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Original article

Protective effects of betulinic acid on intestinal mucosal injury induced by cyclophosphamide in mice

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ABSTRACT

Background: Betulinic acid (BA) is a plant-derived pentacyclic triterpenoid with a variety of biological activities. The purpose of this study was to assess the potential protective role of BA against intestinal mucosal injury induced by cyclophosphamide (CYP) treatment.
Methods: Mice were pretreated with BA daily (0.05, 0.5, and 5.0 mg/kg) for 14 days, then injected intraperitoneally with CYP (50 mg/kg) for 2 days.
Results: BA pretreatment reduced the contents of malondialdehyde (MDA) and glutathione (GSH), decreased the activity of superoxide dismutase (SOD) in small intestine, increased villus hight/crypt depth ratio and restored the morphology of intestinal villi in CYP-induced mice. Moreover, BA pretreatment could significantly down-regulate the levels of pro-inflammatory cytokines interleukin-5 (IL-5), IL-17, IL-12 (P70) and tumor necrosis factor α (TNF-α), reduced production of chemokines macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β) and regulated upon activation, normal T-cell expressed and secreted (RANTES), and enhanced the levels of anti-inflammatory such as IL-2 and IL-10 in serum, and decreased the mRNA expressions of IL-1β and TNF-α in intestine of CYP-induced mice. Furthermore, RT-PCR demonstrated that BA improved intestinal physical and immunological barrier in CYP-stimulated mice by enhancing the mRNA expressions of zonula occluden 1 (ZO-1) and Claudin-1.
Conclusions: BA might be considered as an effective agent in the amelioration of the intestinal mucosal resulting from CYP treatment.

Introduction

Cyclophosphamide (CYP) has been used as a therapeutic agent for treatment of many cancers. It was reported that CYP could indiscriminately injure self-renewing immune cells and intestinal epithelial cells, leading to enteritis by enhancing intestinal permeability and impairing the host immune system, especially the intestinal mucosal immunity [1–3]. The CYP-induced side effects are one of the major obstacles for a successful treatment of cancers and the side effects include vomiting, nausea and diarrhea involved with the intestinal mucosa injury. The oxidative stress and immunological stress have been found to be the major factors of CYP-induced side effects [4–6]. Because of the appearance of antibiotic-resistant bacteria and food safety problems, there is a need to develop viable alternatives to guarantee optimal intestinal health and functions. Therefore, the research and development of a variety of plant-derived antioxidants to minimize its damage and to maximize intestinal health has become increasingly important because of its low side effect and minor drug resistance.

Betulinic acid (BA) is a plant-derived pentacyclic triterpenoids widely distributed in various plants. It possesses many biological activities such as anti-inflammation, anti-tumor, anti-HIV, anti-microbial, anti-parasite. Many of these beneficial effects are due to the antioxidant property of the BA [7–9]. In addition, BA has a very low toxicity and it has been shown to have no overt toxicity at the doses up to 500 mg/kg suggesting a promising prospect for clinical application [10,11]. The antioxidant effects of BA have been widely documented [12–14]. It was reported that BA had a preventive effect on dexamethasone (Dex) induced lymphocyte apoptosis. BA elevated reactive oxygen species (ROS) scavenging capacity, improved the activities of antioxidant enzymes, reduced lipid

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1734-1140/

Table 1
RT-PCR primer list.

Gene	Primer Sequence (5′–3′)		Length	Gene Bank No.
	Forward primer	Reverse primer		
ZO-1	TACCTCTTGAGCCTTGAACTT	CGTGCTGATGTGCCATAATA	248bp	XM_006540785.3
Claudin-1	GGCTTCTCTGGGATGGATCG	TTTGCGAAACGCAGGACATC	134bp	NM_016674.4
IL-1β	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT	138bp	NM_008361.4
IL-6	TGATGGATGCTACCAAACTGGA	TGTGACTCCAGCTTATCTCTTGG	197bp	NM_001314054.1
TNF-α	AGCCGATGGGTTGTACCTTG	AGTACTTGGGCAGATTGACCTC	269bp	NM_001278601.1
β-actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA	171bp	NM_007393

peroxidation, suppressed activation of caspase-9 and caspase-3 and enhanced lymphocyte survival induced by Dex [12,13]. These results suggested that BA could alleviate oxidative stress induced by Dex and might have a positive effect on minimizing the oxidative injury in immunological system. However, it has not been reported whether BA has any effect on intestinal immunity.

In this study, a well-documented model to induce immunological stress for intestine was established in mice by injecting CYP. We hypothesized that BA may alleviate intestinal immunological stress response and ameliorate the intestine tight junction proteins (TJs) in mice exposed to CYP. The purpose of the current study was to explore the possible protective effect of BA on the biochemical indexes, cytokines, redox system and histopathological alterations in intestine of mice induced by CYP and also to investigate the possible mechanisms through which BA exhibits such a protective effect.

Materials and methods

Reagents

CYP (batch number: 12042725) was bought from Jiangsu Hengrui Pharmaceutical Co. Ltd. (Nanjing, Jiangsu, China). Globulin (GLB), aspartate transaminase (AST), alanine transaminase (ALT), triacylglycerides (TG), total cholesterols (TC), total protein (TP), albumin (ALB), calcium ions (Ca^{2+}), urea nitrogen (UREA), creatinine (CREA) and lactic dehydrogenase (LDH) assay kits were from Shenzhen Mindray Bio-medical Electronics Co. Ltd (Shenzhen, China). The 23 cytokines assay kits in serum were purchased from Bio-Rad Laboratories, Inc (CA, USA). BCA protein assay reagent kit and radio immunoprecipitation assay (RIPA) buffer were provided by MultiSciences Biotech Co., Ltd. (Hangzhou, Zhejiang, China). Glutathione (GSH), malondialdehyde (MDA) and superoxide dismutase (SOD) assay kits were obtained from Nanjing Jiancheng Biotech (Nanjing, Jiangsu, China). SYBR Green I fluorescent dyes and Primeseript RTreagent Kit were provided by Takara (Shiga, Japan) and Trizol was obtained from Life Technologies (Carlsbad, CA, USA), respectively. BA was semi-synthesized in accordance with the study with a purity of 96.53% [8].

Animal model

The duration of treatment, and the doses of CYP and BA were selected according to literature data and preliminary experiments [12–14]. Fifty male Kunming mice with body weight (b.w.) 20 ± 2 g, were provided by Hunan Silaikejingda Laboratory Animal Co., Ltd. (Changsha, Hunan, China). The mice were pre-fed for 1 W and then randomly divided into 5 experimental groups (n = 10/group): the control group, the CYP group, the low, medium and high dosage of BA with CYP groups. BA was suspended in 1% starch jelly and administered orally at the doses of 0.05, 0.5, and 5.0 mg/kg b.w. daily for 14 d. The control and the CYP groups received 1% starch

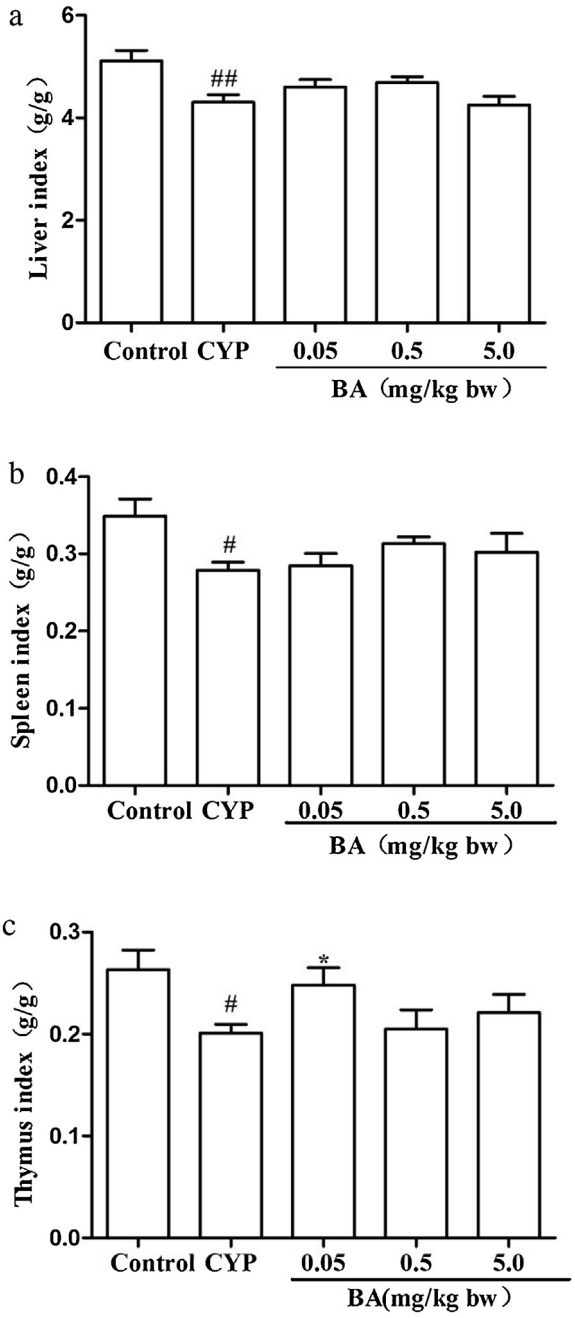

Fig. 1. Effects of BA on organ indexes of liver (a), spleen (b) and thymus (c) in mice treated with CYP. Values are presented as the mean ± SEM. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the control group. $^{*}p < 0.05$ compared to the CYP group.

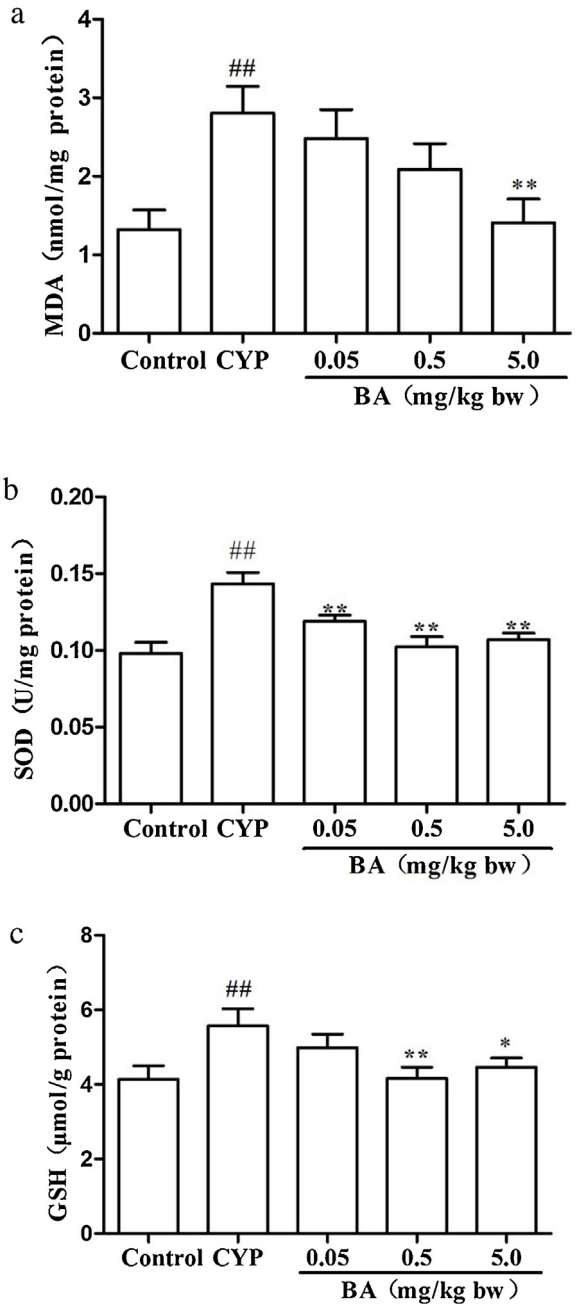

Fig. 2. Effects of BA on the levels of MDA (a), SOD (b) and GSH (c) in intestine of mice treated with CYP. Values are presented as the mean ± SEM. $^{\#\#}p < 0.01$ compared to the control group. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the CYP group.

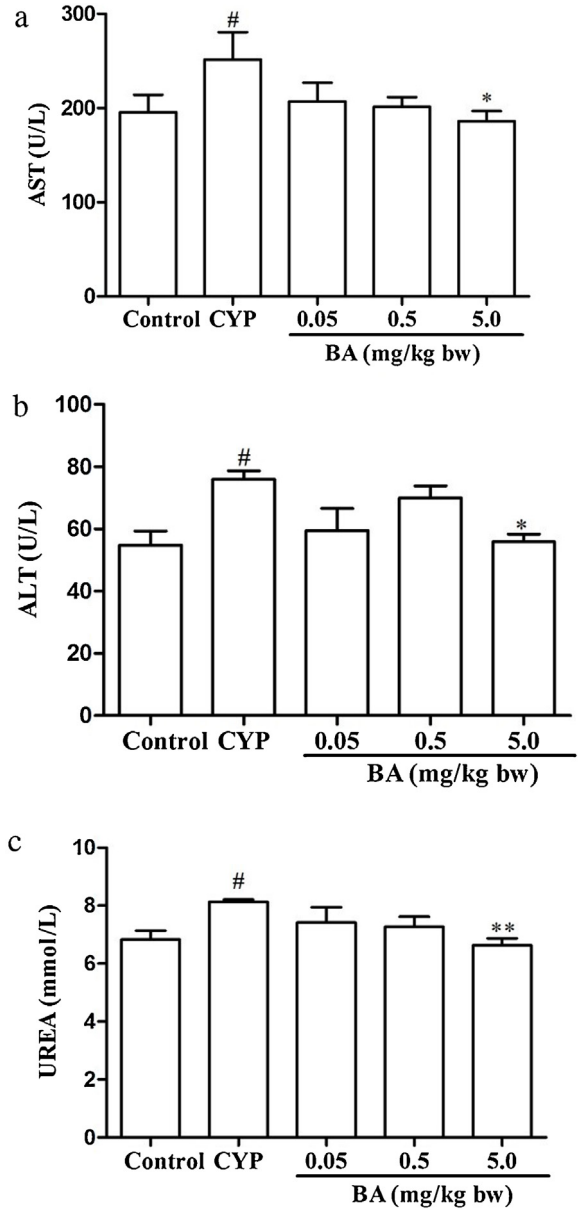

Fig. 3. Effects of BA on the levels of AST (a), ALT (b) and UREA (c) in serum of mice treated with CYP. Values are presented as the mean ± SEM. $^{\#}p < 0.05$ compared to the control group. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the CYP group.

jelly with the same route of administration. Animals were received CYP intraperitoneally at the dose of 50 mg/kg b.w. for 2 days on the 15th and 16th day, while the control group given sterile saline injections of the equal volume.

Mice were anesthetized by diethyl ether (Sinopharm Chemical Reagent, Shanghai, China), and blood were collected in tubes (Eppendorf, Germany) by venous puncture 16 h after the last administration of CYP. The samples were centrifuged for 10 min at 3,000×*g* at 4 °C, and the serum was collected to evaluate blood biochemical parameters and 23 cytokines. Mice were then killed by performing a cervical dislocation, and the thymus, spleen, liver and small intestine were immediately dissected and weighted. The organ indexes were calculated with the following formula: Organ indexes = (organ weight / body weight) * 100 (g/g). 10 cm of jejunum segments were cut and opened longitudinally, and flushed gently with phosphate buffered saline (PBS). 0.5 cm × 0.5 cm jejunum was fixed in 10% neutral buffered formalin for histological analysis, and the rest of jejunum was stored in −80 °C immediately for PCR and antioxidative capacity analysis.

The procedures were complied with the Animal Care and Use Guidelines of China and approved by the Animal Care Committee of Hunan Agricultural University.

Evaluation of antioxidative capacity

10% (w/v) homogenate of jejunum was prepared (Tenbroeck tissue grinders, Wheaton, USA) in PBS (PH 7.4) and centrifuged for 15 min at 2,500×*g* at 4 °C, and the supernatant were collected to evaluate antioxidative capacity. The assay of TP, MDA, GSH and SOD in jejunum was performed using commercial kit for animals

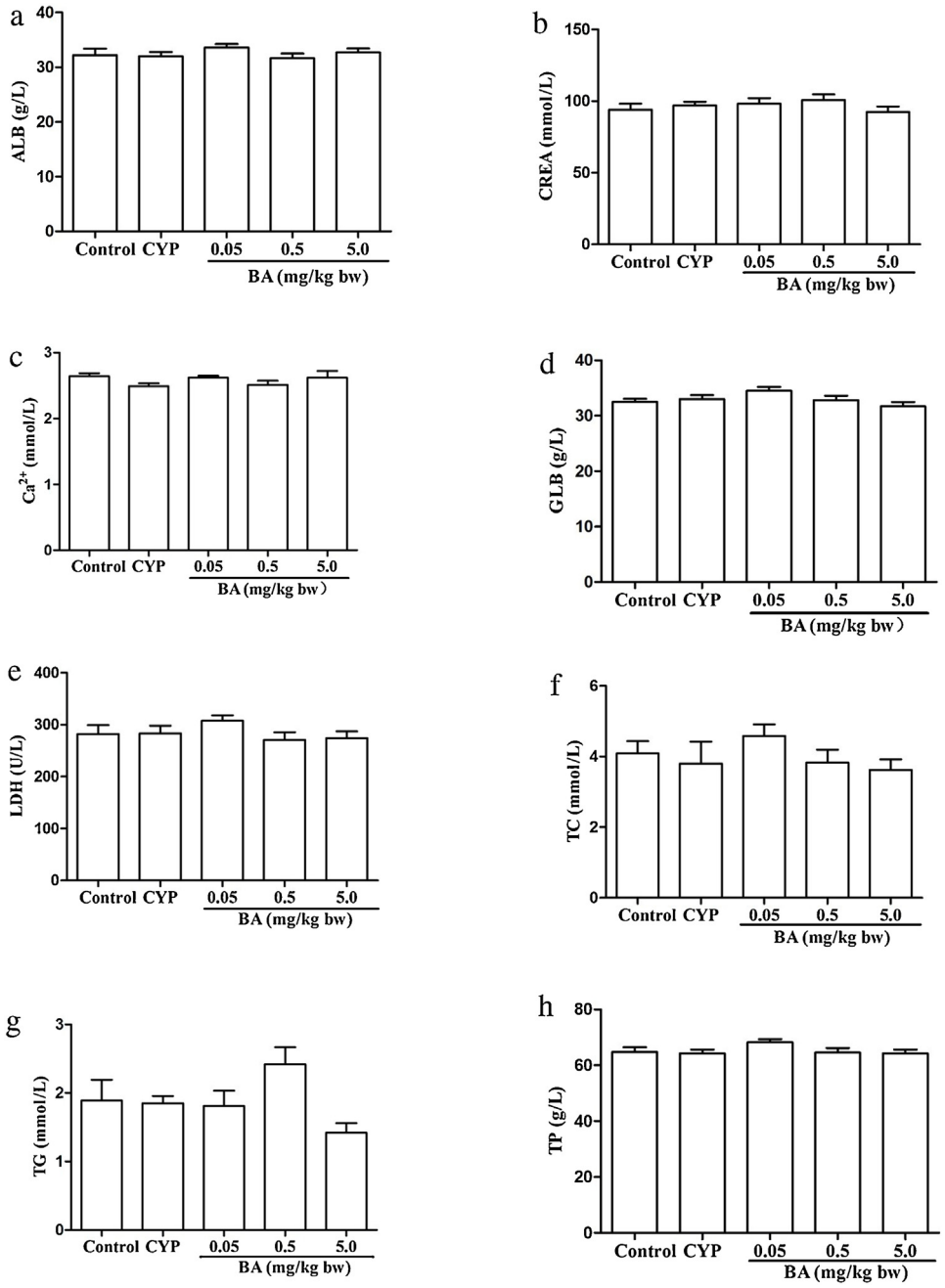

Fig. 4. Effects of BA on serum levels of ALB (a), CREA (b), Ca^{2+} (c), GLB (d), LDH (e), TC (f), TG (g) and TP (h) in mice treated with CYP. Values are presented as the mean ± SEM.

according to the manufacturer's protocols. The content of TP was expressed as milligram per milliliter of protein, and the levels of MDA and GSH were expressed as nanomole per milligram of protein (nmol/mg) and micromole per gram of protein (μmol/g) respectively, while the activity of SOD was expressed as units per milligram of protein (U/mg).

Evaluation of blood biochemical parameters

ALT, ALB, AST, TP, GLB, TC, TG, CREA, UREA, LDH and Ca^{2+} levels in serum were determined by using matched reagents and an automatic biochemistry analyzer (Mindray BS-200, Shenzhen Mindray Bio-Medical Electronics, China).

Evaluation of cytokines in serum

Interleukin 1α (IL-1α), IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(P40), IL-12(P70), IL-13, IL-17, granulocyte colony stimulating factor (G-CSF), eotaxin, interferon γ (IFN-γ), granulocyte-macrophage colony stimulating factor (GM-CSF), monocyte chemotactic protein 1 (MCP-1), keratinocyte chemoattractant (KC), macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), tumor necrosis factor α (TNF-α) and regulated upon activation, normal T-cell expressed and secreted (RANTES) levels were detected using Bio-plex suspension array system analyzer (Bio-Rad, CA, USA) according to the manufacturer's protocols.

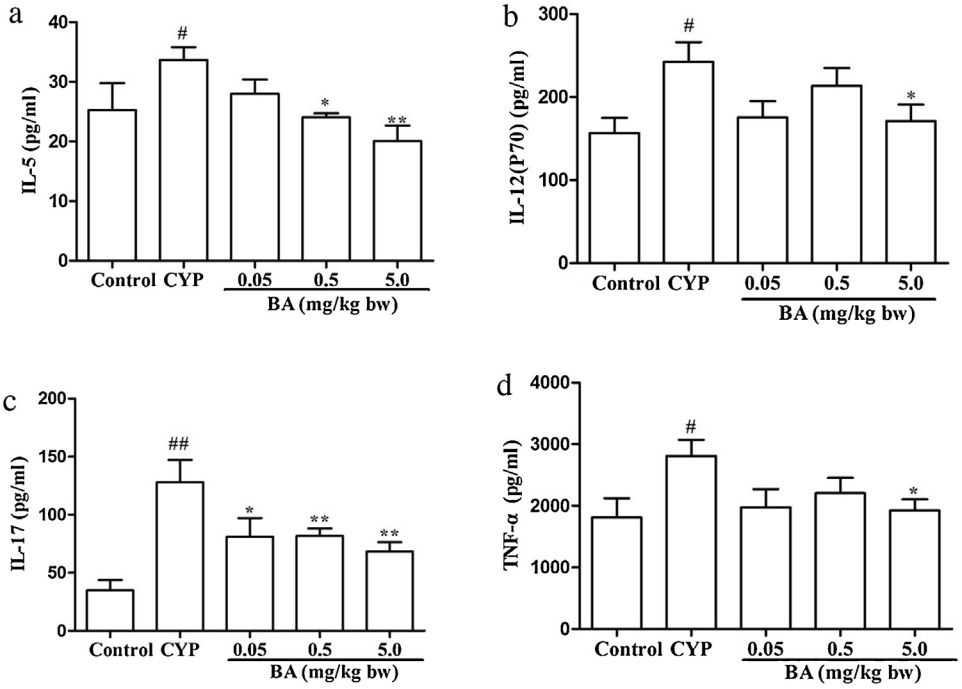

Fig. 5. Effects of BA on serum levels of IL-5 (a), IL-12(p70) (b), IL-17(c) and TNF-α (d) in mice treated with CYP. Values are presented as the mean ± SEM. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the control group. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the CYP group.

Real-time PCR

The mRNA expressions of tight junction proteins and inflammatory cytokines in small intestine were quantified by RT-PCR. The total RNA in small intestine was extracted with Trizol reagent and reversely transcribed with primeseript RTreagent Kit. RT-PCR was performed using SYBR Green I fluorescent dyes kit and a Rotor Gene 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The relative amount of RNA was determined by the $2^{-\triangle\triangle Ct}$ method [13] and β-actin was used as an internal control. Primers sequences were as shown in Table 1:

Histological analysis

Intestines were fixed in 10% neutral buffered formalin, rinsed, dehydrated and embedded in paraffin. Intestines were cut into slices (7-μm-thick) by a rotary microtome (Leica RM2235, Leica Microsystems, Germany), deparaffinized in xylene and ethanol, and then stained with hematoxylin and eosin (Beyotime Institute of Biotechnology, Shanghai, China). Morphological changes of the small intestine, including intestinal villus height (V) and crypt depth (C), were observed using a light microscope (Nikon ECLIPSE 80i, Nikon Corporation, Tokyo, Japan) with a computer-assisted morphometric system.

Statistical analysis

All data were presented as mean ± standard error of mean (SEM). Significance was analyzed by one-way analysis of variance (ANOVA) by using SPSS 17.0 statistic software. Data were considered statistically significant for p value <0.05.

Results

Effect of BA on organ indexes in CYP-treated mice

The organ indexes of thymus, spleen and liver were decreased significantly following induction of CYP (Fig. 1). BA pretreatment had no significant ($p > 0.05$) effects on spleen and liver indexes induced by CYP, but significantly reversed the decreases of thymus index induced by CYP at the dose of 0.05 mg/kg BA.

Effects of BA on antioxidative activity of intestine in CYP-treated mice

To evaluate the effect of BA on the antioxidative capacity in mice, the contents of MDA and GSH, and the activity of SOD in intestine were measured. CYP significantly increased ($p < 0.01$) the levels of MDA, SOD and GSH in intestine as compared with the control group (Fig. 2). In contrast, BA pretreatment significantly decreased the increasing of MDA, SOD and GSH levels in intestine of CYP-induced mice.

Effects of BA on biochemical indexes of blood in CYP-treated mice

The effects of BA on blood biochemical parameters in CYP-challenged mice were shown in Figs. 3 and 4. CYP caused an increase ($p < 0.05$) in ALT, AST and UREA levels in serum as compared to the control group (Fig. 3). Compared to the CYP group, however, a significant decrease ($p < 0.05$, $p < 0.01$) in ALT, AST and UREA levels were recorded in mice treated with BA in combination with CYP, especially BA at the dosage of 5.0 mg/kg. Meanwhile, there were no significant change ($p > 0.05$) on the levels of ALB, CREA, Ca^{2+}, GLB, LDH, TC, TG and TP in serum of mice in all groups (Fig. 4).

Effects of BA on cytokines in serum and intestine of CYP-treated mice

The effects of BA on 23 cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(P40), IL-12(P70), IL-13, IL-17, G-CSF, eotaxin, IFN-γ, GM-CSF, MCP-1, KC, MIP-1α, MIP-1β, TNF-α and RANTES) in serum of CYP-challenged mice were investigated. As shown in Fig. 5, CYP induced a significant increase in the serum levels of IL-5, TNF-α, IL-17 and IL-12(P70). However, pretreatment with BA effectively decreased the levels of IL-5 and IL-17 in a dose-dependent manner. Moreover, BA caused a significant reduction in

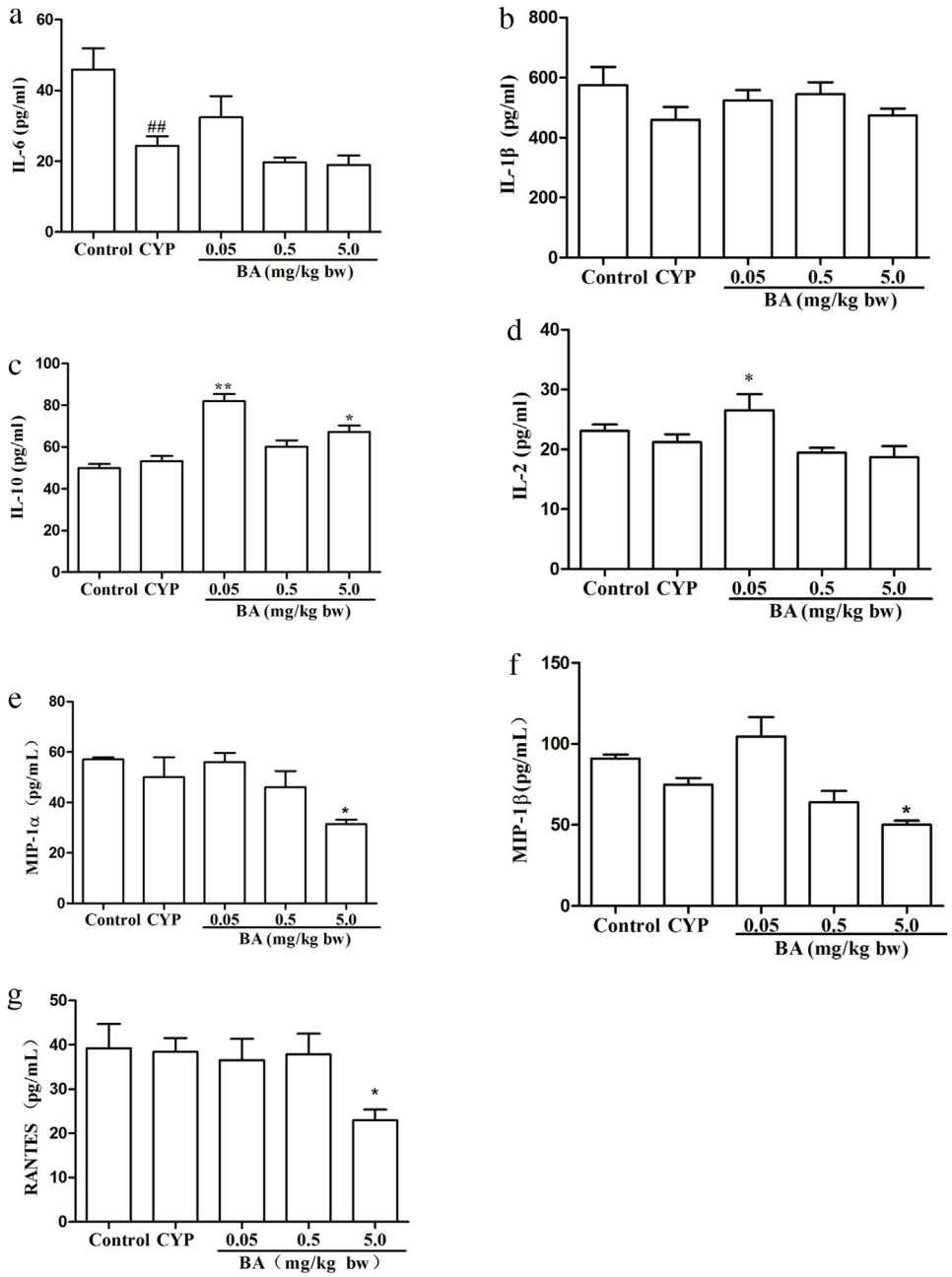

Fig. 6. Effects of BA on serum levels of IL-6 (a), IL-1β (b), IL-10 (c), IL-2 (d) MIP-1α (e), MIP-1β (f) and RANTES (g) in mice treated with CYP. Values are presented as the mean ± SEM. $^{\#\#}p < 0.01$ compared to the control group. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the CYP group.

serum IL-12(P70) and TNF-α levels at the dosage of 5.0 mg/kg. Fig. 6 showed that IL-6 level was significantly decreased ($p < 0.01$), while the changes of IL-10, IL-1β, and IL-2 were not significant ($p > 0.05$) in the CYP group. However, pretreatment with BA reduced production of chemokines such as MIP-1α, MIP-1β and RANTES, and enhanced the levels of anti-inflammatory such as IL-2 and IL-10 in CYP-induced mice, while the IL-6 and IL-1β levels had no significant change ($p > 0.05$).

As shown in Fig. 7, CYP significantly increased ($p < 0.01$) gene expressions of IL-1β and TNF-α compared with the control group, while had no significantly effect on IL-6. However, pretreatment with BA reduced ($p < 0.01$) gene expressions of IL-1β and TNF-α. At the same time, BA at the dosage of 0.05 mg/kg and 5.0 mg/kg significantly increased the IL-6 gene expression as compared with the CYP group.

BA alleviated CYP-induced intestinal morphological changes in mice

To explore the protective role of BA on intestinal impairment, H&E staining was performed. The CYP group showed a shortening and thickening of intestinal villi with a disruption of the epithelial cell layer and an increase in C, and the ratio of V/C in intestine was decreased as compared with the control group ($p < 0.05$, Figs. 8 and 9). On the contrary, pretreatment with BA groups showed that the intestinal C was significantly decreased ($p < 0.05$), and the V and V/C ratio were significantly elevated in a dose-dependent

manner. These results indicated that BA markedly prevented CYP-induced histopathological alterations and tissue injury in intestine of mice.

BA increased ZO-1 and Claudin-1 gene expressions of intestine in CYP-treated mice

Gene expression analysis of the intestine tight junction proteins that are important for the intestinal barrier functions were performed. As shown in Fig. 10, CYP reduced gene expressions of ZO-1 with Claudin-1 showing a downward trend in small intestine compared with the control group. However, BA at the dosage of 5 mg/kg pretreatment markedly increased ZO-1 and Claudin-1 mRNA expressions in small intestine of CYP-challenged mice, thus counteracted against CYP-imposed suppression of the barrier gene expressions.

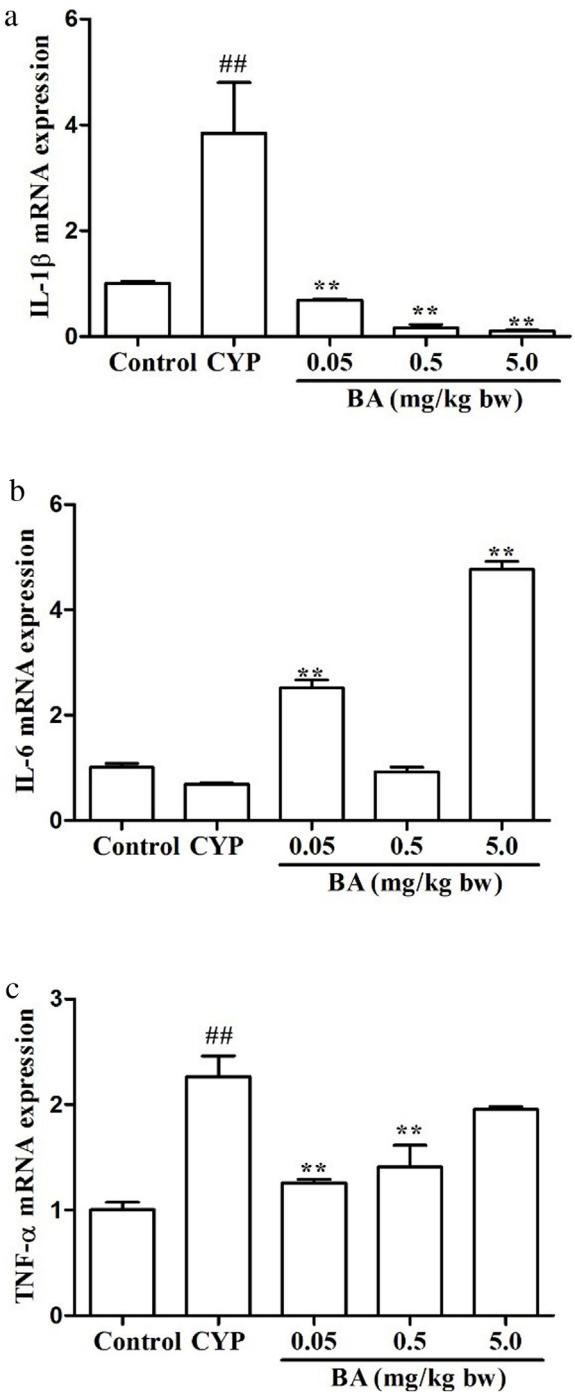

Fig. 7. Effects of BA on mRNA expression of IL-1β (a), IL-6 (b) and TNF-α (c) in intestine of CYP-induced mice. Values are presented as the mean ± SEM. $^{\#\#}p < 0.01$ compared to the control group. $^{**}p < 0.01$ compared to the CYP group.

Discussion

Although CYP, as a chemotherapy drug, is a widely used in the treatment of cancer and autoimmune diseases, it exerts severe cytotoxic effect on normal tissues because of the lack of tumor specificity [15]. CYP induces overproduction of ROS, interferes with antioxidant systems, and results in lipid peroxidation and severe inflammatory reaction during drug metabolism due to the generation of acrolein [16]. Oxidative stress induced by CYP plays a great role in the development from the pathogenesis of injury to various tissues. Intestinal epithelial cells are especially susceptible to CYP, which produced intestinal oxidative stress and immunosuppression, leading to intestinal mucosal barrier dysfunction [17,18]. In the past decades, more attention has been paid to the antioxidants from plants in maintaining an appropriate balance between pro-oxidants and antioxidants, and protecting intestine against CYP-induced toxicity due to their powerful antioxidant and immunomodulatory properties, and their minimal side effects associated with its use [19,20]. BA, as an antioxidant and anti-inflammatory agent, is widely distributed throughout the plant kingdom, but there is little research on intestinal mucosal barrier. Hence, the current study explored the potential effect of BA in preventing against CYP-induced intestinal mucosal injury in mice.

Thymus and spleen, as important immune organs, reflect immune function of the organism. The present study showed that CYP markedly reduced thymus and spleen indexes suggesting that the immune function went down in CYP-treated mice. Pretreatment with BA, the thymus index remarkably increased indicating that BA could get over the inhibitive effect of CYP on immunity. Coincidentally, Yi et al. have observed that pretreatment with BA significantly raised thymus and spleen indexes in the Dex-induced oxidative stress mice [12,13].

An overdose of CYP causes intestine and liver injury, and alters blood biochemical parameters due to increase of oxidative stress [19–21]. Membrane lipids are primary targets for oxidative stress, and the production of free radicals by CYP can attack lipids and give rise to severe alteration of membrane structure and function resulting in lipid peroxidation [19–21]. Therefore, MDA reflects the degree of lipid peroxide of tissue injury and oxidative stress. Significant increase the level of UREA in serum is an indication of abnormal renal function which observed only with marked damage to functioning nephrons due to intrinsic renal lesions [22,23], and meanwhile AST and ALT levels in serum are important markers for the evaluation of liver injury due to their leakage into serum [14,19,21]. Consistent with previous studies [19–21], this study showed that large amounts of MDA in intestine were generated, and the levels of UREA, ALT and AST in serum were increased in CYP-treated mice, suggesting that oxidative stress was induced by CYP which resulted in tissues damage. However, the levels of MDA, UREA, ALT and AST in BA-pretreated mice were significantly reduced, suggesting membrane stabilizing and potent protective effect of BA which could overcome CYP-induced oxidative stress. Similar results were found in previous report in which BA administration exhibited protective effect on the alcohol-induced liver damage and effectively improved the antioxidant ability by reducing the content of MDA in liver, decreasing the activities of ALT and AST in serum [14]. Moreover, the findings of present study are supported by other study which shown that BA decreased MDA content in kidney to ameliorate cecal ligation puncture-induced renal oxidative stress and

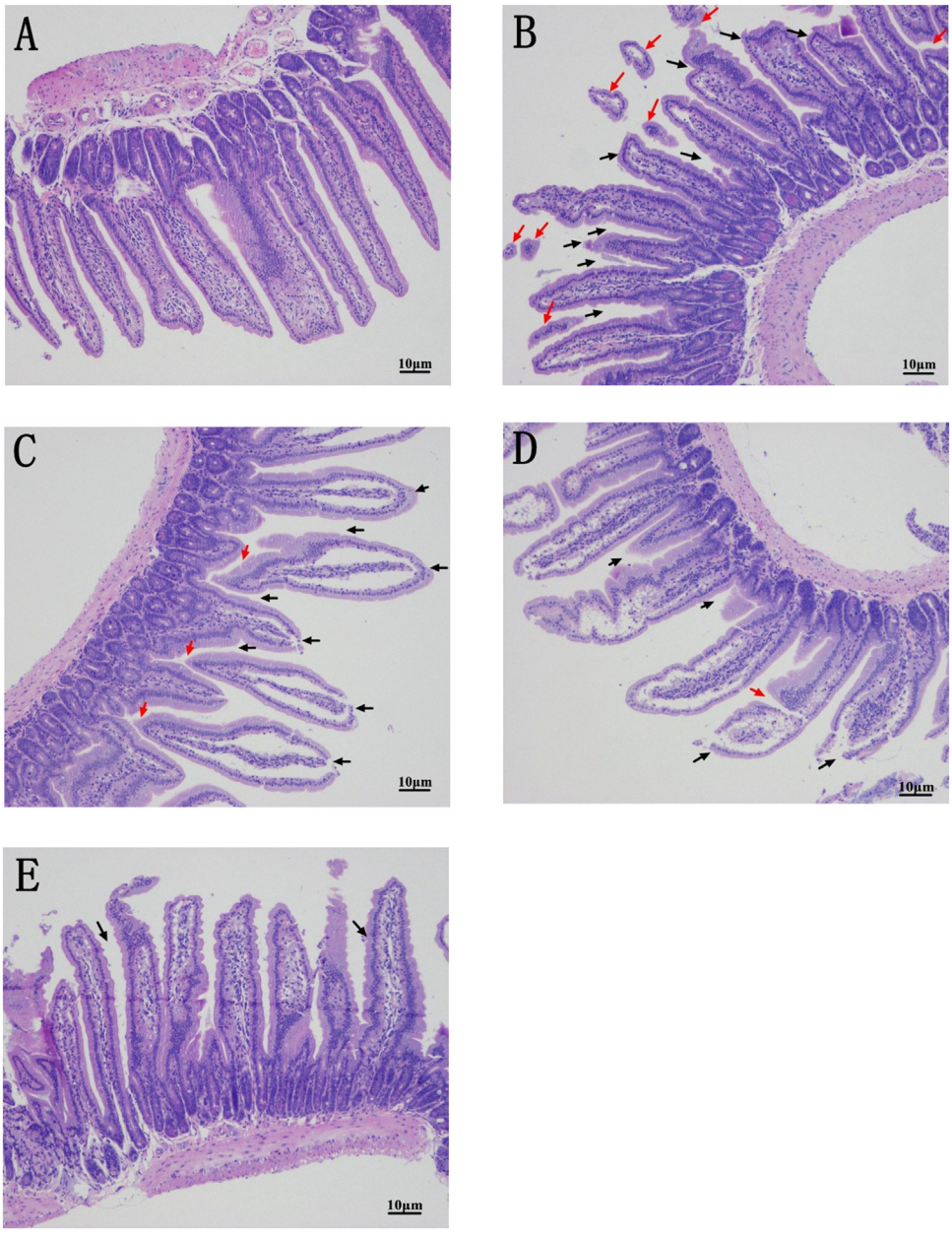

Fig. 8. Effect of BA on intestinal morphological structure in mice treated with CYP by H&E staining (magnification: 100×). No obviously intestinal morphological changes were observed in the control group (A). A shortening and thickening of intestinal villi with a disruption of the epithelial cell layer was observed in intestine of CYP-treated mice (B). Intestinal morphological changes were alleviated in a dose-dependent manner by pretreatment with 0.05 mg/kg (C), 0.5 mg/kg (D), and 5 mg/kg (E) of BA. Black arrows showed the shortening and thickening of intestinal villi, and red arrows showed the breaking, losing, disorganizing of the villus. Scar bar: 10 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

ischemia reperfusion kidney injury model [24]. Decreased content of MDA by BA in CYP-challenged mice was further supported by another report which shown that BA suppressed lipid peroxidation in colon of dextran sulfate sodium (DSS)-induced mice [25].

One of the major mechanisms that accounts for CYP-induced cytotoxic effects is the overproduction of ROS with subsequent depression in the antioxidant defense system. A lot of studies have shown that CYP treatment induced tissue toxicity with a decrease in GSH and SOD levels which interfered with the antioxidant functions [19,21,26,27]. Interestingly, results from the current study revealed that CYP injection increased GSH content and SOD activity in intestine. The increase in the levels of SOD and GSH might be a compensatory response of the body caused by a CYP-induced acute injury which stimulated secretion of excessive SOD and GSH, and maintained the balance of the body oxidation system. The level of GSH was changed during tissue injury, which was increased or declined in a short term according to different diseases [28–31]. It was reported that the content of GSH was reduced in blood of head and neck cancer patients and elevated in tissues when compared to the respective controls [29]. Many clinical trials showed remarkable increase in GSH content in tumors compared to normal controls and peritumoral tissue [28]. In thyroid tissue, enhanced GSH content was observed in adenomas and papillary carcinomas but not follicular carcinoma [30]. Based on this finding, it is acceptable to presume that GSH is quickly degraded by glutamyl transpeptidase to resist the injury caused by oxidative stress during tissue injury, and the body captures the correlated signals which induce GSH to be

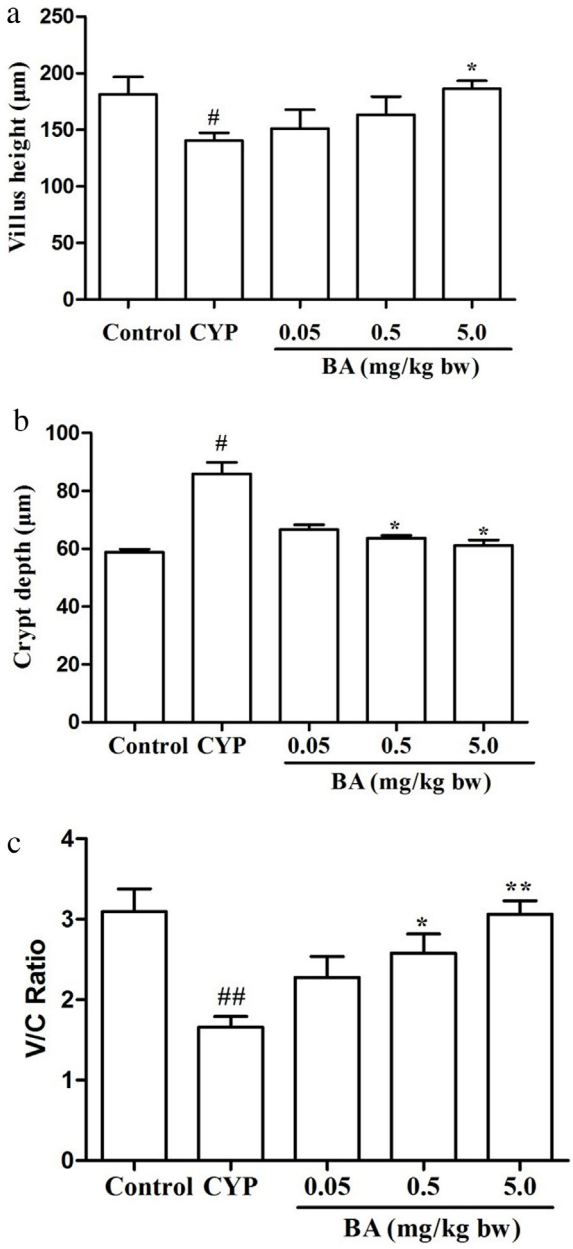

Fig. 9. Effects of BA on intestinal villus height (a), crypt depth (b) and V/C ratio (c) of CYP-challenged mice. Values are presented as the mean ± SEM. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the control group. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the CYP group.

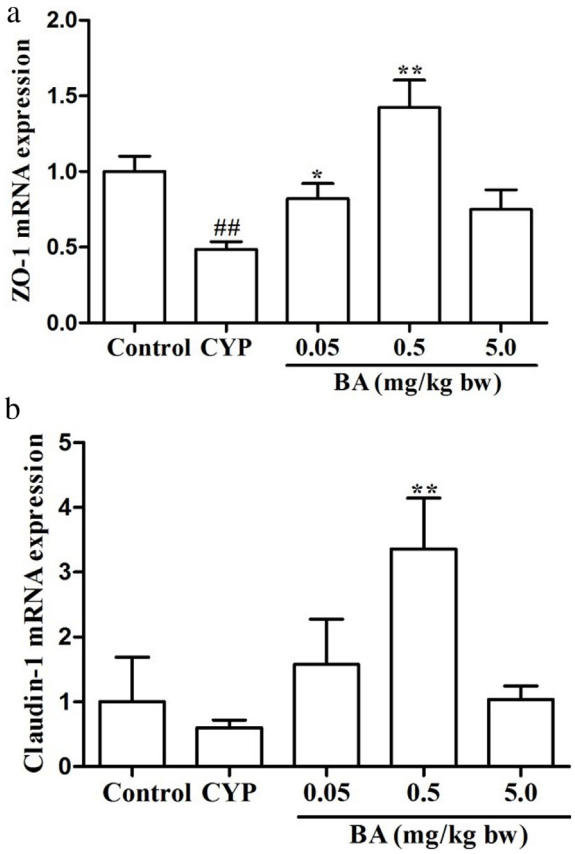

Fig. 10. Effects of BA on mRNA expression of ZO-1 (a) and Claudin-1 (b) in intestine of CYP-induced mice. Values are presented as the mean ± SEM. $^{\#\#}p < 0.01$ compared to the control group. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the CYP group.

synthesized rapidly, and release it to adapt to environmental change. With the development of the disease and the lesion of tissue, the rate of GSH consumption is higher than the synthetic rate. Therefore, the level of GSH in injury tissue is higher than normal one in the early stage of tissue injury caused by oxidative stress, but the validity of this postulate remains to be further studied. BA pretreatment significantly reversed the elevated GSH and SOD levels induced by CYP, suggesting potent protective effect of BA which could alter antioxidant status and maintain the balance of redox system by restoring GSH and SOD levels in intestine.

Along with oxidative stress, inflammatory cytokines were significantly increased in CYP-induced animals which leading to further tissue injury. CYP-administration to rats generated significant increase in serum levels of IL-1β, TNF-α and IFN-γ, demonstrating inflammation [19,21]. The present study was found that CYP resulted in overproduction of pro-inflammatory cytokines IL-5, IL-12(P70), IL-17, and TNF-α levels in serum, and IL-1β and TNF-α mRNA expressions in intestine. The finding was consistent with clinical study that CYP induced immune dysfunction by increasing IL-1β, TNF-α, IL-17 and IL-21 levels [19,21,31]. However, our study showed IL-6 level of serum in CYP-treated mice was markedly decreased, while the mRNA expression of IL-6 in intestine had no significant difference from those of the control, which was inconsistent with previous researches [20,21]. Interestingly, there were some discrepancies existed in literatures regarding the effect of CYP on inflammation cytokines. Although CYP was proved to show pro-inflammatory effects in animals, it was found to inhibit TNF-α and IL-12 secretion [32] or no effect on IL-6, IL-10, TNF-α, IFN-γ and IL-2 production in animals [3,33]. Furthermore, CYP was shown to both promote and descend the levels of IL-6, IL-10, IFN-γ and IL-1β in mice [19,34]. Such paradoxical effect of CYP might be due to its various kinds of cytotoxic effects on both infection and chronic inflammation, and the impact of CYP on cytokine production relies heavily on the cell that is producing the cytokine and the stimulus. It is also possible that several signaling pathways may be involved in this process, which warrant further investigation. IL-6 level in serum was significantly reduced in CYP-treated mice which may be part of counter mechanism to inhibit the pro-inflammatory reaction. The pro-inflammatory state of CYP will eventually incur a compensatory anti-inflammatory response involving control of mediator such as IL-6. Besides, it was reported that the IL-6 level of CYP-treated mice was increased in whole brain and hippocampus, while no change in serum and prefrontal cortex [35], which indicated

that IL-6 level induced by CYP was different in different tissues of the same animal. The present study revealed that, through its anti-inflammatory effect, BA suppressed circulating pro-inflammatory cytokines IL-5, IL-17, IL-12(P70) and TNF-α, reduced production of chemokines MIP-1α, MIP-1β and RANTES, enhanced the levels of anti-inflammatory such as IL-2 and IL-10 in serum, and decreased mRNA expression of IL-1β and TNF-α in intestine of CYP-induced mice. Similar effect of BA has been reported in previous studies [36,37], which confirmed BA induced a decrease in TNF-α and IL-1β levels *in vivo* and *in vitro*, while enhancing IL-10 level in serum of LPS-treated mice. However, in this study, pretreatment with 0.5 mg/kg BA had no effect on IL-6 mRNA expression in intestine induced by CYP which was consistent with the study of Costa et al. [37], whereas pretreatment with BA at the dosage of 0.05 mg/kg and 5.0 mg/kg significantly increased IL-6 mRNA expression, which may be caused by complex enteral environment and individual differences in mice or simply due to its U-shape dose-response [38]. The current findings suggest that BA exerts its protective effects against tissue damage by reducing CYP-induced cytokine dysregulation and improving the balance between inflammatory and anti-inflammation. In accordance, several studies have demonstrated the anti-inflammatory effect of BA [37,39,40].

Intestinal V and C, and the ratio of V/C are important indices for intestinal health and function [41]. C is strongly associated with tissue turnover, and V can be an index of the absorptive surface. Short crypts, long villi and a high V/C ratio indicate a healthy digestive function with high brush border enzyme activity [42,43]. In the current study, BA pretreatment significantly increased the V and the ratio of V/C and reduced the depth of the crypt in mice, suggesting that BA could act as a protective agent against the intestine mucosal injury induced by CYP.

Intestinal physical barrier plays one of the most important roles in the intestinal health of animals which diffuses toxins and pathogens from luminal environment to blood circulation [44,45]. The intestinal physical barrier function relies on the integrity of the intercellular junctions, which are dependent on TJs. TJs are composed by integral membrane proteins including claudins and occludins, and peripheral membrane proteins including zonula occludens (ZO-1, ZO-2, and ZO-3), which play critical functions in regulating intestinal mucosa permeability and physical barrier function [46–48]. In the current study, BA promoted the mRNA expressions of ZO-1 and Claudin-1 in the small intestine of CYP-challenged mice indicating that BA could favorable influence on integrity of the intercellular junctions and barrier function in intestine. The favorable effect of BA on intestine was further verified through improving intestinal mucosa morphology by simultaneously decreasing C, enhancing V and the V/C ratio. The improved intestinal integrity and morphology found in this study was in accordance with other findings that BA alleviated diarrhea and colonic morphological changes *via* ameliorating inflammatory cell infiltration with minimal damage and loss of epithelial cells, villi and crypts in DSS-challenged mice [25]. Apart from this study, it has been reported that BA inhibited pro-inflammatory cytokines expression in colon of DSS-induced murine colitis [49]. Above findings indicated that the preventive role of BA on CYP-induced intestinal mucosal injury achieved by increasing TJs expressions, improving intestinal morphology and inhibiting the infiltration of inflammatory cells.

In summary, BA has a protective effect on CYP-induced intestinal mucosal barrier injury. This protective effect is due, at least in part, to the ability of BA to reduce lipid peroxidation, restore the antioxidant capacity, regulate immune cytokines and increase TJs expressions which contribute to the strengthened intestinal villus structure, improvement of the intestinal barrier functions and enhancement of intestinal immunity, thereby protecting the intestinal mucosal barrier. BA could be applied to food and pharmaceutical industries because of its gut health benefit. However, further studies will be needed to validate the exact mechanism of BA on gut health.

Author contributions

Jine Yi, Bozena Obminska-Mrukowicz and Jing Wu conceived and designed the studies. Jine Yi and Zhuliang Tan wrote the paper and were responsible for the acceptance of final manuscript version. Xihong Wang, Lijuan Zhu, Xianglian Yi and Zhaoping Ou participate in performing the analysis and collecting data. Besides, Zhihang Yuan and Jing Wu were responsible for data interpretation. Meanwhile, Xihong Wang, Zhihang Yuan, Lijuan Zhu, Rongfang Li and Blazej Pozniak were responsible for literature search.

Conflict of interest

The authors declare no conflict of interest.

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