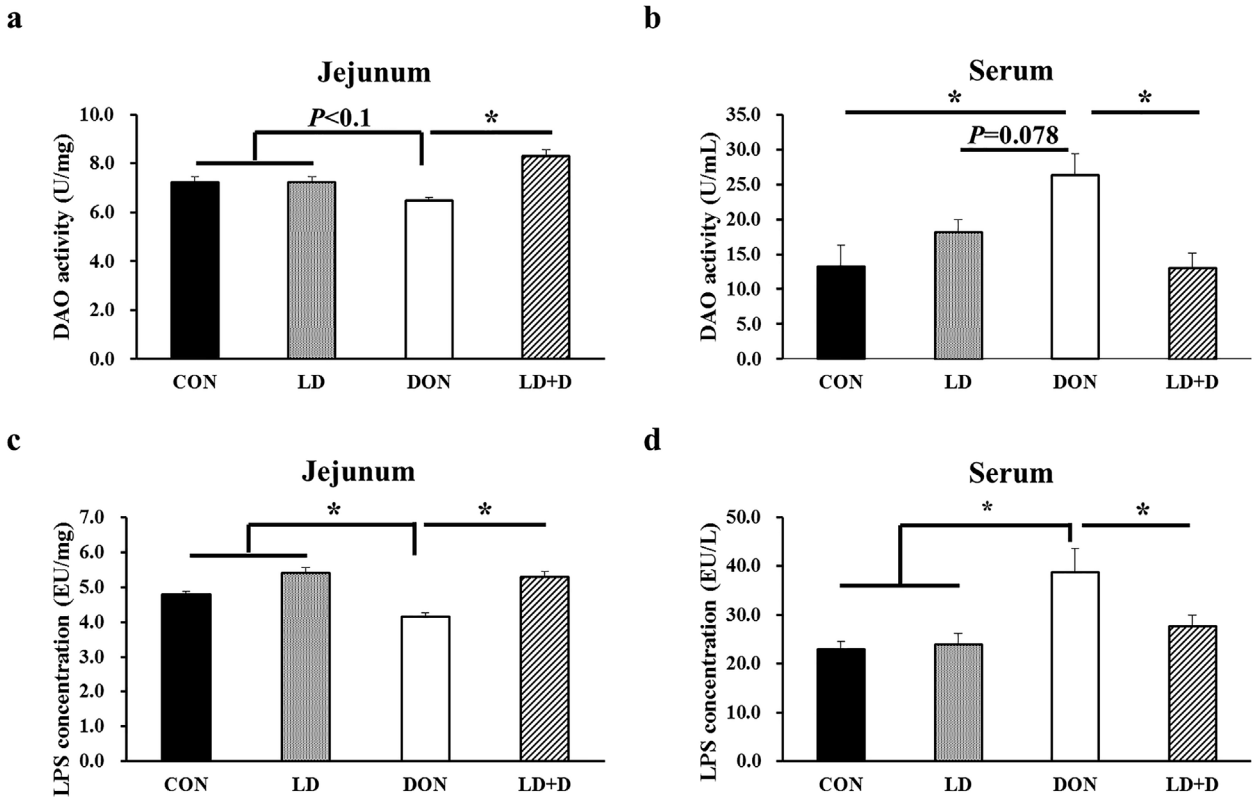

Fig. 3. Effect of HWG on DON-induced disturbances in jejunum and serum DAO and LPS of mice. (a) The activity of DAO in jejunum, (b) the activity of DAO in serum, (c) the concentration of LPS in jejunum and (d) the concentration of LPS in serum.

3. Results

3.1. HWG increases the weight gain and water intake of mice exposed to DON

To investigate the effect of HWG on mice with DON poisoning, weight gain, feed intake and water intake were monitored during the experiment. The experimental procedure is shown in Fig. 1a, compared with the CON group, the average daily gain (ADG) of mice in DON group was decreased significantly; however, compared with the DON group, HWG significantly increased the ADG and average water intake (ADWI) of mice ($P < 0.05$) (Fig. 1b and c) but had no effect on the average daily feed intake (ADFI) ($P > 0.05$) (Fig. 1d). These results indicated that HWG increased the weight gain and water intake of mice exposed to DON.

3.2. HWG stimulates the restitution of intestinal epithelial injury

To explore whether the growth-promoting effect of HWG was due to the repair of intestinal injury induced by DON, the weight of intestine was recorded and it was found that DON significantly reduced the jejunum weight ($P < 0.05$) (Fig. 2a) without affecting the duodenum and ileum (Supplementary Figs. 1a and 1b), as well as the small intestine length (Supplementary Fig. 1c). Interestingly, this reduction was significantly modulated by administration of HWG ($P < 0.05$).

Correspondingly, the HWG significantly increased ($P < 0.05$) the villous height (Fig. 2b and c) and crypt depth (Fig. 2d), resulting in a greater ratio of villus to the crypt in the jejunum ($P < 0.05$) (Fig. 2e). In addition, the jejunum of DON-treated mice showed severe and diffuse villi atrophy and multifocal villi apical necrosis accompanied by a remarkable amount of cell debris on the intestinal surface; however, DON plus low-dose or high-dose HWG mice presented well-delineated villi, showing no atrophy or apical necrosis (Fig. 2f). These results indicated that HWG stimulated the restitution of intestinal epithelial injury induced by DON.

3.3. HWG prevents the DON-induced disruption of the intestinal barrier

Because the intestine of mice in the LD + D group had exhibited a complete epithelial morphology which is conducive to reducing production costs, especially for animal husbandry, thus, the DAO activity and LPS concentration in jejunum and serum of the CON, LD, DON and LD + D groups were detected. The DON-induced decreased diamine oxidase activity (DAO) in jejunum (Fig. 3a) and increased its activity in serum (Fig. 3b). These disturbances in DAO were significantly reversed by HWG supplementation ($P < 0.05$). In accordance with the concentration of lipopolysaccharide (LPS) in jejunum and serum was also significantly reduced and increased, respectively ($P < 0.05$) (Fig. 3c and d). Furthermore, Western blotting analysis showed that the exposure of mice to DON resulted in a significant decrease in the Claudin-1 protein level, which was significantly upregulated with HWG supplementation (Supplementary Fig. 2). These results indicated that HWG prevented the DON-induced disruption of the intestinal barrier.

3.4. HWG promotes the expansion of enteroids exposed to DON

Considering that ISCs drive intestinal epithelial renewal and regeneration, the low-dose HWG showed a better effect against DON-induced intestinal injury. The jejunal crypts were isolated from the mice in the CON, LD, DON and LD + D groups and cultured *ex vivo*. It was observed that the crypts were rod-shaped in the DON group, but Y-shaped in other groups (Fig. 4a). In the subsequent processes of crypt cells, including ISC expansion, we found that crypt stem cell expansion was suppressed by DON treatment, with a significant decrease in enteroid forming efficiency and area ($P < 0.05$) (Fig. 4b and c), and HWG supplementation not only significantly increased enteroid

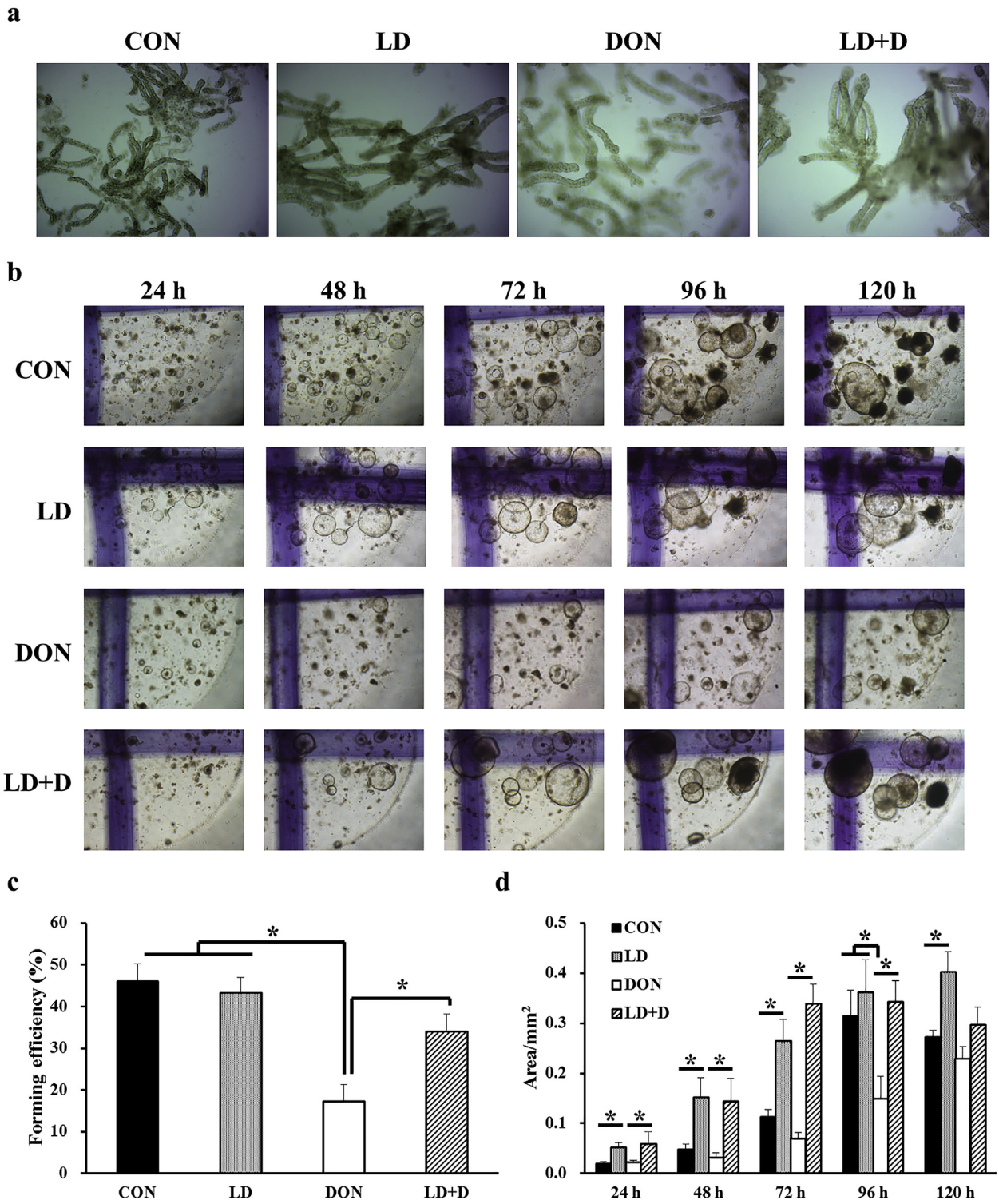

Fig. 4. Effect of HWG on the expansion of crypt stem cells of jejunum of mice treated with DON. (a) Images of jejunal crypts (×100), (b) images of enteroids expanded from crypt stem cells (×40), (c) enteroid formation efficiency and (d) enteroid area.

forming efficiency, but also significantly enlarged the area at 24, 48, 72 and 96 h in both the presence or absence of DON ($P < 0.05$) (Fig. 4d). These results indicated that HWG promoted the expansion of enteroids exposed to DON.

3.5. HWG rescues the DON-induced disturbance of Wnt/β-catenin signaling

The changes in Wnt/β-catenin signaling in the jejunum (Fig. 5a and b), crypt (Fig. 5c and d) and enteroid (Fig. 5e and f) were detected by Western blotting or WES. The results showed that DON significantly inhibited the β-catenin, TCF4 and Lgr5 protein expression and that HWG prevented the downregulation of TCF4 and Lgr5 ($P < 0.05$) and was effective in preventing the DON-induced β-catenin downregulation ($P = 0.095$) in the crypt. These data suggested that HWG might increase the activity of Lgr5-positive stem cells by activating the Wnt/β-catenin signaling pathway postinjury. These results indicated that HWG

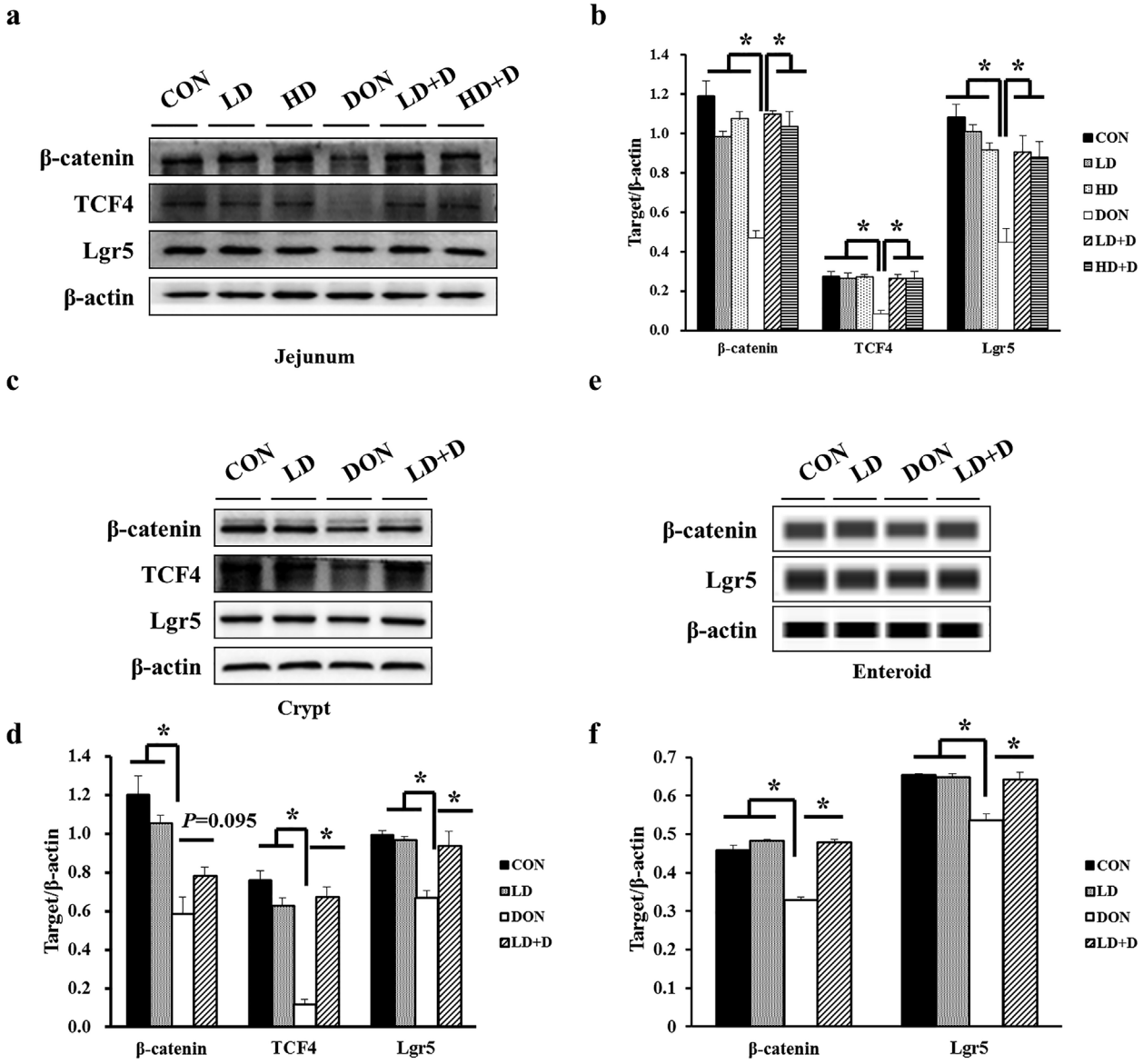

Fig. 5. Effect of HWG on the disturbance of Wnt/β-catenin signaling induced by DON in the intestinal of mice. (a–b) The expression of β-catenin, TCF4 and Lgr5 in jejunum, (c–d) the expression of β-catenin, TCF4 and Lgr5 in crypt and (e–f) the expression of β-catenin and Lgr5 in enteroid.

rescued the DON-induced disturbance of Wnt/β-catenin signaling.

3.6. HWG improves intestinal cell proliferation in mice exposed to DON

Same as above, compared with expression in the DON group, the expression of PCNA and Ki67, markers of the proliferating cell, significantly increased in the jejunum (Fig. 6a and b), crypt (Fig. 6c and d) and enteroid (Fig. 6e and f) in the LD + D group. These results indicated that HWG improved intestinal cell proliferation in mice exposed to DON.

3.7. HWG improves intestinal cell differentiation in mice exposed to DON

The effect of HWG on intestinal cell differentiation induced by DON revealed that HWG significantly increased the number of goblet cells in the villi (Fig. 7a and b) and the number of Paneth cells in the crypt (Fig. 7c and d) of the jejunum. Moreover, the expression of KRT20, a marker of terminal differentiation, was significantly downregulated by DON and this effect was mitigated by HWG (Fig. 7e–j). These results indicated that HWG improved intestinal cell differentiation in mice exposed to DON.

4. Discussion

An important function of the intestinal epithelium is to digest and absorb nutrients to provide the necessary energy for the body. Dietary exposure to DON has been shown to reduce feed intake and weight gain (Dänicke et al., 2012; Pestka and Smolinski, 2005). In the present study, The ADG, not the ADFI, was negatively affected by the given dose of DON; thus, it is not difficult to suggest that DON might decrease the absorption and utilization of nutrients in the intestine. Marc et al. (2002) found DON inhibited the activities of intestinal transporters, including D-glicose/D-galactose sodium-dependent transporter (SGLT1), D-fructose transporter GLUT5. Typically, the nutrient transport and absorption of the intestinal epithelium are positively correlated with its area (Ghareeb et al., 2015). The prominent decrease in villous atrophy and corresponding reduced height and surface area in the jejunum could at least partly explain the decrease in absorption efficiency in the DON-exposed mice. Strikingly, most of these toxic effects were eliminated by HWG, which might be related to its rich active

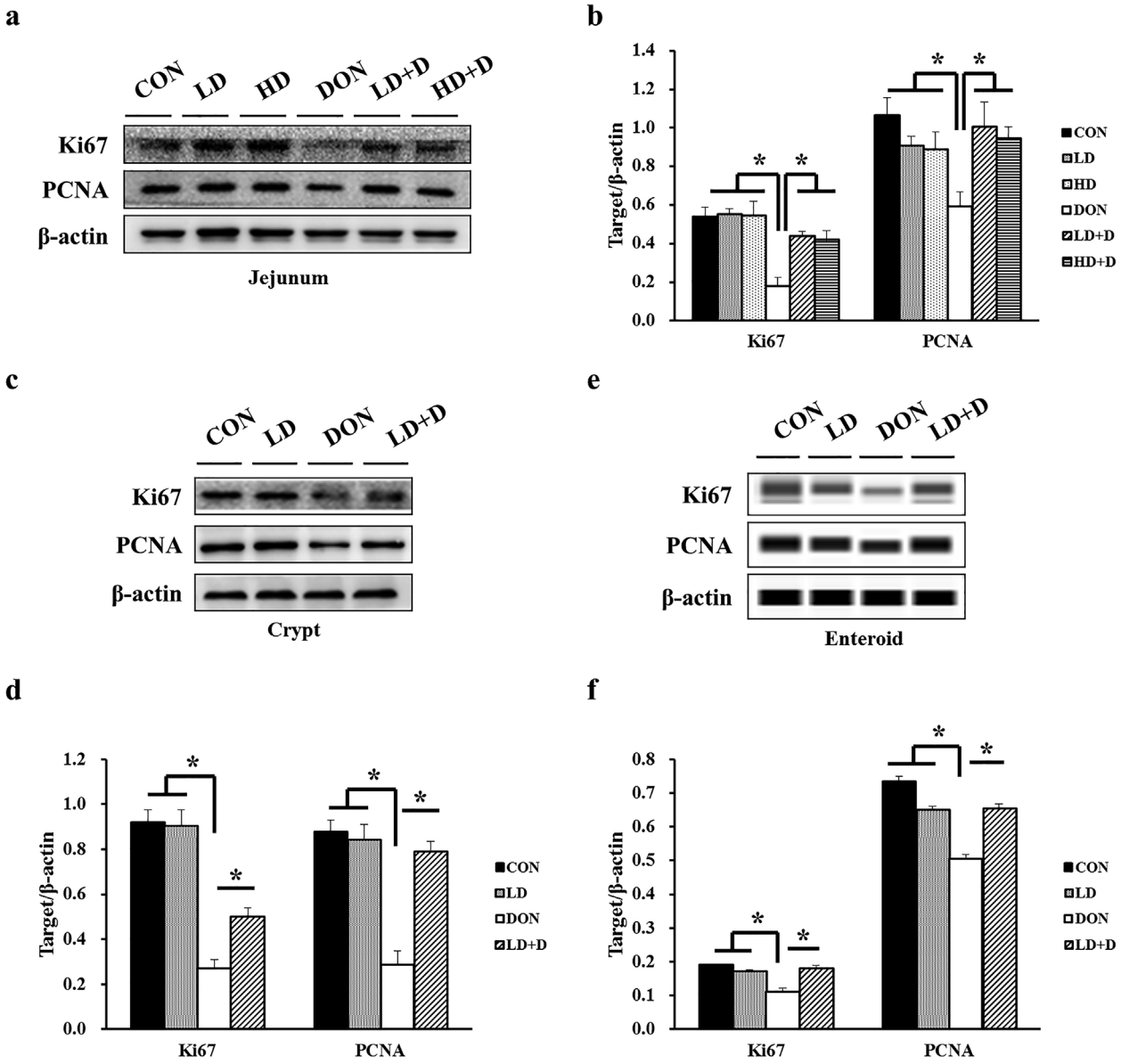

Fig. 6. Effect of HWG on intestinal cell proliferation in mice exposed to DON. (a–b) The expression of Ki67 and PCNA in jejunum, (c–d) the expression of Ki67 and PCNA in crypt and (c–d) the expression of Ki67 and PCNA in enteroid.

components, including glutamate, glutamine and wheat gluten peptides (e.g., glutamine peptides, antimicrobial peptides). Growing evidence suggests that glutamate and glutamine are important functional nutrients for intestinal integrity, mainly reflected in protein synthesis, energy supply for intestinal epithelial cells and improved intestinal recovery postinjury (Jeong et al., 2018; Jiang et al., 2017; Jiao et al., 2015; Kim and Kim, 2017). In addition, wheat gluten peptides have also been demonstrated to have a positive correlation between intestinal trophic effects and against injury effect under non-steroidal anti-inflammatory, drug-induced oxidative stress model *in vivo* and *in vitro* (Hong et al., 2014; Yin et al., 2014). Similar phenomena were observed in the current investigation, which in turn increased the ADG of mice.

Another important function of the intestinal epithelium is to provide a barrier against the penetration of food contaminants and pathogens present in the intestinal lumen. The disruption of the intestinal barrier allows increased penetration of normally excluded luminal substances that may promote intestinal disorders (Drolia et al., 2018). In the present investigation, we demonstrated that DON impaired the intestinal barrier by altering claudin-1 expression. Pinton et al. (2009) also demonstrated it in IPEC-1 or human Caco-2 cell. DON-mediated changes in tight junctions (TJs) increase paracellular permeability to other substances. This study confirmed that hyperpermeability, which causes a significant increase in the translocation of LPS from the intestinal lumen to blood circulation was observed in the DON-treated mice. This is consistent with the high permeability for FITC-dextran exposed to DON found by Akbari et al. (2014). Furthermore, DAO, as an enzyme found in high concentrations in the intestinal mucosa, is used to assess the intestinal integrity (Deng et al., 2016). We found that DAO activity decreased in the intestine and increased in serum indicating the dysfunction of the intestinal barrier in mice exposed to DON. Morphological alterations of the jejunum, including necrosis of intestinal cells, also supported this result. The capability of HWG to modulate Claudin-1 expression has been shown to prevent barrier injury in the present investigation. This effect might be related to the abundant glutamate and glutamine in HWG, which stimulates the growth of the small intestinal mucosa and protects the intestinal barrier by increasing TJs and eliminating the intercellular space from atrophy and injury under various stress conditions (Wang et al., 2015; Xiao et al., 2014).

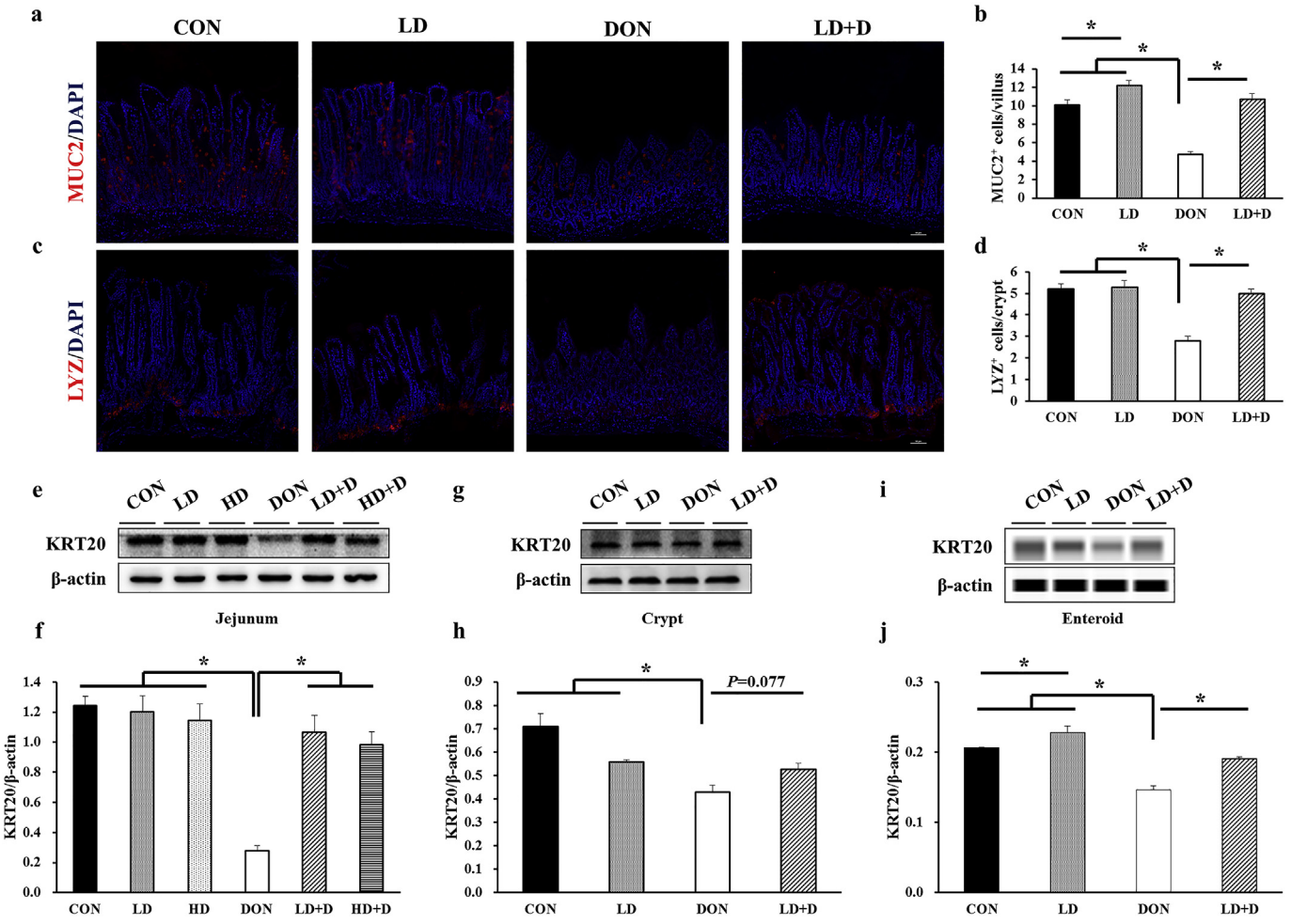

Fig. 7. Effect of HWG on intestinal cell differentiation in mice exposed to DON. (a) images of immunohistochemistry (IHC) staining with the MUC2 antibody in jejunum (×200), (b) statistical analysis of the number of MUC2$^+$ cells in each villus, (c) images of IHC staining with LYZ antibody in jejunum (×200), (d) statistical analysis of the number of LYZ$^+$ cells in each crypt (e–f) the protein expression of KRT20 in jejunum, (g–h) the protein expression of KRT20 in crypt and (i–j) the protein expression of KRT20 in enteroid.

Given that ISCs are the driving force behind the perpetual renewal and regeneration of the intestinal epithelium and are sensitive to external stimuli, it was not surprising for us to find that DON inhibited ISC expansion. ISCs reside within a local Wnt niche and are dependent on Wnt/β-catenin signaling for survival (Andersson-Rolf et al., 2017; Fan et al., 2017; Gong et al., 2016; Koch, 2017). Accordingly, Wnt/β-catenin inhibition in the intestine blocks ISC activity. The result that Lgr5 expression was downregulated, accompanied by inactivation of the Wnt/β-catenin pathway after DON administration in this investigation, strongly supported this view. Previous study has shown that high levels of β-catenin in regenerating intestinal crypts are important for intestinal homeostasis (Cordero and Sansom, 2012). And the normally quiescent +4 ISCs (marked by Bmi1) or daughters of the ISC are activated to fall back into the ISC niche and replenish the stem cell pool after the Lgr5$^+$ stem cells are ablated following injury (Li et al., 2014, 2018; Montgomery et al., 2011). Our data argue that HWG increased a robust regenerative response by upregulating Wnt/β-catenin signaling and stem cell activity in jejunum during DON-induced injury.

The process of intestinal regeneration is achieved by a marked increase in proliferation within the crypt and differentiation within the villi which is not only regulated by Wnt signaling, but also by Notch and mTORC1 signaling (Liang et al., 2019; Zhou et al., 2019). In the present study, we found that HWG reversed the inhibitory effect of DON on the expression of Ki67/PCNA (proliferative cell markers) and KRT20 (terminally differentiated cell marker), which suggests that HWG might improve ISC proliferation and differentiation to promote intestinal epithelial regeneration in mice. Specific dietary factors, such as glutamate, can regulate stem cell activity and induce ISC division in *Drosophila* (Deng et al., 2015). However, it is not clear whether other components of HWG have this effect.

Moreover, the numbers of MUC2$^+$ cells (goblet cell) and LYZ + cells (Paneth cell) in the jejunum were also significantly increased with the enhancement of differentiation ability. There is mounting evidence that Paneth cells are a rich source of Wnt/β-catenin pathway activators, which can dynamically shape Wnt/β-catenin signaling to control epithelial proliferation and restitution (Sato et al., 2011). Thus, the idea that HWG improved the Paneth cell generation in the DON-treated mice, which in turn provided Wnts to promote ISC differentiation, should be acceptable. Meanwhile, MUC2 protein secreted from goblet cells limits the exposure of individual epithelial cells to DON and ensures swift repair of the epithelial barrier.

Taken together, our results demonstrated that HWG supplementation effectively alleviates intestinal injury induced by DON in mice and prevents the disruption of intestinal integrity by increasing Wnt/β-catenin signaling, stimulating the proliferation and differentiation of ISCs, and reinforcing the intestinal barrier. Therefore, this study will be valuable in providing effective means for daily protection and clinical intervention against DON.

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Appendix A. Supplementary data

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Transparency document

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contribution statement

Jia-yi Zhou was involved in conception and design, collection of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; Sai-wu Zhang and Hua-lin Lin were involved in the collection of data, and manuscript writing; Chun-qi Gao and Hui-chao Yan were involved in data analysis and interpretation; Xiu-qi Wang was involved in data interpretation, and final approval of manuscript.

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