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Chlorogenic acid attenuates cadmium-induced intestinal injury in Sprague–Dawley rats

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ABSTRACT

Chronic exposure to cadmium (Cd) causes remarkable damage to the liver and gastrointestinal tract. Previous studies have revealed that chlorogenic acid (CGA) could improve the intestinal barrier integrity for weaned rats. Hence, this study sought to investigate the protective effects of CGA from pure reagent and sunflower seed extract (SSE) on growth performance, antioxidant indicators, inflammatory biomarkers and intestinal barrier function in Cd treated rats. A total of 32 Sprague–Dawley female rats with similar weights were randomly allotted to four treatments: control, $CdCl_2$ (6 mg/kg BW), co-treatment of Cd (6 mg/kg BW) and pure CGA (50 mg/kg BW), and co-treatment of Cd (6 mg/kg BW) and SSE (50 mg/kg BW) for 14 days. The data indicated that, CGA or SSE with Cd sequestration and good antioxidative ability decreased Cd absorption and accumulation in the jejunum and increased fecal Cd levels in Cd-exposed rats. Compared with the Cd group, co-treatment with CGA or SSE also alleviated inflammation, ameliorated the villus damage, reversed the disruption of tight junctions, and recovered weight gain of rats. These results suggest that CGA or SSE can protect the intestinal barrier, which is related to the alleviation of Cd-induced oxidative stress and growth decrease.

1. Introduction

Cadmium (Cd) is a toxic and carcinogenic metal widely distributed in the environment. Cadmium exposure induces renal, intestinal, reproductive and cardiovascular dysfunction both in experimental animals and humans (Breton et al., 2013; Chen et al., 2016a; Hong et al., 2004; Tellez-Plaza et al., 2008; Thompson and Bannigan, 2008). In the general population, the primary sources of Cd exposure are cigarette smoke, ambient air particularly in urban areas and in the vicinity of industrial settings, and food intake (shellfish, offal, certain vegetables), which represents the major route of human exposure.

Following oral exposure, only a minor part of the ingested Cd (ranging from 2 to 12%) is absorbed in mammals, preferentially in the duodenum and proximal jejunum, where it directly impacts on the complex gut ecosystem and its physiology during the entire transit time (Andersen et al., 1994; Cardin et al., 2009; Zalups and Ahmad, 2003). The intestinal epithelium is the first protective barrier against Cd toxicity following oral exposure (Jumarie et al., 1999; Suzuki et al., 2008). Previous studies have shown that the intestinal barrier plays a crucial role in limiting Cd absorption (Jumarie et al., 1999), and Cd exposure causes an inflammatory response, oxidative stress, death of epithelial cells, and damage to tight junctions in the intestines, leading to the disruption of the intestinal barrier and the amplification of Cd absorption (Blais et al., 1999; Breton et al., 2013; Ninkov et al., 2015). After uptake into the organism, Cd is transported via the blood to various tissues, particularly to the liver where it induces the synthesis of metallothionein (MT), a ubiquitous metal-binding detoxification protein that converts toxic ionized Cd into non-toxic bound Cd (Blais et al., 1999; Klaassen and Liu, 1997; Saito and Kojima, 1997).

Cadmium favors the production of oxygen species leading to

Abbreviations: ALB, albumin; ALT, alanine transaminase; AST, aspartate aminotransferase; CAT, catalase; Cd, cadmium; CGA, chlorogenic acid; CHOL, cholesterol; DMT1, divalent metal transporter 1; Fe, iron; GLU, glucose; GPx, glutathione peroxidase; HDL, high density lipoprotein; ig, intragastric administration; IL-6, interleukin-6; JAM-A, junctional adhesion molecule A; LDLC, low density lipoprotein cholesterol; MDA, malondialdehyde; MT, metallothionein; ROS, reactive oxygen species; SOD, superoxide dismutase; SSE, sunflower seed extract; TG, triglyceride; TNF-α, tumor necrosis factor alpha; TP, total protein; ZO-1, Zonula occludens protein 1

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oxidative stress (Wang et al., 2004). The formation of reactive oxygen species (ROS) is thought to be mediated mainly by superoxide production in the mitochondria following Cd-induced inhibition of the electron transfer chain (Wang et al., 2004). Cadmium can bind to the sulfhydryl groups of specific proteins and release iron from its binding sites, with the subsequent Fenton reactions (Souza et al., 2004).

Natural products are known to exert protective effects against heavy metal-mediated injury by scavenging free radicals and modulating the antioxidant defense system (Kim et al., 2018). Chlorogenic acid (CGA) is an ester formed between caffeic and quinic acids, and is one of the most abundant hydroxy cinnamic acid (phenolic acids) compounds in the human diet, such as sunflower seed kernel, coffee and tea (Ruan et al., 2014b). Accumulating evidence shows that CGA exhibits many biological effects, including anti-bacterial, anti-oxidant, anti-inflammation, anti-cancer, anti-diabetes, anti-hypertension and anti-obesity actions (Lou et al., 2011; Ruan et al., 2014b; Yun et al., 2012; Zhao et al., 2012). Several papers report the complexing ability of CGA toward different metal ions in aqueous solution, such as iron (Fe), copper, manganese, zinc, and aluminum (Cornard et al., 2008). Chlorogenic acid decreases intestinal permeability and increases intestinal expression of tight junction proteins in weaned rats challenged with lipopolysaccharide (Ruan et al., 2014a). However, the protective effects of CGA against chronic exposure in Cd-induced intestinal injury have not been fully investigated. We hypothesized that the administration of CGA would influence the intestinal barrier function and through this change might ameliorate Cd-induced toxicity in Sprague–Dawley rats. Therefore, the objective of the present study was to test the above hypothesis by investigating the effect of CGA (from pure reagent or sunflower seed extract) on the growth performance, biochemical parameters, antioxidant abilities, inflammatory response, mRNA and protein-based biomarkers associated with enteric barrier function of Cd-treated rats.

2. Materials and methods

2.1. Experimental design and treatments and animals

A completely randomized design of treatments was used in this experiment. A total of 32 Sprague–Dawley female rats with similar weights were randomly allotted to 4 treatments with 8 replicates per treatment; namely.

(i) Group I serves as the control and rats of this group received daily administration of distilled water via intragastric administration (ig).
(ii) Group II received daily administration of $CdCl_2$ (6 mg/kg BW) ig.
(iii) Group III received daily administration of $CdCl_2$ (6 mg/kg BW) plus pure CGA (50 mg/kg BW) ig.
(iv) Group IV received daily administration of $CdCl_2$ (6 mg/kg BW) plus sunflower seed extract (SSE, 50 mg/kg BW, calculated by CGA content) ig.

The experiment lasted for 14 days. All experimental procedures were approved by the Nanchang University Animal Experiment Ethics Committee [NUAEEC (Gan) 2015–0052], and performed at the Animal Laboratory of Jiangxi Province Center for Disease Control and Prevention (Nanchang, China). Thirty two Sprague-Dawley female rats weighing 215 ± 0.8 g were obtained from Changsha Tianqin Biotechnology Co, Ltd. (Changsha, China) and housed in a controlled room. The rats were acclimatized for 7 days in plastic cages at temperature (22 ± 2 °C), humidity (55 ± 5%) and 12 h light/dark cycle and given laboratory chow (formulated to meet China General Quality Standards for Animal Feed, GB14924.1–2001, Table 1) and tap water ad libitum before the experiment. After the adaption, the remaining feed and body weight of each rat was recorded daily.

Table 1
The composition and nutrient levels of the experimental diet (As-fed basis).

Ingredients, %		Nutrient levels, % unless noted	
Wheat	14	Metabolizable energy (Mcal/kg)	3.40
Corn	43	Crude protein	21.0
Soybean meal	24	Crude fat	4.5
Defatted soybean	8.0	Calcium	1.0
Soybean oil	1.4	Total phosphorus	0.7
Whey powder	3.0	Sodium	0.3
Fish meal	3.2	Methionine + Cystine	0.78
Limestone	1.3	Lysine	1.35
Dicalcium phosphate	1.1	Threonine	0.88
Vitamin-mineral Premix[a]	1.0		

[a] Provided per kilogram of experimental diet: vitamin A 14,000 IU; vitamin D3 1500 IU; vitamin E 5 mg; vitamin K 5 mg; thiamine 13 mg; riboflavin 12 mg; pyridoxine 12 mg; vitamin B12 0.022 mg; niacin 60 mg; pantothenic acid 24 mg; biotin 0.2 mg; folic acid 6 mg; choline 350 mg; Fe ($FeSO_4$•$7H_2O$) 120 mg; Copper ($CuSO_4$•$5H_2O$) 10 mg; Manganese ($MnSO_4$•H_2O) 75 mg; Zinc ($ZnSO_4$•$7H_2O$) 30 mg; Iodine (KI) 0.5 mg; Selenium (Na_2SeO_3) 0.2 mg.

2.2. Chemicals

Cadmium chloride ($CdCl_2$) was purchased from West long chemical Co., LTD. (Shantou, Guangdong,China), CGA(> 98%, HPLC)was purchased from Aladdin Bio-Chem Technology Co., LTD. (Shanghai, China). All the other solvents and chemicals used in the study were of analytical grade.

2.3. Preparation sunflower seed extract

The ethanol extract of CGA from sunflower seed kernels was performed by our laboratory according to the method described by Liu et al. (2005) with slight modifications. Briefly, the dried sunflower seed kernels were ground into fine powder and then degreased in petroleum ether at room temperature for 48 h. The supernatant was discarded and the precipitate was dried at 60 °C. Two gram dried samples were mixed with 64 mL 66% ethanol and were water bathed at 64°Cfor 28 min. The extracts were evaporated using a rotary evaporator (R211L, SENCO, China) under a reduced pressure (100 psi) at a controlled temperature (40 °C) to remove the solvent and obtain the soluble components of the samples. The concentrated extract was stored in a sterile container and preserved in a refrigerator at 4 °C for further use.

2.4. High performance liquid chromatography (HPLC) analysis of extracted chlorogenic acid of sunflower seed kernels

The polyphenol analyses were carried out on an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto-sampler, a binary pump, a column oven and a photodiode array detector. The column was a reverse phase ALLTIMA C18 (Alltech, 5 μm, 250 mm × 4 nm id). The mobile phase consisted of acetonitrile and 2% (15:85, v/v) acetic acid in water. The injection volume of all samples was 6 μL. Phenolic compounds were monitored with SPD-6AV ultraviolet detector at 327 nm, at a flow rate of 1.0 mL/min. The calibration curves were constructed for the CGA concentration ranging 0.05–0.35 mg/mL and the CGA content in the sunflower seed extract was accounted for 33.27%, calculated from the linear regression equations.

2.5. Sample collections and preparations

On day 14 of the experiment, the rats were fasted overnight and anesthetized with pentobarbital sodium. Blood was collected from the orbit and centrifuged for 10 min at 1500 g. The serum samples were then immediately frozen and stored at −20 °C until analyses. The rats were then killed by cervical dislocations. The liver, kidney, jejunum and

Table 2
Primer sequences of target genes.

Gene name	Accession number	Product length (bp)	Primer sequence (5,- 3)
ZO-1	XM_017588936.1	192	Forward: CTGCACGATTTCTGCTTCCTC Reverse: CCTCCATTGCTGTGCTAGTGA
Occludin	NM_031329.2	252	Forward: CTAAATTGGCATCCAGCCCAG Reverse: TCCTTTCCACTCGGGCTCA
Cadherin 1	NM_031334.1	155	Forward: TTGAGAATGAGGTCGGTGCC Reverse: CAGAATGCCCTCGTTGGTCT
Plakophilin1	NM_001107181.1	274	Forward: ACTATGACTGCCCACTCCCT Reverse: CCTGATTCCCGCACCACATC
DMT1	NM_013173.2	130	Forward: CTTGGGTTGGCAGTGTTTGA Reverse: AGGCTGACCCAGTGTTTACT
MT	NM_138826.4	234	Forward: ACCGTTGCTCCAGATTCACC Reverse: GCAGCACTGTTCGTCACTTC
β-actin	NM_031144.3	101	Forward: TGTCCACCTTCCAGCAGATGT Reverse: AGCTCAGTAACAGTCCGCCTAGA

ZO-1, Zonula occludens protein 1; DMT1, divalent metal transport 1; MT, metallothionein.

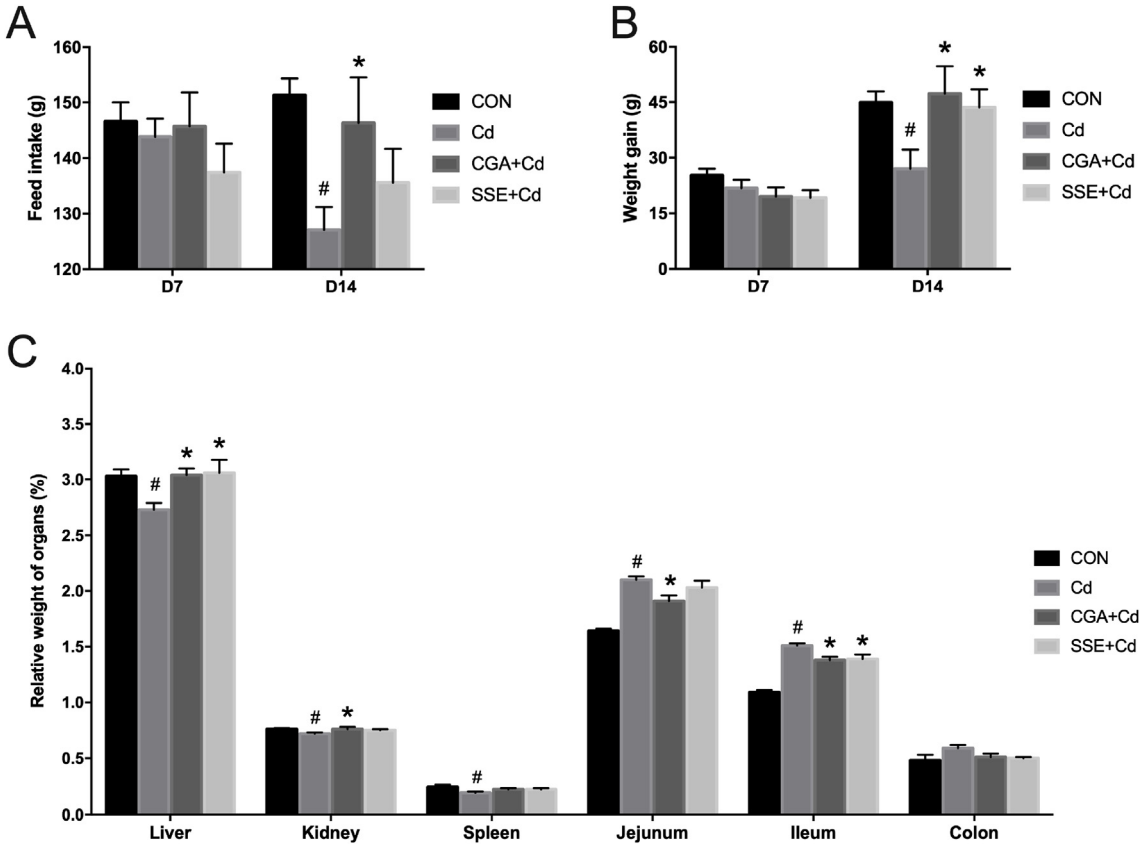

Fig. 1. Effects of chlorogenic acid on growth performance and relative weight of organs of cadmium-treated rats. A, feed intake; B, weight gain; C, relative weight of organs.
CON, control; Cd, cadmium chloride; CGA + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and pure chlorogenic acid (50 mg/kg BW); SSE + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and extracted chlorogenic acid from sunflower seed kernels (50 mg/kg BW).
Significant difference from the CON group at $P < 0.05$.
* Significant difference from the Cd-treated group at $P < 0.05$.
All values represented the mean ± SE of 8 replicate cages (n = 8).

feces were collected and weighed. One part of jejunum from each rat was collected and frozen in liquid nitrogen and then stored at −80 °C until analyses. Another part of each jejunum (approximately 2 cm length) was flushed with a 0.9% sodium chloride solution, fixed with 10% formaldehyde-phosphate buffer, and kept at 4 °C for a microscopic assessment of the mucosal morphology. The relative weight of each organ was calculated with relation to body weight (final body weight before euthanasia).

2.6. Jejunal morphology

The histological analyses of jejunum stained with hematoxylin and eosin (H&E) was performed as described by Nabuurs et al. (1993) and Ruan et al. (2014a). The degree of intestinal pathological damage was graded according to the method described by Chiu et al. (1970) and Chen et al. (2016b): Grade 0, normal mucosa and villi; Grade 1, the epithelial gap is enlarged, blood vessels are congested; Grade 2, the epithelial gap is expanded obviously, the epithelium and lamina propria

Table 3
Effects of chlorogenic acid on serum biochemical parameters of cadmium-treated rats.

Item	CON	Cd	CGA + Cd	SSE + Cd
ALT (U/L)	64.7 ± 3.51	76.2 ± 2.21[#]	66.2 ± 3.48[a]	62.8 ± 2.75[a]
AST (U/L)	215 ± 12.6	266 ± 17.0[#]	215 ± 7.40[a]	213 ± 16.1[a]
TP (g/L)	65.6 ± 0.86	64.1 ± 0.87	64.4 ± 1.04	63.1 ± 1.47
ALB (g/L)	35.8 ± 0.35	36.0 ± 0.32	35.7 ± 0.49	34.5 ± 0.81
TG (mmol/L)	0.83 ± 0.07	0.75 ± 0.07	0.72 ± 0.06	0.73 ± 0.06
CHOL (mmol/L)	2.45 ± 0.12	2.30 ± 0.16	2.25 ± 0.19	2.44 ± 0.12
LDLC (mmol/L)	0.37 ± 0.03	0.38 ± 0.03	0.38 ± 0.02	0.37 ± 0.04
HDL (mmol/L)	1.97 ± 0.10	1.80 ± 0.12	1.84 ± 0.16	1.98 ± 0.10

CON, control; Cd, cadmium chloride; CGA + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and pure chlorogenic acid (50 mg/kg BW); SSE + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and extracted chlorogenic acid from sunflower seed kernels (50 mg/kg BW); ALT, alanine transaminase; AST, aspartate aminotransferase; ALB, albumin; CHOL, cholesterol; TG, triglyceride; TP, total protein; GLU, glucose; HDL, high density lipoprotein; LDLC, low density lipoprotein cholesterol.
[#] Significant difference from the CON group at $P < 0.05$.
All values represented the mean ± SE of 8 replicate cages (n = 8).
[a] Significant difference from the Cd-treated group at $P < 0.05$.

are separated; Grade 3, mucosal epithelium and part of the tip of the villi peeling off; Grade 4, the villi peeling off, the lamina propria and the dilated blood vessels expose; Grade 5, the lamina propria show decomposition, bleeding and ulcers.

2.7. Determinations of biochemical parameter, antioxidants indicators and endotoxin level in the serum

Serum alanine transaminase (ALT) and aspartate aminotransferase (AST) activities, albumin (ALB), cholesterol (CHOL), triglyceride (TG), total protein (TP), glucose (GLU), high density lipoprotein (HDL) and low density lipoprotein cholesterol (LDLC) and contents were measured using a HITACHI 7180 automatic biochemical analyzer (Hitachi Ltd., Tokyo, Japan) with detection kits (Nanjing JianCheng Bioengineering Institute, Nanjing, China), respectively. The superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activity were determined using commercial assay kits (Cat no. were A001-1-1,A005 and A007-1-1, respectively, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The level of malondialdehyde (MDA) and endotoxin were determined using a commercial assay kit (Cat no. were A003-1 and E039-1-1, respectively, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.8. Measurement of inflammatory cytokines

The levels of interleukin-6 (IL-6) (Cat no. 900-K86, Shanghai Wen Yue Biotechnology, Shanghai, China), and tumor necrosis factor alpha (TNF-α) (Cat no.500850, Shanghai Wen Yue Biotechnology, Shanghai, China) in jejunum were determined using commercial ELISA kits, according to the manufacturers' instructions.

2.9. RNA extractions, reverse transcriptions and real-time PCR

The total RNA in the jejunum was isolated using Trizol reagent (Cat no. 15596018, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocols. The concentration of each isolated RNA sample was determined using a NanoDrop Spectrophotometer (ND-2000; Gene Company Ltd) and the integrity of the RNA was checked using denatured RNA electrophoresis. A total of 1 μg of RNA was used to obtain complementary DNA (cDNA) by reverse transcription using PrimeScript™ RT reagent kit with gDNA eraser (Cat no. RR047A, TaKaRa, Kusatsu, Shiga, Japan) according to the manufacturer's instructions. Real-time PCR reactions were performed on a Bio-Rad CFX96 real-time PCR detection system using TB Green™ Premix Ex Taq™ (Tli RNaseH Plus) (Cat no. RR420A, TaKaRa, Kusatsu, Shiga, Japan). The protocol for PCR was as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. All the primers are listed in Table 2. The β-actin was used as reference to normalize the expressions of the target genes. Relative mRNA expression levels of target genes were calculated using the $2^{-\triangle\triangle CT}$ method as described by Zhou et al. (2016).

2.10. Tissue preparations and western blotting

The frozen tissue samples (40 mg) were ground in liquid nitrogen and homogenized in 0.7 mL of ice-cold RIPA lysis buffer (Cat no. P0013B, Beyotime Institute of Biotechnology) supplemented with protease inhibitor (Cat no. 4693159001, Roche). The homogenates were centrifuged at 12000 g for 10 min at 4 °C and then the cytosolic fractions were collected. Total protein concentration was determined using a BCA Protein Assay kit. Protein extract (30 μg) from each sample was then loaded onto 10% SDS–PAGE gels and the separated proteins were transferred onto PVDF membranes (Cat no. IPVH00010, Merck-Millipore). After the transfer, membranes were blocked for 2 h at room temperature in blocking buffer with 5% nonfat milk and then incubated overnight at 4 °C with the following primary antibodies purchased from Bioss: claudin-1 (1:800, Cat no. bs-13754R), desmoglein2 (1:500, Cat no. bs-10152R), E-cadherin (1:500, Cat no. bs-10009R), junctional adhesion molecule A (JAM-A) (1:800, Cat no. bs-3651R), plakophilin1 (1:500, Cat no. bs-7505R), zonula occludens protein 1 (ZO-1) (1:800, Cat no. bs-1329R), and β-actin (1:5000, Cat no. bs-10966R); Invitrogen: occludin (1:800, Cat no. 33–1500); Abcam: divalent metal transport 1 (DMT1) (1:400, Cat no. ab55735), MT (1:400, Cat no. ab12228). After 5 washes for 5 min with tris-buffered saline containing Tween-20, membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5000, Cat no. CW0103A, ComWin Biotech) for 2 h at room temperature. After 5 washes for 5 min each, bands were visualized by chemiluminescence using Pierce™ ECL Western Blotting Substrate (Cat no. 32106, Thermo). The signals were recorded with a ChemiDoc™ Touch Imaging System (Bio-Rad) and analysed with Image-Pro Plus 6.0 software.

2.11. Measurements of cadmium

The concentrations of Cd in jejunum, feces, liver and kidney samples were measured by inductively coupled plasma emission spectroscope (model IRIS Intrepid II, Thermal Jarrell Ash, Waltham, MA) after wet digestions with HNO_3 and $HClO_4$ as described by Huang et al. (2009).

2.12. Statistical analyses

The data are expressed as the mean ± SE. Data analyses were carried out using IBM SPSS 21. Data were analysed using general descriptive statistics, one-way analysis of variance (ANOVA) at 95%. Differences among means were tested by the least significant difference (LSD) method, and statistical significance was set at $P < 0.05$.

3. Results

3.1. Growth performance and relative weight of organs

The feed intake, weight gain and organ index of Cd-treated rats are shown in Fig. 1. Compared to the CON group, the oral administration of Cd had no influence on growth performance on day 7, but caused a reduction in feed intake and weight gain on day 14 (Fig. 1A and B). However, the co-treatment with CGA significantly attenuated the adverse effects of Cd by restoring the changes in feed intake and weight gain on day 14 (Fig. 1A and B). The co-treatment with SSE (50 mg/kg, oral) also increased the weight gain while compared with the Cd-

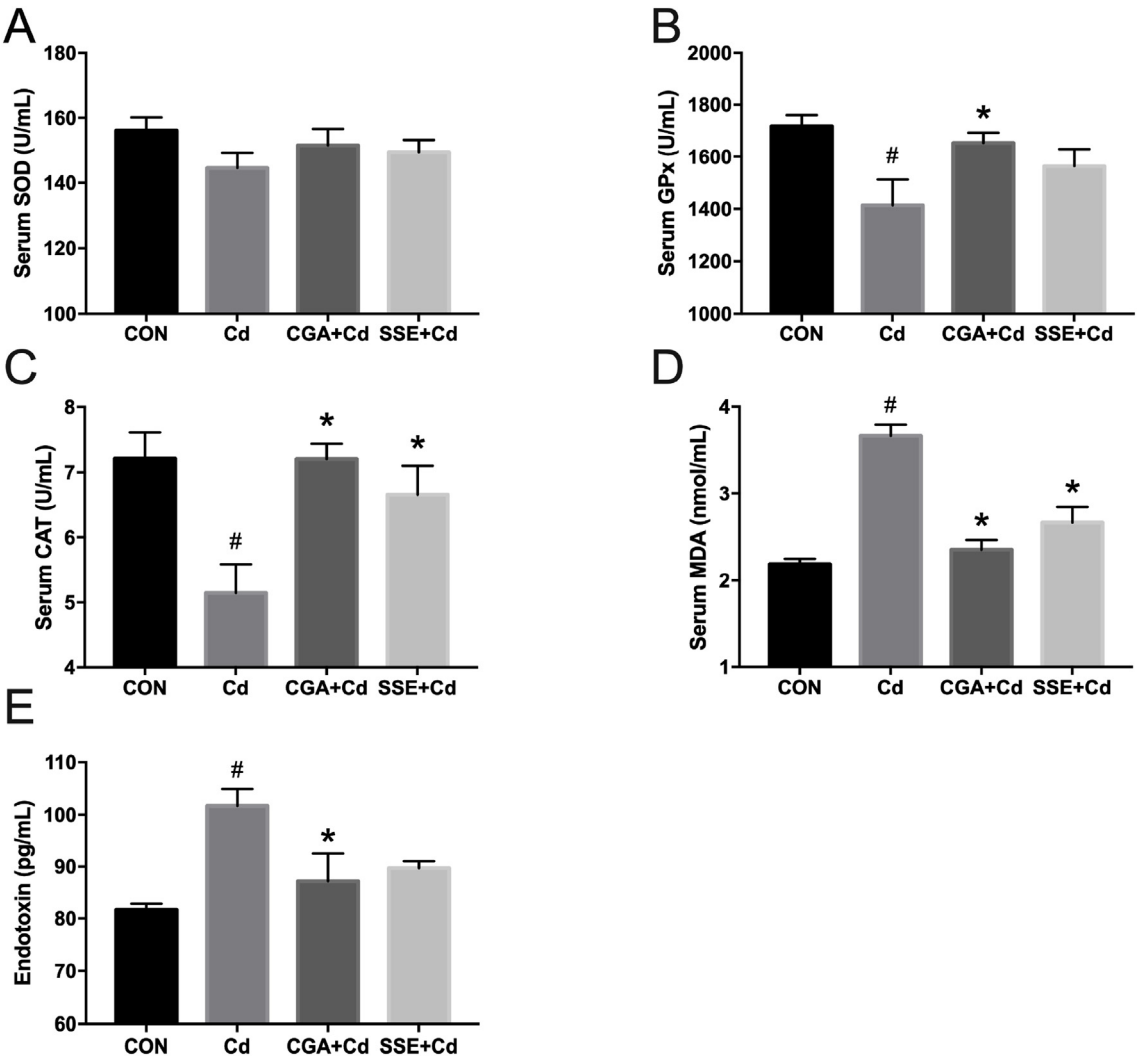

Fig. 2. Effects of chlorogenic acid on antioxidant indices and the content of endotoxin in the serum of cadmium-treated rats. A, superoxide dismutase (SOD) activity; B, catalase (CAT) activity; C, glutathione peroxidase (GPx) activity; D, malondialdehyde (MDA) level; E, endotoxin level.
CON, control; Cd, cadmium chloride; CGA + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and pure chlorogenic acid (50 mg/kg BW); SSE + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and extracted chlorogenic acid from sunflower seed kernels (50 mg/kg BW).
$^{\#}$ Significant difference from the CON group at $P < 0.05$.
* Significant difference from the Cd-treated group at $P < 0.05$.
All values represented the mean ± SE of 8 replicate cages (n = 8).

treated group (Fig. 1B).

The relative weights of liver, kidney and spleen were reduced, but the relative weights of jejunum and ileum were increased, in comparison with those in the CON group. Compared to the Cd group, the co-treatment with CGA increased the relative weight of liver and kidney, decreased the relative weight of jejunum and ileum (Fig. 1C). Compared to the Cd group, the co-treatment with SSE increased the relative weight of liver and decreased the relative weight of ileum (Fig. 1C).

3.2. Changes in serum biochemical parameters

The changes of serum biochemical parameters are presented in Table 3. Serum ALB, CHOL, HDL, LDLC, TG and TP levels were not influenced in the Cd group compared with those in the CON group (Table 3). Serum ALT and AST activities were increased in the Cd group compared with those of the CON group. But the co-treatment with CGA or SSE significantly reduced serum ALT and AST activities.

3.3. Antioxidant indicators and the contents of endotoxin in the serum

The antioxidant indicators and endotoxin level in the serum are shown in Fig. 2. Serum CAT and GPx activities were significantly decreased and the MDA level was increased, but serum SOD activity was not altered in the Cd group in comparison with the corresponding values of the CON group. The co-treatment with CGA or SSE restored the CAT activity and MDA level to approximately the same level as in the CON group. In the rats of co-treated with CGA, the increase in GPx activity was much higher than that observed in the Cd group. As shown in Fig. 2E, Cd exposure significantly increased the endotoxin content, in comparison with that of the CON. However, the administration of CGA markedly reduced endotoxin content in Cd-exposed rats.

3.4. Histological examinations

We next evaluated the effect of CGA on intestinal mucosal morphology in Cd-treated rats. Jejunum sections were observed by H&E staining. As shown in Fig. 3A, the control group showed intact jejunal mucosa, neatly aligned villi and compact arrangement of lamina

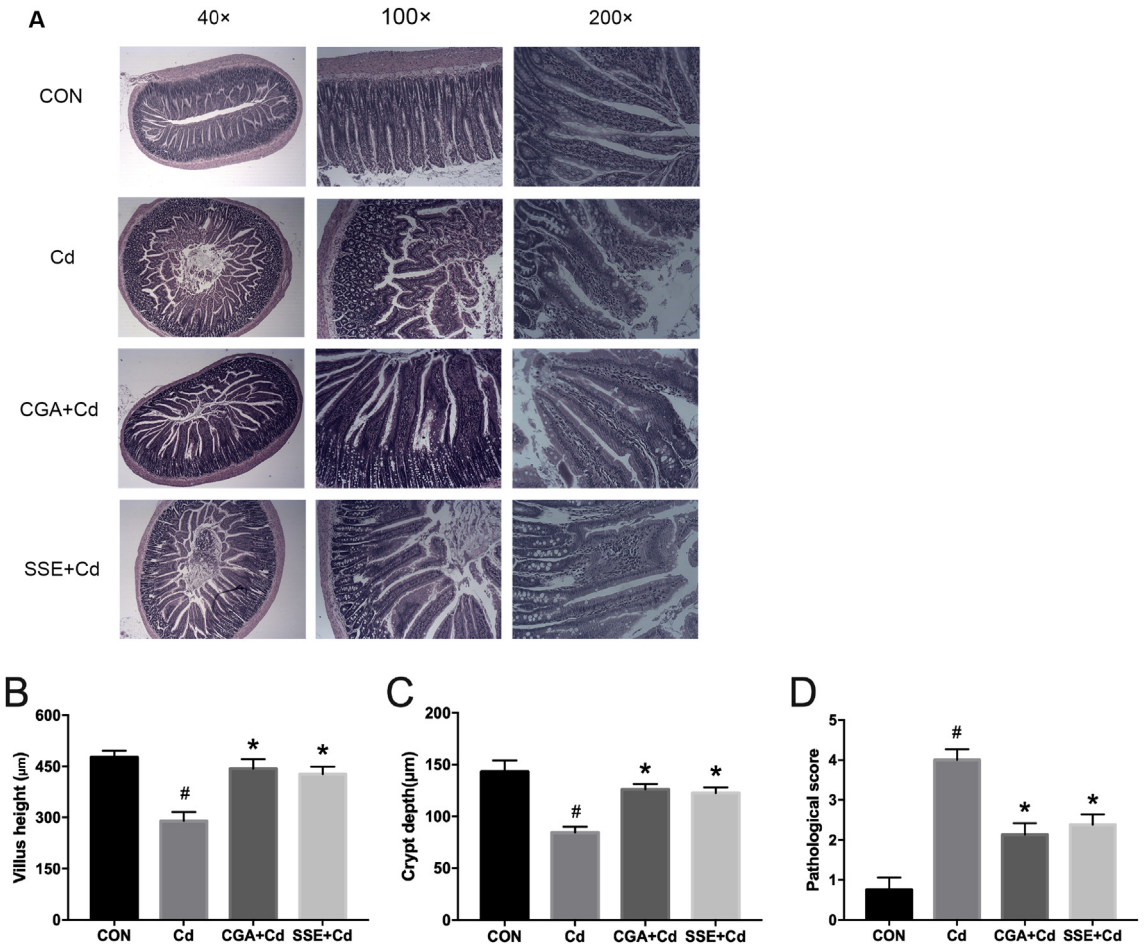

Fig. 3. Effects of chlorogenic acid on histological microphotographs, villus height, crypt depth and Chiu score in the jejunum of cadmium-treated rats. A, representative figures of histological sections of the jejunum (original magnification of 40 ×, 100 × and 200 × , respectively); B, villus height; C, crypt depth; D. Chiu score.

CON, control; Cd, cadmium chloride; CGA + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and pure chlorogenic acid (50 mg/kg BW); SSE + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and extracted chlorogenic acid from sunflower seed kernals (50 mg/kg BW).

$^{\#}$ Significant difference from the CON group at $P < 0.05$.

* Significant difference from the Cd-treated group at $P < 0.05$.

All values represented the mean ± SE of 8 replicate cages (n = 8).

propria. In contrast, the jejunal mucosa tissue was seriously damaged in Cd group, which was characterized by inflammatory cell infiltration, loss of partial villi, exposed lamina propria, degeneration, necrosis, extensive capillary hemorrhage and ulceration. The morphologies of intestinal mucosal were improved in CGA or SSE groups when compared with Cd group. In CGA or SSE groups, the intestinal mucosa was relatively complete, and inflammatory cell infiltration was negligible. The villus height and crypt depth were significantly decreased in the Cd group in comparison with the corresponding values of the CON group (Fig. 3B and C). The co-treatment with CGA or SSE restored the villus height and crypt depth to approximately the same level as in the CON group. Chiu's pathology grading scores was shown in Fig. 3D, indicating that pathological damage caused by Cd was alleviated by CGA or SSE.

3.5. Contents of cadmium in jejunum, feces, liver and kidney

The Cd concentration in the jejunum, feces, liver and kidney were significantly increased in rats treated with Cd compared to that in the CON group (Fig. 4). Co-treatment with CGA or SSE markedly reduced the Cd accumulation in the jejunum of Cd-exposed rats, but a significant elevation in Cd level was observed in the feces. Co-treatment with SSE decreased the Cd content in the liver of rats treated with Cd. No changes in Cd level of kidney occurred in rats that were administered CGA or SSE compared to that in the Cd group.

3.6. Levels of proinflammatory cytokines in jejunum

As shown in Fig. 5, Cd exposure significantly increased the IL-6 and TNF-α contents, in comparison with that of the CON. However, the administration of CGA or SSE markedly reduced these biomarkers in Cd-exposed rats.

3.7. mRNA and protein expressions of divalent metal transporter 1 and metallothionein in the jejunum of cadmium-treated rats

As shown in Fig. 6, real-time PCR and western blotting analyses revealed significant increases in the mRNA and protein expressions of DMT1 and MT in the jejunum in the Cd-treated group compared with those in the CON group. The co-treatment with CGA caused a significant decrease in the mRNA and protein expressions of DMT1 and MT compared to those in the Cd group. And the administration of SSE caused a significant decrease in the mRNA and protein expression of DMT1 compared to those in the Cd group.

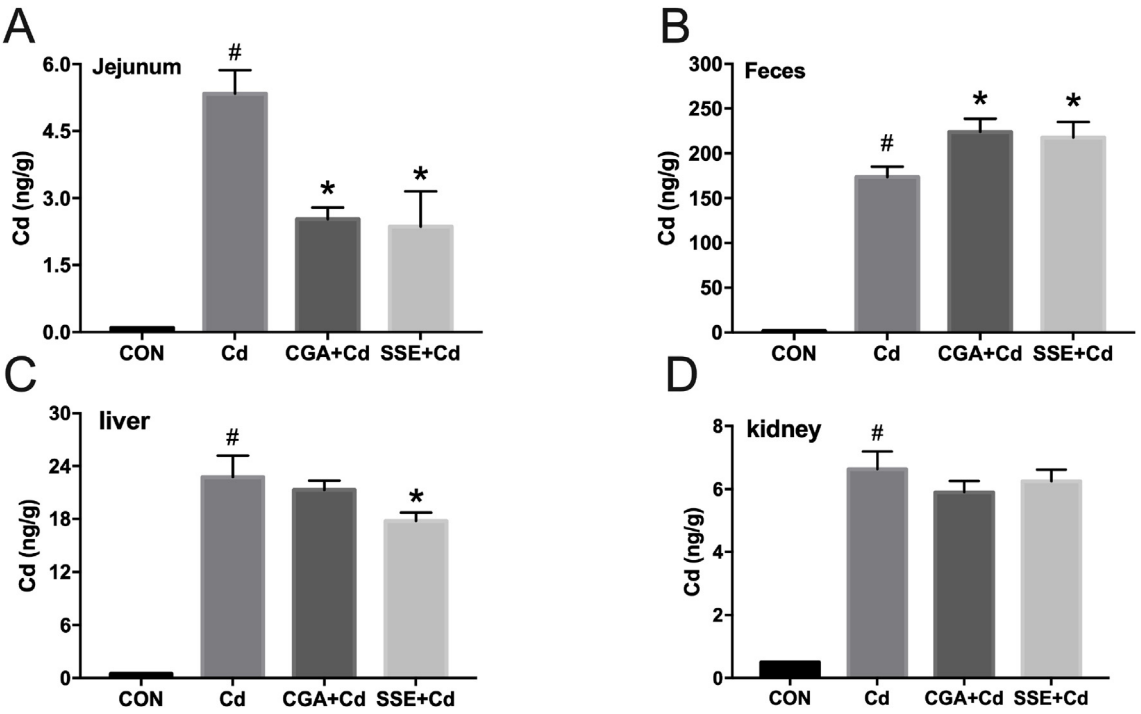

Fig. 4. Effects of chlorogenic acid on cadmium content in the tissues and feces of cadmium-treated rats. A, jejunum; B, feces; C, liver; D, kidney.
CON, control; Cd, cadmium chloride; CGA + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and pure chlorogenic acid (50 mg/kg BW); SSE + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and extracted chlorogenic acid from sunflower seed kernels (50 mg/kg BW).
Significant difference from the CON group at $P < 0.05$.
* Significant difference from the Cd-treated group at $P < 0.05$.
All values represented the mean ± SE of 8 replicate cages (n = 8).

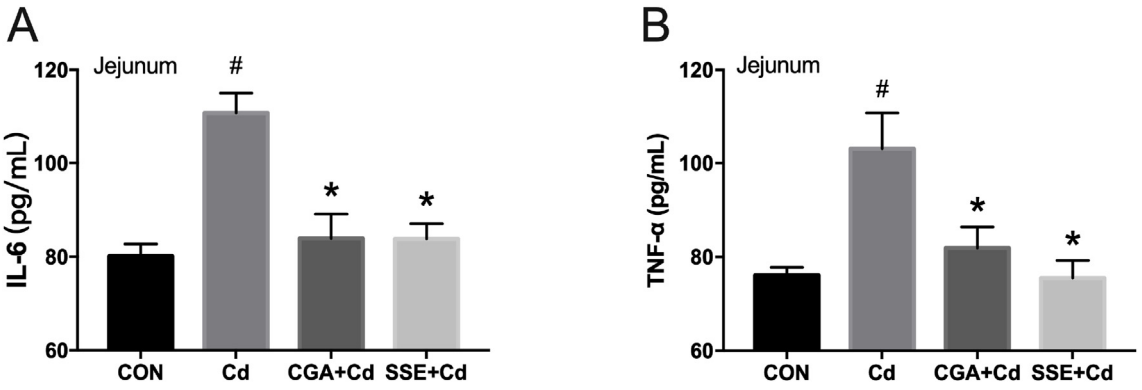

Fig. 5. Effects of chlorogenic acid on proinflammatory cytokines levels in the jejunum of cadmium-treated rats. A, interleukin-6 (IL-6) levels; B, tumor necrosis factor alpha (TNF- α) levels.
CON, control; Cd, cadmium chloride; CGA + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and pure chlorogenic acid (50 mg/kg BW); SSE + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and extracted chlorogenic acid from sunflower seed kernels (50 mg/kg BW).
Significant difference from the CON group at $P < 0.05$.
* Significant difference from the Cd-treated group at $P < 0.05$.
All values represented the mean ± SE of 8 replicate cages (n = 8).

3.8. mRNA and protein expressions of intestinal mechanical barrier genes in the jejunum of cadmium-treated rats

As show in Fig. 7, the mRNA and protein expressions of ZO-1 and occludin in the jejunum were decreased in the Cd group compared with those in the CON group. The co-treatment with CGA caused a significant increase in the mRNA and protein expressions of ZO-1 and occludin compared to those in the Cd group, and the co-treatment with SSE caused a significant increase in the mRNA and protein expression of ZO-1, and the mRNA expression of occludin compared to those in the Cd group. No significant changes in mRNA expression levels of *plakophilin1* and *cadherin-1,* protein expressions levels of plakophilin1, E-cadherin, claudin1, JAM-A and desmoglein2 were observed in the jejunum of rats in the Cd group compared with those in the CON group.

4. Discussion

The hypothesis that administration of CGA would influence the intestinal barrier function and through this change might protect Sprague–Dawley rats against the Cd toxicity was supported by the results of the present study. To the best of our knowledge, this is the first study to measure intestinal biomarkers to evaluate the protective effects

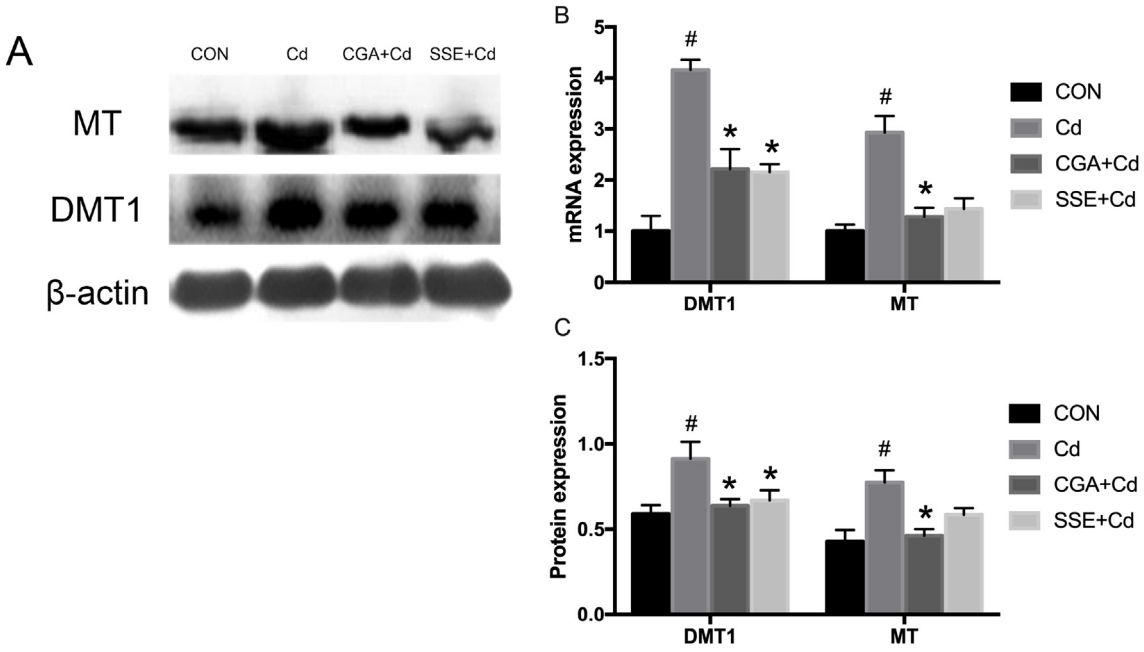

Fig. 6. Effects of chlorogenic acid on mRNA and protein expressions of divalent metal transporter 1 and metallothionein in the jejunum of cadmium-treated rats. A, Representative immunoblots of the indicated proteins were shown; B, the mRNA expressions of DMT1 and MT in the jejunum; C, the protein expressions of DMT1 and MT in the jejunum.

CON, control; Cd, cadmium chloride; CGA + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and pure chlorogenic acid (50 mg/kg BW); SSE + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and extracted chlorogenic acid from sunflower seed kernels (50 mg/kg BW); DMT1, divalent metal transport 1; MT, metallothionein.

Significant difference from the CON group at $P < 0.05$.

* Significant difference from the Cd-treated group at $P < 0.05$.

All values represented the mean ± SE of 8 replicate cages (n = 8).

of CGA against Cd-induced toxicity.

The results showed that exposure to Cd (6 mg/kg body weight) for 14 days negatively affected the body weight gain, while treatment with CGA or SSE offered protection against Cd induced weight loss. We observed that the daily feed intake significantly differed across the groups, suggesting that the reduced body weight gain observed in Cd treated group is connected to decreased feed intake. Our finding for body weight gain is consistent with previous reports (Kim et al., 2018; Nna et al., 2017). The relative weights of the liver, kidney and spleen decreased in the Cd exposed group, but the very slight reductions were insufficient to support a conclusion of a Cd-induced relationship in the context of the current study. The above organ weight changes might be related to the lower feed intake. The reasons for organ mass decrease and associated functional implications are worthy of future studies.

In this study, the administration of Cd might exert hepatic injure as verified by the increase in serum ALT and AST activities, which are known as important markers of hepatocellular damage (Abdel-Moneim et al., 2011). The elevation in liver enzymes has been well documented to Cd-mediated toxicity (Renugadevi and Prabu, 2010). In this context, the protective effect of CGA in liver injury has already been reported by Zhou et al. (2016) and Yun et al. (2012). Chlorogenic acid or SSE may stabilize the hepatic cellular membrane and protect the hepatocytes against toxic effects of Cd, which may decrease the leakage of the enzymes into the blood stream.

It is known that oxidative stress is one mechanism for Cd-induced toxicity. Not only that Cd is able to stimulate the production of ROS and reactive nitrogen species, but also that Cd suppresses the activities of antioxidant enzymes in vivo (Abib et al., 2011; Dkhil et al., 2014). Malonaldehyde is a lipid peroxidation product and usually used as an indicator for organ oxidative injury. In the liver of mice, acute Cd intoxication also caused a marked increase of MDA and a decrease of SOD and CAT (Zhai et al., 2013). Chlorogenic acid may up-regulate cellular antioxidant enzymes and suppress ROS-mediated nuclear factor kappa-B, activator protein-1, and mitogen-activated protein kinase activation, and protect against environmental carcinogen-induced carcinogenesis (Feng et al., 2005). These were inline with our findings. Oral administrated with Cd for 14 days diminished the serum CAT and GPx activities and enhanced the levels of MDA. However, daily treated with CGA or SSE effectually exerted protective effects against the Cd-induced acute oxidative damage.

Since the gastrointestinal mucosa is the first target of Cd exposure and the intestinal inflammatory response provides indispensable protection against aggressive injury from pathogens or chemicals, we investigated whether oral exposure to Cd would trigger the inflammatory response in the intestines. An oral administration of Cd to rat significantly increased the levels of IL-6 and TNF-α in the jejunum. IL-6 and TNF-α have been well characterized as pivotal inflammatory cytokines (Låg et al., 2010; Tracey, 2002). Studies have reported a Cd-induced inflammatory response in the liver, brain and spleen (Akinyemi and Adeniyi, 2018; Liu et al., 2015; Xie et al., 2018). Cadmium exposure significantly increased the levels of proinflammatory cytokines, including TNF- and IL-6 in HT-29 cells (Zhai et al., 2016). Oral administration of low and high concentrations of Cd for 2 and 3 weeks led to a remarkable elevation in the level of TNF- as compared to that of the control group in the colon of mice (Liu et al., 2014). It is interesting to note that co-treatment with CGA or SSE prevents the boost in the level of these proinflammatory cytokines when compared with Cd exposed group. In our study, administration of Cd to rats daily for a period of 14 days was found to elevate the relative weight of the jejunum and ileum tissues leading to organomegaly or hypertrophy. Similar observations were reported in earlier studies suggesting inflammation as the possible cause of organomegaly (Bhattacharjee et al., 2019). Co-treatment of rats with CGA provided protection against intestinal enlargement with consequent reduction. Chlorogenic acid exerted immunomodulatory

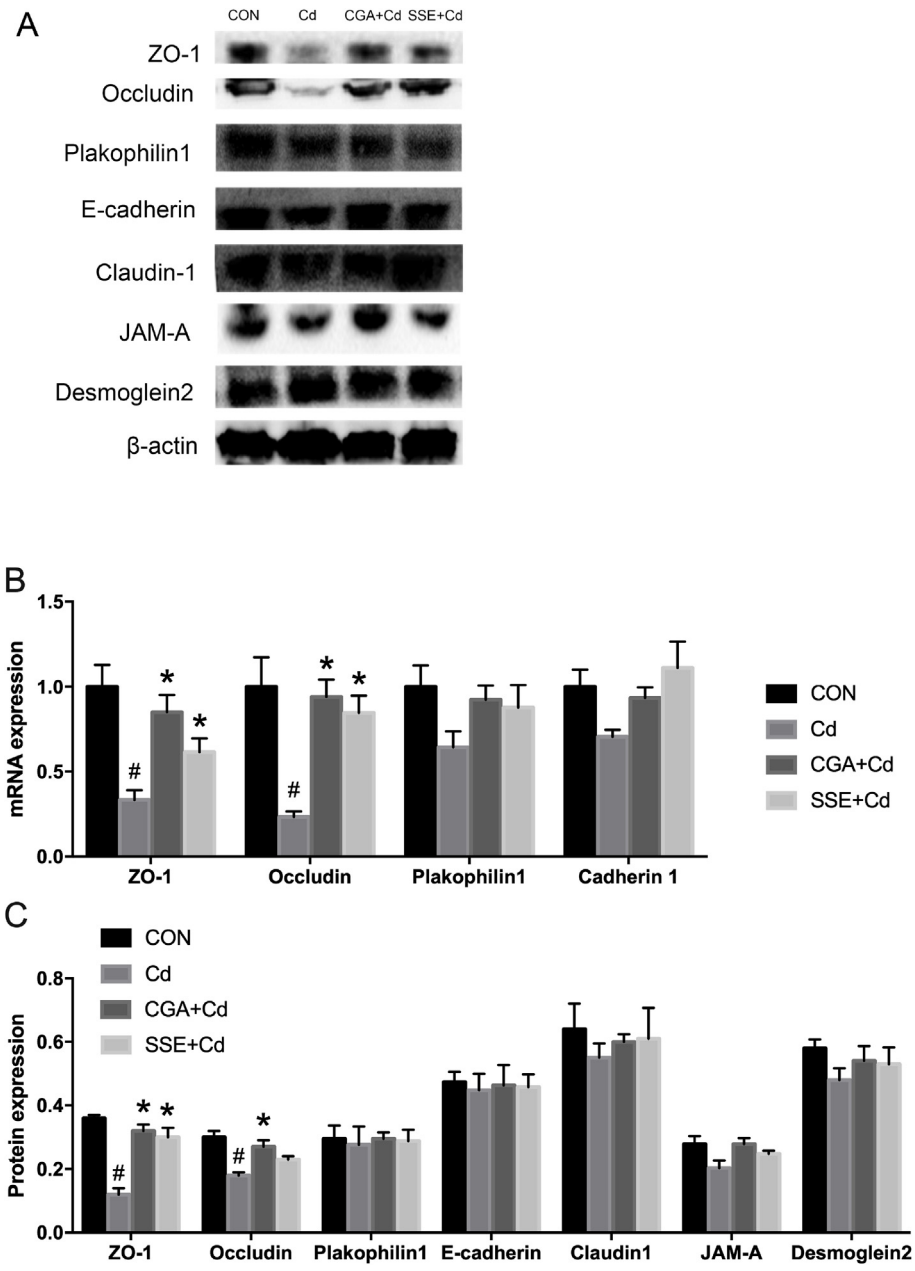

Fig. 7. Effects of chlorogenic acid on mRNA and protein expressions of gut barrier genes in the jejunum of cadmium-treated rats.

A, Representative immunoblots of the indicated proteins were shown; B, the mRNA expressions of gut barrier genes in the jejunum; C, the protein expressions of gut barrier genes in the jejunum.

CON, control; Cd, cadmium chloride; CGA + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and pure chlorogenic acid (50 mg/kg BW); SSE + Cd, intragastric administration of cadmium chloride (6 mg/kg BW) and extracted chlorogenic acid from sunflower seed kernels (50 mg/kg BW); ZO-1, Zonula occludens protein 1; JAM-A, junctional adhesion molecule A.

$^{\#}$ Significant difference from the CON group at $P < 0.05$.

* Significant difference from the Cd-treated group at $P < 0.05$.

All values represented the mean ± SE of 8 replicate cages (n = 8).

activity and offered enteric protection against Cd-induced inflammatory processes. This activity could be attributed to its anti-inflammatory properties, which has been extensively studied (Ruan et al., 2014a; Shi et al., 2013; Xu et al., 2010).

Excessive secreted inflammatory mediators may damage the epithelial cells (Macdonald and Monteleone, 2005). The jejunal histological analysis confirmed that Cd induced wilder villus damage, which were consistent with those of Zhao et al. (2006). In the present study, treatment with CGA or SSE inhibited villus damage when compared with Cd exposed group. Supplementation of CGA increased villus height and the ratio of villus height to crypt depth in the jejunal mucosae under conditions of lipopolysaccharide challenge (Ruan et al., 2014a). These results highlight the pivotal role of CGA in inflammation and protection of intestinal mucosa in the Cd-induced cell injury.

As a consequence of the villus damage, the epithelial cells may die and the epithelial layer may leak, resulting in disruption of the intestinal barrier (Baumgart and Dignass, 2002). The intercellular tight junction proteins are positioned around the apical end of the lateral cell membrane, such as occludin and zonula occludens-1 (ZO-1) (Noth et al., 2011), and play major roles in maintaining the intestinal barrier function. In the Cd-exposed HT-29 cell monolayers, the fluorescence associated with the tight-junction proteins, including ZO-1and claudin-1, were more dispersed, with discontinuous and irregular distribution at sites of cell-to-cell contact (Zhai et al., 2016). In Caco-2 cells, after 4 h Cd exposure, there were a decrease in transepithelial electrical resistance, increased permeability of mannitol and PEG-4000, and changes in intercellular localization of ZO-1, occludin, and e-cadherin (Duizer et al., 1999). In the present study, Cd decreased expression of ZO-1 and occludin in the jejunum of Cd-exposed rats. But these effects induced by Cd were attenuated by CGA or SSE. Previous study confirmed that CGA can decrease intestinal permeability and attenuate intestinal barrier damage by decreases the non-phosphorylation of occludin and claudin-1 to promote assembly of epithelial tight junctions in colitis rats (Ruan et al., 2016).

In the present study, our results demonstrated co-treatment of CGA or SSE can effectively reduce jejunal Cd accumulation and increase Cd excretion in feces, compared to the Cd exposed rats. This protective effect may be more positive than antioxidant and immunomodulatory

activity, as it decreases the intestinal absorption at an early phase of Cd intoxication to prevent the transportation of a high level of this toxic metal into the body. The decreased intestinal absorption of Cd can reduce Cd accumulation in jejunum, in accordance with the reduction of the Cd-bound MT level, which in turn alleviates intestinal inflammation and injure of barrier function. The protective mechanism is partly due to the intestinal sequestration by the Cd-binding ability of the CGA. Polyphenol compounds have metal chelating properties (Kumamoto et al., 2001). Similar observation was reported in earlier study. Chlorogenic acid is a multisite ligand that presents several metal complexing sites, notably the carboxylic and catechol moieties (Cornard et al., 2008). Chlorogenic acid prevents the formation of the hydroxyl radical by forming a chelate with Fe whose complex cannot catalyze the Fenton-type reaction (Kono et al., 1998). Chlorogenic acid has neuroprotective effects against aluminum-induced cytotoxicity by chelation (Wang et al., 2017). Milić et al. (2011) determined that CGA could chelate lead and copper species and form the complexes detected by simultaneous polarographic surveying. Our in vitro study reveals strong scavenging activities of CGA or SSE to chelate Cd^{2+} (Supplementary Fig. 1). Administration of CGA or SSE could reduce its absorption through chelation and could significantly increase the excretion of Cd.

The DMT1 can transport ions such as Cd^{2+} and Fe^{2+} into cells, normally expressed in large amounts in the small intestine of animals and has been shown to significantly promote Cd uptake in xenopus oocytes (Gunshin et al., 1997; Okubo et al., 2003). The DMT1 knockdown cell lines displayed much lower levels of DMT1 mRNA and uptake for Cd decreased 50% (Bannon et al., 2003). Increased energy demands of inflamed enterocytes leads to an hypoxic microenvironment, which favors an increase of ROS, leading to oxidative damage which further promotes inflammation (McGarry et al., 2018). Hypoxia induces DMT1 expression at the apical surface of enterocytes (Hentze et al., 2010). Intestinal villus damage and inflammatory responses in the Cd exposure rats increased the DMT1 expression, and then more toxic Cd was absorbed. Co-treatment of CGA or SSE can effectively alleviate Cd-induced intestinal inflammatory responses, thereby reducing the expression of DMT1 and limiting the Cd absorption.

5. Conclusion

In conclusion, this study has demonstrated that treatment with CGA or SSE can alleviate the inflammatory response and villus damage, reverse tight-junction disruption, decrease the Cd absorption, attenuate oxidative stress and the weight lose. We also confirmed that along with the initial intestinal Cd sequestration, the antioxidative ability and immunoregulation of CGA or SSE are important for protection against Cd induced disruption of the gut barrier.

Author disclosure statement

The authors declare that there are no conflicts of interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110751.

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