Int J Clin Exp Pathol 2016;9(9):9018-9027
www.ijcep.com /ISSN:1936-2625/IJCEP0027735

Original Article

Salvianolic acid B ameliorates hydrogen peroxide-induced barrier disruption in monolayer Caco-2 cells

Chi Ma[1*], Liangang Shi[1*], Hongshuo Zhang[2*], Yujie Zhao[2], Zhenlong Yu[3], Jingbo Yu[1], Changkai Sun[3,4], Dong Wang[1]

[1]Dalian Central Hospital Affiliated to Dalian Medical University, Dalian, China; [2]Department of Biochemistry, Institute of Glycobiology, Dalian Medical University, Dalia, China; [3]School of Pharmacy, Dalian Medical University, Dalian, China; [4]Research Institute of Integrated Traditional and Western Medicine, Dalian Medical University, Dalian, China. [*]Equal contributors.

Received March 8, 2016; Accepted July 19, 2016; Epub September 1, 2016; Published September 15, 2016

Abstract: Disruption of intestinal epithelial barrier is crucial for the pathogenesis of a variety of intestinal and systemic disorders. Salvianolic acid B (Sal B), a natural bioactive component from Salvia miltiorrhiza, has been reported to exert protection in various types of cells. In the present study, we investigated the protective effects of Sal B on the barrier function of Caco-2 cell monolayers exposed to hydrogen peroxide (H_2O_2). Cell metabolic activity and barrier integrity were assessed by measuring lactate dehydrogenase (LDH) release, cellular morphology change, transepithelial electrical resistance (TEER), and the expressional levels of the tight junction proteins including Claudin-1, Occludin and zonula occludens-1 (ZO-1). Administration of Sal B prevented H_2O_2-induced changes in cell morphology, LDH release, production of glutathione (GSH), superoxide dismutase (SOD), TEER and the localization of Claudin-1, Occludin and ZO-1 proteins. These results suggest that Sal B protects the barrier of intestinal epithelia from oxidative stress-induced damage. In this study, we have demonstrated for the first time that Sal B was protective against H_2O_2-induced barrier disruption in Caco-2 cell monolayer model. We determined the functional roles of Sal B from multiple levels, including cellular morphology, ultrastructure, and metabolisms, which were all collectively support the hypothesis that Sal B ameliorates oxidative stress-induced barrier disruption in intestinal epithelia.

Keywords: Salvianolic acid B, hydrogen peroxide, intestine, epithelial barrier, Caco-2 cells, tight junctions

Introduction

Intestinal epithelial barrier is a highly selective permeability barrier restricting the passage of harmful pro-inflammatory and toxic molecules into the mucosa and systemic circulation. The integrity of intestinal epithelial barrier is maintained by intracellular junctional complexes, including tight junction proteins (TJs), adherent junctions, and desmosomes [1-5]. TJs, a multifunctional complex, consists of at least three types of transmembrane proteins (claudin, occluding, and junctional adhesion molecule), forming the molecular basis of tight junctions in epithelial cells. These proteins interact with cytoplasmic proteins zonula occludens (ZO), F-actin filaments, and myosin II to attach the tight junction strands to the cytoskeleton [6-8]. Disruption of TJs and elevated permeability to luminal toxins, allergens, and pathogens play a critical role in the pathogenesis of a number of gastrointestinal diseases such as inflammatory bowel disease, celiac disease, and alcohol liver disease [9-11]. Pro-inflammatory factors such as reactive oxygen species [12-14], cytokines [15, 16], and toxins [17] disrupt the TJs and the barrier function of the intestinal epithelium. Hydrogen peroxide (H_2O_2) disrupts TJs in the Caco-2 cell monolayers by the mechanisms involving activation of phosphatidylinositol 3-kinase and c-Src [14], and phosphorylation of TJs proteins [18]. Preventing the inflammation-mediated disruption of TJs and barrier function may provide potential therapeutic approaches for the treatment of many gastrointestinal diseases.

Salvianolic acid B (Sal B), a pure bioactive compound extracted from Salvia miltiorrhiza, is known for its broad pharmacological potential, including neuro- and cardio-protective properties by inhibiting lipid peroxidation and superoxide anion production [19, 20]. Sal B suppresses platelet aggregation, inhibits tumor necrosis factor-induced matrix metallo-proteinase-2 up-regulation, and improves coronary microcirculation and cerebral blood flow, as well as inhibiting myocardial ischemia [20-23] via modulation of Mtor-4EBP1, MKK3/6-p38, MAPK-ATF2 and ERK1/2 signaling pathways [24]. However, the functional role of Sal B on H_2O_2-induced intestinal epithelial barrier disruption still remains unclear.

Caco-2 cell, a human intestinal epithelial cell line, is a well-known monolayer model for investigating the intestinal epithelial barrier integrity and function *in vitro* [25-27]. In the present study, we determined the effects of Sal B against H_2O_2-induced Caco-2 cell injury and barrier disruption. To our knowledge, this is the first report showing that Sal B acts directly on intestinal epithelial cells to protect the barrier function against oxidative stress.

Materials and methods

Materials

Sal B was obtained from Dr. Xiaochi Ma. Caco-2 cells were provided by Dr. Zhonggui He. Millicell transwell inserts were purchased from Millipore (Minneapolis, USA). Cell culture reagents were from Gibco (Grand Island, NY, USA).

Caco-2 cell culture

Caco-2 cells were cultured as described previously [18] with minor modifications. Briefly, Caco-2 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acid solution, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. All cell cultures were maintained in a humidified incubator at 37°C with an atmosphere of 5% carbon dioxide in air. Culture medium was changed every other day and cells were plated on 24-well transwell inserts at a density of 5×10^5 cells/well when the cells reached confluence. The cells were treated on day 21 after seeding onto the transwells.

Assessment of cell damage

Caco-2 cells were pre-treated with Sal B (1, 10, 100 μmol/L) or PBS (control) for 12 h, followed by H_2O_2 (800 μmol/L) treatment for 12 h. Epithelial monolayers were then photographed with a Nikon camera under an inverted phase contrast microscope (Leica, Germany).

Determination of epithelial cell viability

The cell viability was determined using MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, 5 mg/ml) reduction assay. Caco-2 cells were seeded in 96-wells plates at a concentration of 1×10^4 cells/well in DMEM culture medium and cultured at 37°C for 24 h. Subsequently, the cells were treated with Sal B (1, 10, 100 μmol/L) for 12 h, followed by H_2O_2 treatment for 24 h (800 μmol/L). PBS buffer was employed as a negative control. After 24 h of incubation at 37°C, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, 0.5 mg/ml) was added to the cells and incubated for another 4 h. The media was discarded and 100 μL of DMSO was added to dissolve the formazan crystals formed in intact cells. The absorbance of formazan was measured with a microplate reader at 570 nm. Experiments were repeated independently three times. The results are expressed as percentage of cell viability.

Measurement of lactate dehydrogenase (LDH) activity

Caco-2 cells were pretreated with Sal B (1, 10, 100 μmol/L) or PBS for 12 h prior to the exposure of H_2O_2 (800 μmol/L, 12 h). LDH in the cell culture media, an indicator of cell injury, was detected by chromatometry with an assay kit according to the manufacturer's instruction (Nanjing Jiancheng Biochemical Reagent Co., Nanjing, China).

Measurement of glutathione (GSH) content and superoxide dismutase (SOD) activity

Caco-2 cells were pre-treated with Sal B (1, 10, 100 μmol/L) or PBS for 12 h, followed by H_2O_2 exposure (800 μmol/L) for 12 h. The GSH levels and SOD activity in Caco-2 cells were deter-

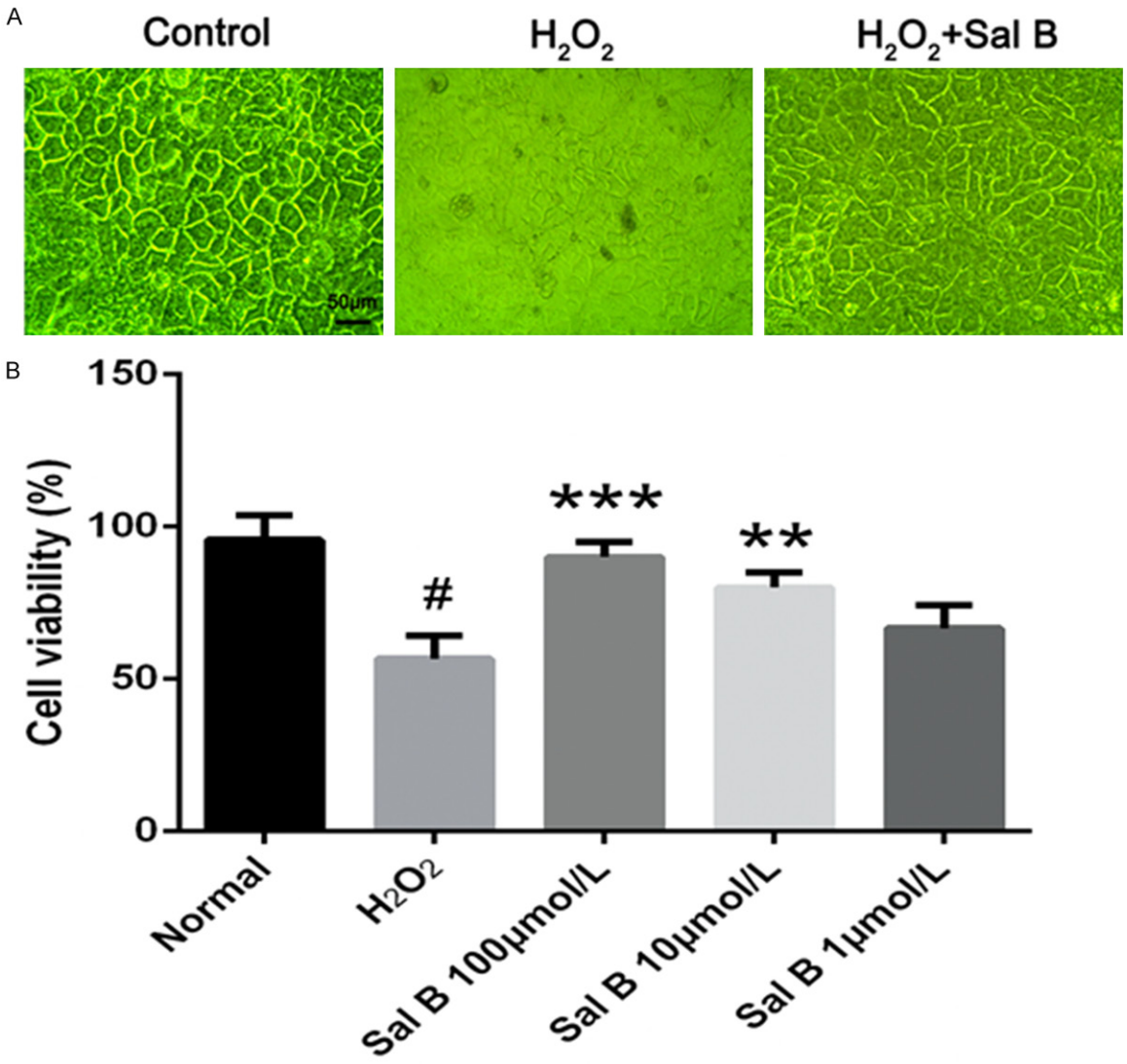

Figure 1. A. Effects of Sal B on H_2O_2-induced cell injury. Caco-2 cells were treated with H_2O_2 (800 μmol/L) for 12 h with or without pretreatment of Sal B (100 μmol/mL). Epithelial monolayers were then photographed with a Nikon camera under an inverted phase contrast microscope (200×). Data are representative and are from three independent experiments. B. Effects of Sal B on H_2O_2-induced loss of cell viability. Caco-2 cells were exposed to 800 μmol/L H_2O_2 for 12 h and cell viability was measured by MTT assay. Data are expressed as mean ± S.D. monolayers n=3. #P<0.05 vs. control, **P<0.01 vs. H_2O_2, ***P<0.001 vs. H_2O_2.

mined using commercial kits (Nanjing Jiancheng Biochemical Reagent Co. Nanjing, China) according to the manufacturer's instructions.

Measurement of transepithelial electrical resistance (TEER)

Monolayers of Caco-2 cells were grown on the membrane of transwells (0.4 μm pore size; 0.6 cm^2 effective membrane area). At 100% confluence, TEER was measured using Millicell-ERS (Millipore Corporation, Bedford, MA, USA) when the resistance was stable (>240 Ωcm^2). The resistance was calculated as follows: TEER (Ω·cm^2) = (Total resistance - Blank resistance) (Ω) × Area (cm^2) (13).

Transmission electron microscopy

Monolayers of Caco-2 cells grown on transwell membranes were fixed with 2.5% glutaraldehyde for 2 h at 4°C. After washing in 0.2 M Na cacodylate/HCL (pH 7.4) for three times, cells were fixed with 1% OSO_4-0.15 M Na cacodyl-

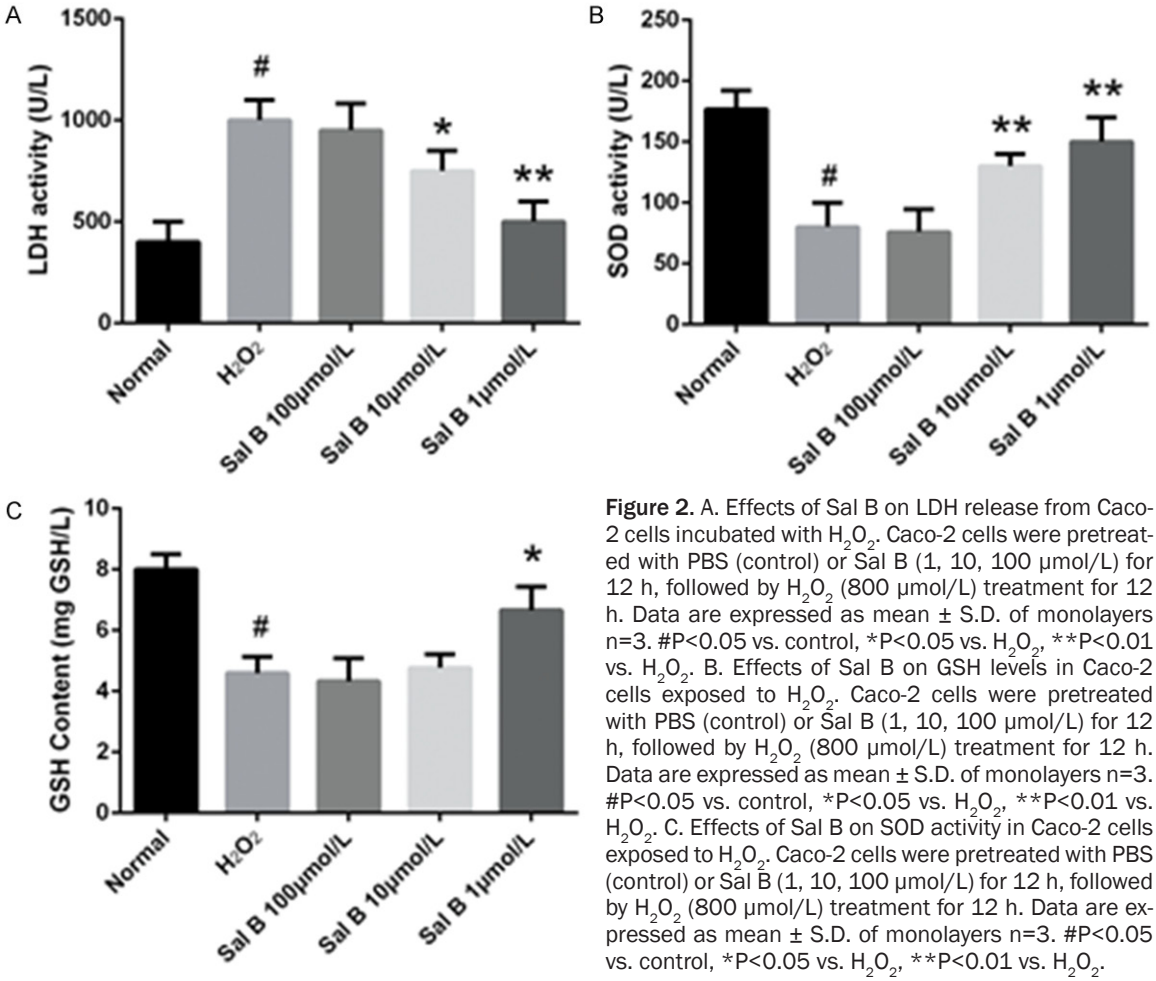

Figure 2. A. Effects of Sal B on LDH release from Caco-2 cells incubated with H_2O_2. Caco-2 cells were pretreated with PBS (control) or Sal B (1, 10, 100 μmol/L) for 12 h, followed by H_2O_2 (800 μmol/L) treatment for 12 h. Data are expressed as mean ± S.D. of monolayers n=3. #P<0.05 vs. control, *P<0.05 vs. H_2O_2, **P<0.01 vs. H_2O_2. B. Effects of Sal B on GSH levels in Caco-2 cells exposed to H_2O_2. Caco-2 cells were pretreated with PBS (control) or Sal B (1, 10, 100 μmol/L) for 12 h, followed by H_2O_2 (800 μmol/L) treatment for 12 h. Data are expressed as mean ± S.D. of monolayers n=3. #P<0.05 vs. control, *P<0.05 vs. H_2O_2, **P<0.01 vs. H_2O_2. C. Effects of Sal B on SOD activity in Caco-2 cells exposed to H_2O_2. Caco-2 cells were pretreated with PBS (control) or Sal B (1, 10, 100 μmol/L) for 12 h, followed by H_2O_2 (800 μmol/L) treatment for 12 h. Data are expressed as mean ± S.D. of monolayers n=3. #P<0.05 vs. control, *P<0.05 vs. H_2O_2, **P<0.01 vs. H_2O_2.

ate/HCL (pH 7.4) for 30 min. The cells were then dehydrated in graded ethanol and embedding in Araldite epoxy resin. Thin sections (1 μm) were cut and stained with 1% toluidine blue in 1% sodium borate. Areas selected for ultrastructural observations were cut into ultra-thin sections (80 nm in thickness) and stained with saturated uranyl acetate and Reynold's lead citrate. Images were taken with an H7000 (Hitachi, Japan) transmission electron microscope. At least three images from each sample were analyzed by three people in a blinded fashion [22].

Immunocytochemical study of TJs protein expression in Caco-2 cells

Caco-2 cell monolayers on transwell membranes were permeated with 0.5% NP-40, and blocked with 5% normal goat serum (Gibco BRL Co. Ltd., USA). Cells were fixed with 3.5% paraformaldehyde in PBS and incubated with a specific primary antibody (Santa Cruz Biotechnology, USA) for ZO-1, Occludin, or claudin-1 for 30 min at room temperature, washed, and then incubated with HRP-conjugated goat anti-rabbit IgG antibody. Primary antibodies (rabbit monoclonal anti-human ZO-1, Occludin, and Claudin-1, Santa Cruz Biotechnology, USA) were diluted 1:20 to 1:100 in 2% bovine serum albumin-PBS. Monolayers were washed four times with saline and then color developed using diaminobenzidine solution and stained with hematoxylin. The transwell membranes were mounted onto the slides using DPX (BDH Laboratories; Poole, UK).

Immunoblot study of TJs protein expression in Caco-2 cells

Caco-2 cells, and Caco-2 cells which were pretreated with Sal B (100 μmol/L) or PBS for 12 h, followed by H_2O_2 exposure (800 μmol/L) for 12 h, were prepared for western blot analysis. St-

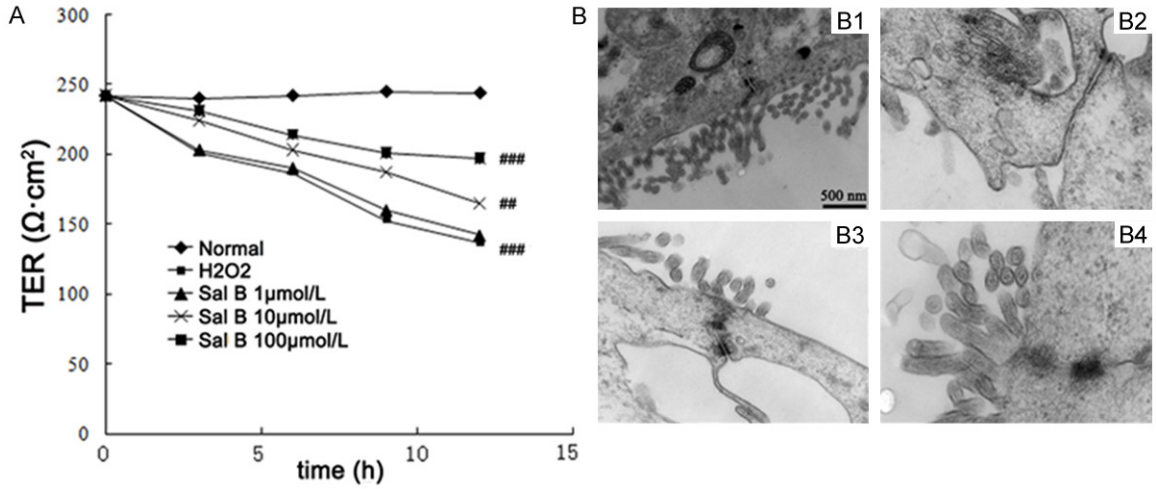

Figure 3. A. Effects of Sal B on H_2O_2-induced alteration of TEER in Caco-2 cells. Caco-2 monolayers were treated with H_2O_2 (800 μmol/L) for 3 h, 6 h, 9 h, and 12 h with or without pretreatment with Sal B (1, 10, 100 μmol/L). Data are expressed as mean ± S.D. of monolayers n=3. ##P<0.01, ###P<0.001 compared with H_2O_2 treatment. B. Effects of Sal B on the changes of ultrastructure of TJs induced by H_2O_2. Caco-2 cell monolayers were treated with H_2O_2 (800 μmol/L) for 12 h, with or without pretreatment of Sal B (100 μmol/L). Images were taken with an H7000 transmission electron microscope (15000×). Data are representative of 3 independent experiments. (B1: control; B2: H_2O_2 treatment; B3 & B4: Sal B & H_2O_2 treatment).

andard Western blotting was performed using a rabbit antibody against human ZO-1 (1:1000), Occludin (1:1000), and Claudin-1 (1:1000), and then incubated with HRP-conjugated goat anti-rabbit IgG antibody. Equal protein sample loading was monitored by probing the same membrane filter with an anti-GAPDH antibody (Santa Cruz Biotechnology, USA).

Statistical analysis

Statistical analysis was performed with SPSS 13.0 software. Results are shown as means ± SD. The data were statistically evaluated using one-way ANOVA followed by Student's t test when only two value sets were compared, and Dunnett's test when the data involved three or more groups. P<0.05 was considered to be statistically significant.

Results

Sal B inhibits cell morphology and cell viability change induced by H_2O_2

As shown in **Figure 1A**, most Caco-2 cells are round in shape with decreased attaching ability to the culture surface after exposed to H_2O_2 for 12 h. Pretreatment with Sal B significantly prevented the morphology change of Caco-2 cells induced by H_2O_2. After exposed to H_2O_2, Caco-2 cells viability dramatically decreased as compared to control group (P<0.05) (**Figure 1B**). Sal B significantly increased cell viability in a dose-dependent manner (P<0.05). At the concentration of 100 μmol/mL, Sal B rescued the cell viability close to the levels of control.

Sal B inhibits H_2O_2-induced LDH release, changes of GSH levels, and regulates SOD activity

To further investigate the protective effects of Sal B on H_2O_2-induced cell damage, the levels of LDH in cultured media of Caco-2 cells were determined. As shown in **Figure 2A**, there was significantly increase in LDH levels after the exposure to H_2O_2 for 12 h. Pre-incubation of Caco-2 cells with Sal B attenuated H_2O_2-induced LDH release in a dose-dependent manner. H_2O_2-induced cell damage was usually accompanied with the increase of lipid peroxides. As shown in **Figure 5**, the levels of GSH in Caco-2 cells were significantly decreased after the stimulation with 800 μmol/L H_2O_2 for 12 h. However, H_2O_2-induced reduction of GSH was remarkably inhibited by Sal B in a dose-dependent manner (**Figure 2B**). The activity of SOD, an important antioxidant enzyme preventing cellular damage caused by ROS, significantly

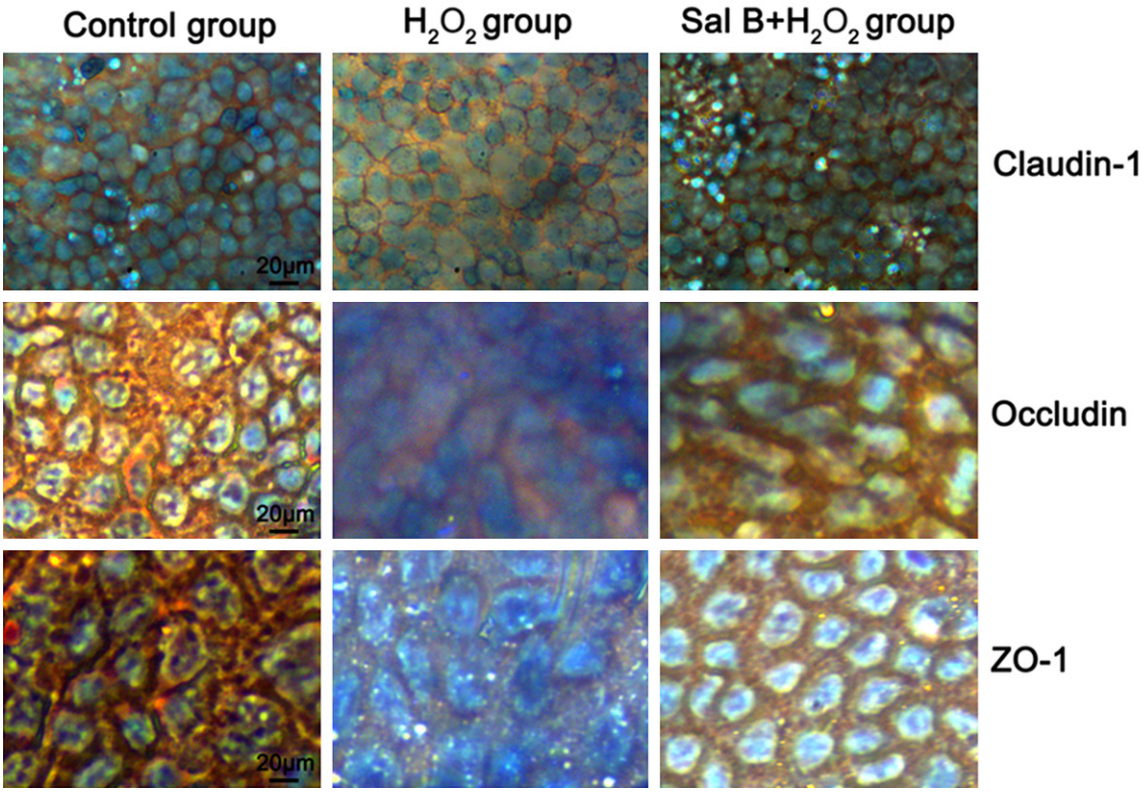

Figure 4. Effects of Sal B on H_2O_2-induced re-localization of TJs proteins. Caco-2 cells were pretreated with PBS (control) or Sal B (100 μmol/L) for 12 h, followed by H_2O_2 (800 μmol/L) treatment for 12 h. Expression of TJs proteins (Claudin-1, Occludin, ZO-1) were detected by immunohistrochemistry. Images shown were representative of at least 5 regions observed on the same slide, and 2 different sections were analyzed for each condition (400×). Results were based on a double-blinded experiment.

decreased when the Caco-2 cells were exposed to H_2O_2 (800 μmol/L) for 12 h. Pretreatment with Sal B inhibited the decline of SOD activity in the setting of H_2O_2 exposure (**Figure 2C**).

Sal B attenuates H_2O_2-induced decrease of TEER and morphology disruption of TJs

To investigate the protective effects of Sal B on intestinal barrier function, we determined the values of TEER in monolayer Caco-2 cells, an indicator of epithelial paracellular permeability to ions. H_2O_2 (800 μmol/L) treatment of monolayer of Caco-2 cells caused significantly decrease in TEER at 9 and 12 h after stimulation (**Figure 3A**). Pretreatment of the Caco-2 cells with Sal B (10 μmol/L, and 100 μmol/L) significantly inhibited the H_2O_2-induced reduction of TEER. H_2O_2 exposure resulted in the disruption of TJs morphology of Caco-2 monolayers. After stimulation with H_2O_2, TJs complexes in Caco-2 cells were reduced and TJs became markedly "open" with less electron-dense material, although there is no obvious disruption of desmosomes (**Figure 3B**). Sal B treatment significantly prevented the disruption of TJs in the setting of H_2O_2 exposure.

Sal B prevents H_2O_2-induced redistribution of Claudin-1, Occludin and ZO-1 proteins

Previous studies have shown that intestinal barrier dysfunction induced by ROS is associated with the morphological tight junction disruption and the relocalization of tight junction proteins. To determine the effects of Sal B on the localization of TJs proteins, immunohistochemistry method was used to investigate the expression of ZO-1, occludin and claudin-1. As shown in **Figure 4**, ZO-1, occludin, and claudin-1 were expressed at intercellular tight junctions under control conditions. Administration of Sal B alone did not alter the localization of these TJs proteins in Caco-2 cells (data was not shown). After exposed to H_2O_2, the distribution of TJs became irregular and discontinuous. However, pretreatment of Sal B significantly prevented there distribution of ZO-1, occludin

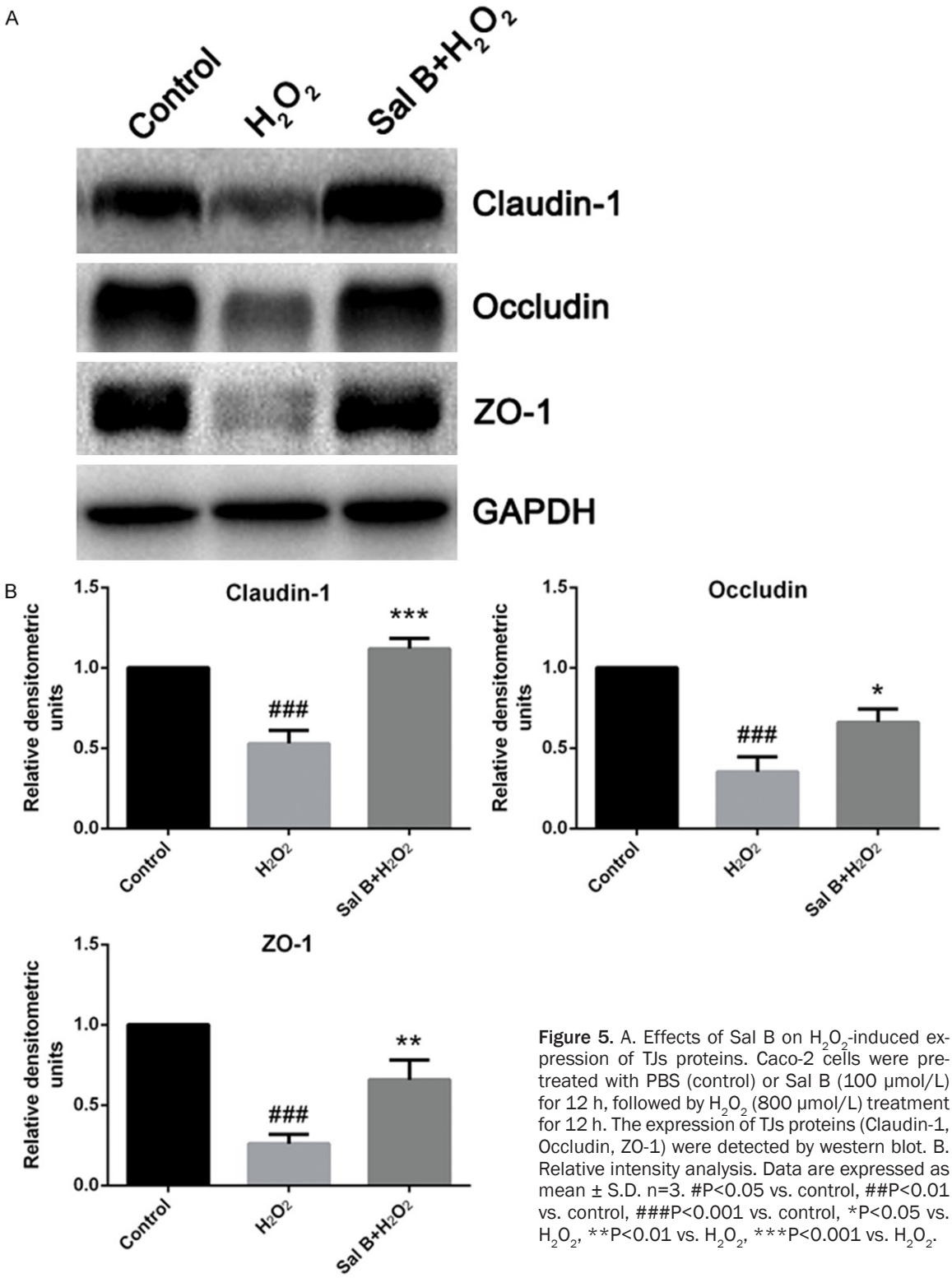

Figure 5. A. Effects of Sal B on H_2O_2-induced expression of TJs proteins. Caco-2 cells were pretreated with PBS (control) or Sal B (100 μmol/L) for 12 h, followed by H_2O_2 (800 μmol/L) treatment for 12 h. The expression of TJs proteins (Claudin-1, Occludin, ZO-1) were detected by western blot. B. Relative intensity analysis. Data are expressed as mean ± S.D. n=3. #P<0.05 vs. control, ##P<0.01 vs. control, ###P<0.001 vs. control, *P<0.05 vs. H_2O_2, **P<0.01 vs. H_2O_2, ***P<0.001 vs. H_2O_2.

and claudin-1 induced by H_2O_2 in Caco-2 cells. And to determine the effects of Sal B on the expression of TJs proteins, we used western blot to investigate the expression of TJs at proteins level, as shown in **Figure 5**.

Discussion

Intestinal epithelial cell layers are the first line of defense against the entry of luminal antigens. The importance of maintaining the integ-

rity of TJs is underscored by large body of studies indicating that increased intestinal permeability contributes to the pathogenesis of several intestinal disorders, including inflammatory bowel disease [3, 28, 29]. Preventing oxidative stress-induced disruption of the integrity of intestinal barrier will provide new therapeutic alternatives in multiple gastrointestinal diseases. In the present study we demonstrated that Sal B plays a critical role in preventing H_2O_2-induced cell damage, alteration of TEER, morphology change of TJs, and relocation of Claudin-1, Occludin and ZO-1 proteins in Caco-2 monolayer model.

Human colon cancer-derived Caco-2 monolayer model is a well-established *in vitro* model applied to determine the alteration of cell permeability and distribution of TJs proteins [30]. Oxidative stress is one of the major insults inducing intestinal barrier disruption. Previous study showed that H_2O_2 causes a robust decrease in TEER in Caco-2 cells monolayers [31], which is consistent with our present study showing that H_2O_2 treatment reduced TEER in a time-dependent manner. H_2O_2 disrupts the TJs and increases paracellular permeability [26, 31, 32] by enhancing tyrosine phosphorylation of TJs proteins in Caco-2 cells monolayers [32]. Some studies also demonstrated that p38 MAP kinase [33-35] and ERK1/2 [36] mediate H_2O_2-induced TJ barrier disruption. In addition, H_2O_2 also induces the re-distribution of TJs proteins, such as Claudin-1, Occludin and ZO-1 leading to the disruption of barrier, which is consistent with our current study.

Preventing oxidative stress-induced intestinal barrier disruption will provide more effective treatment regimens for these oxidative inflammatory disorders. Previous studies have shown that epidermal growth factor (EGF) protects intestinal barrier integrity and microtubule cytoskeleton against oxidant [37-39]. Nitric oxide also has been shown to attenuate H_2O_2-induced barrier disruption via preventing tyrosine phosphorylation of TJs proteins [40]. Salvianolic acid is one of the bioactive compounds of Salvia miltiorrhiza. Among salvianolic acids, Sal A and Sal B are the most abundant components. The therapeutic potential of salvianolic acids on hepatic protection [41], neural protection [42], cancer treatment [43, 44], and cardiovascular protection [45] have been proposed in recent years. However, Sal B was thought to have much more commercial value for the food and medicine purposes due to the containment of the highest amounts in Salvia miltiorrhiza [46]. Sal B has been shown to exhibit strong antioxidant activity by scavenging reactive oxygen species including HO., O2.-, DPPH radicals, and H_2O_2 [47]. Our present study demonstrates that Sal B prevented H_2O_2-induced decrease in GSH levels and SOD activity and inhibited cellular LDH leaking as a result of lipid peroxidation. In addition, Sal B has been reported to inhibit cardiovascular inflammation via regulating MMP2/9 and ERK1/2 [48, 49], which could be one of the potential mechanisms accounting for the protective effects of Sal B on Caco-2 cells permeability under the challenge of H_2O_2. In the present study, we observed that Sal B prevented H_2O_2-induced morphology change of TJs and the relocation of TJs proteins, including ZO-1, occludin and claudin-1. The protect effects of Sal B could arise from the inhibition of protein phosphorylation of TJs [18], which needs to be further investigated in future.

To our knowledge, this is the first report demonstrating that Sal B protects the integrity of intestinal epithelial barrier in the setting of H_2O_2 stimulation. Further studies are required to elucidate the mechanisms employed by Sal B, *in vitro* and *in vivo,* to protect the intestinal epithelial barrier function against oxidative stress. The protective effects of Sal B on intestinal barrier function will provide a new therapeutic approach in the treatment of inflammatory bowel diseases.

Disclosure of conflict of interest

None.

Address correspondence to: Dong Wang, Dalian Central Hospital Affiliated to Dalian Medical University, Dalian, China. E-mail: wangdong_2016@126.com

References

[1] Edelblum KL and Turner JR. The tight junction in inflammatory disease: communication breakdown. Curr Opin Pharmacol 2009; 9: 715-720.

[2] Jankowski JA, Bedford FK, Boulton RA, Cruickshank N, Hall C, Elder J, Allan R, Forbes A, Kim YS, Wright NA and Sanders DS. Alterations in classical cadherins associated with progression in ulcerative and Crohn's colitis. Lab Invest 1998; 78: 1155-1167.

[3] Schmitz H, Barmeyer C, Fromm M, Runkel N, Foss HD, Bentzel CJ, Riecken EO and Schulzke

JD. Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. Gastroenterology 1999; 116: 301-309.
[4] Kinugasa T, Sakaguchi T, Gu X and Reinecker HC. Claudins regulate the intestinal barrier in response to immune mediators. Gastroenterology 2000; 118: 1001-1011.
[5] North AJ, Bardsley WG, Hyam J, Bornslaeger EA, Cordingley HC, Trinnaman B, Hatzfeld M, Green KJ, Magee AI and Garrod DR. Molecular map of the desmosomal plaque. J Cell Sci 1999; 112: 4325-4336.
[6] Tsukita S and Furuse M. Occludin and claudins in tight-junction strands: leading or supporting players. Trends Cell Biol 1999; 9: 268-273.
[7] Anderson JM and Van Itallie CM. Tight junctions and the molecular basis for regulation of paracellular permeability. Am J Physiol 1995; 269: G467-475.
[8] Wittchen ES, Haskins J and Stevenson BR. Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. J Biol Chem 1999; 274: 35179-35185.
[9] Macintire DK and Bellhorn TL. Bacterial translocation: clinical implications and prevention. Vet Clin North Am Small Anim Pract 2002; 32: 1165-1178.
[10] Hollander D. The intestinal permeability barrier. A hypothesis as to its regulation and involvement in Crohn's disease. Scand J Gastroenterol 1992; 27: 721-726.
[11] Keshavarzian A, Holmes EW, Patel M, Iber F, Fields JZ and Pethkar S. Leaky gut in alcoholic cirrhosis: a possible mechanism for alcohol-induced liver damage. Am J Gastroenterol 1999; 94: 200-207.
[12] Rao RK, Baker RD, Baker SS, Gupta A and Holycross M. Oxidant-induced disruption of intestinal epithelial barrier function: role of protein tyrosine phosphorylation. Am J Physiol 1997; 273: G812-823.
[13] Rao RK, Basuroy S, Rao VU, Karnaky JKJ and Gupta A. Tyrosine phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-beta-catenin complexes from the cytoskeleton by oxidative stress. Biochem J 2002; 368: 471-481.
[14] Sheth P, Basuroy S, Li C, Naren AP and Rao RK. Role of phosphatidylinositol 3-kinase in oxidative stress-induced disruption of tight junctions. J Biol Chem 2003; 278: 49239-49245.
[15] Clayburgh DR, Musch MW, Leitges M, Fu YX and Turner JR. Coordinated epithelial NHE3 inhibition and barrier dysfunction are required for TNF-mediated diarrhea in vivo. J Clin Invest 2006; 116: 2682-2694.
[16] Utech M, Ivanov AI, Samarin SN, Bruewer M, Turner JR, Mrsny RJ, Parkos CA and Nusrat A. Mechanism of IFN-gamma-induced endocytosis of tight junction proteins: myosin II-dependent vacuolarization of the apical plasma membrane. Mol Biol Cell 2005; 16: 5040-5052.
[17] Shifflett DE, Clayburgh DR, Koutsouris A, Turner JR and Hecht GA. Enteropathogenic E. coli disrupts tight junction barrier function and structure in vivo. Lab Invest 2005; 85: 1308-1324.
[18] Rao R, Baker RD and Baker SS. Inhibition of oxidant-induced barrier disruption and protein tyrosine phosphorylation in Caco-2 cell monolayers by epidermal growth factor. Biochem Pharmacol 1999; 57: 685-695.
[19] Tang MK, Ren DC, Zhang JT and Du GH. Effect of salvianolic acids from Radix Salviae miltiorrhizae on regional cerebral blood flow and platelet aggregation in rats. Phytomedicine 2002; 9: 405-409.
[20] Zhang HS and Wang SQ. Salvianolic acid B from Salvia miltiorrhiza inhibits tumor necrosis factor-alpha (TNF-alpha)-induced MMP-2 up-regulation in human aortic smooth muscle cells via suppression of NAD(P)H oxidase-derived reactive oxygen species. J Mol Cell Cardiol 2006; 41: 138-148.
[21] Li M, Zhao C, Wong RN, Goto S, Wang Z and Liao F. Inhibition of shear-induced platelet aggregation in rat by tetramethylpyrazine and salvianolic acid B. Clin Hemorheol Microcirc 2004; 31: 97-103.
[22] Xia QG, Reinecke A, Dorenkamp M, Storz C, Bitterling H, Penz S, Cleutjens J, Daemen MJ, Simon R and Unger T. Comparison of cardioprotective effects of mibefradil and ramipril in stroke-prone spontaneously hypertensive rats. Acta Pharmacol Sin 2004; 25: 763-768.
[23] Jiang RW, Lau KM, Hon PM, Mak TC, Woo KS and Fung KP. Chemistry and biological activities of caffeic acid derivatives from Salvia miltiorrhiza. Curr Med Chem 2005; 12: 237-246.
[24] Tang Y, Jacobi A, Vater C, Zou X and Stiehler M. Salvianolic acid B protects human endothelial progenitor cells against oxidative stress-mediated dysfunction by modulating Akt/mTOR/4EBP1, p38 MAPK/ATF2, and ERK1/2 signaling pathways. Biochem Pharmacol 2014; 90: 34-49.
[25] Tanida S, Mizoshita T, Mizushima T, Sasaki M, Shimura T, Kamiya T, Kataoka H and Joh T. Involvement of oxidative stress and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in inflammatory bowel disease. J Clin Biochem Nutr 2011; 48: 112-116.
[26] Basuroy S, Seth A, Elias B, Naren AP and Rao R. MAPK interacts with occludin and mediates EGF-induced prevention of tight junction disruption by hydrogen peroxide. Biochem J 2006; 393: 69-77.

[27] Audus KL, Bartel RL, Hidalgo IJ and Borchardt RT. The use of cultured epithelial and endothelial cells for drug transport and metabolism studies. Pharm Res 1990; 7: 435-451.
[28] Farhadi A, Banan A, Fields J and Keshavarzian A. Intestinal barrier: an interface between health and disease. J Gastroenterol Hepatol 2003; 18: 479-497.
[29] Bjarnason I, Williams P, Smethurst P, Peters TJ and Levi AJ. Effect of non-steroidal anti-inflammatory drugs and prostaglandins on the permeability of the human small intestine. Gut 1986; 27: 1292-1297.
[30] Quaroni A, Wands J, Trelstad RL and Isselbacher KJ. Epithelioid cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria. J Cell Biol 1979; 80: 248-265.
[31] Oshima T, Sasaki M, Kataoka H, Miwa H, Takeuchi T and Joh T. Wip1 protects hydrogen peroxide-induced colonic epithelial barrier dysfunction. Cell Mol Life Sci 2007; 64: 3139-3147.
[32] Jepson MA. Disruption of epithelial barrier function by H_2O_2: distinct responses of Caco-2 and Madin-Darby canine kidney (MDCK) strains. Cell Mol Biol (Noisy-le-grand) 2003; 49: 101-112.
[33] Kevil CG, Oshima T and Alexander JS. The role of p38 MAP kinase in hydrogen peroxide mediated endothelial solute permeability. Endothelium 2001; 8: 107-116.
[34] Lui WY, Lee WM and Cheng CY. TGF-betas: their role in testicular function and Sertoli cell tight junction dynamics. Int J Androl 2003; 26: 147-160.
[35] Yamamoto T, Kojima T, Murata M, Takano K, Go M, Hatakeyama N, Chiba H and Sawada N. p38 MAP-kinase regulates function of gap and tight junctions during regeneration of rat hepatocytes. J Hepatol 2005; 42: 707-718.
[36] Savkovic SD, Ramaswamy A, Koutsouris A and Hecht G. EPEC-activated ERK1/2 participate in inflammatory response but not tight junction barrier disruption. Am J Physiol Gastrointest Liver Physiol 2001; 281: G890-898.
[37] Banan A, Fields JZ, Talmage DA, Zhang Y and Keshavarzian A. PKC-beta1 mediates EGF protection of microtubules and barrier of intestinal monolayers against oxidants. Am J Physiol Gastrointest Liver Physiol 2001; 281: G833-847.
[38] Banan A, Choudhary S, Zhang Y, Fields JZ and Keshavarzian A. Ethanol-induced barrier dysfunction and its prevention by growth factors in human intestinal monolayers: evidence for oxidative and cytoskeletal mechanisms. J Pharmacol Exp Ther 1999; 291: 1075-1085.
[39] Banan A, Fields JZ, Zhang Y and Keshavarzian A. Key role of PKC and Ca2+ in EGF protection of microtubules and intestinal barrier against oxidants. Am J Physiol Gastrointest Liver Physiol 2001; 280: G828-843.
[40] Katsube T, Tsuji H and Onoda M. Nitric oxide attenuates hydrogen peroxide-induced barrier disruption and protein tyrosine phosphorylation in monolayers of intestinal epithelial cell. Biochim Biophys Acta 2007; 1773: 794-803.
[41] Yan X, Zhou T, Tao Y, Wang Q, Liu P and Liu C. Salvianolic acid B attenuates hepatocyte apoptosis by regulating mediators in death receptor and mitochondrial pathways. Exp Biol Med (Maywood) 2010; 235: 623-632.
[42] Wang SX, Hu LM, Gao XM, Guo H and Fan GW. Anti-inflammatory activity of salvianolic acid B in microglia contributes to its neuroprotective effect. Neurochem Res 2010; 35: 1029-1037.
[43] Zhou ZT, Yang Y and Ge JP. The preventive effect of salvianolic acid B on malignant transformation of DMBA-induced oral premalignant lesion in hamsters. Carcinogenesis 2006; 27: 826-832.
[44] Liu X, Yang Y, Zhang X, Xu S, He S, Huang W and Roberts MS. Compound Astragalus and Salvia miltiorrhiza extract inhibits cell invasion by modulating transforming growth factor-beta/Smad in HepG2 cell. J Gastroenterol Hepatol 2010; 25: 420-426.
[45] Wang SB, Tian S, Yang F, Yang HG, Yang XY and Du GH. Cardioprotective effect of salvianolic acid A on isoproterenol-induced myocardial infarction in rats. Eur J Pharmacol 2009; 615: 125-132.
[46] Liu GT, Zhang TM, Wang BE and Wang YW. Protective action of seven natural phenolic compounds against peroxidative damage to biomembranes. Biochem Pharmacol 1992; 43: 147-152.
[47] Ishige K, Schubert D and Sagara Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. Free Radic Biol Med 2001; 30: 433-446.
[48] Chen YL, Hu CS, Lin FY, Chen YH, Sheu LM, Ku HH, Shiao MS, Chen JW and Lin SJ. Salvianolic acid B attenuates cyclooxygenase-2 expression in vitro in LPS-treated human aortic smooth muscle cells and in vivo in the apolipoprotein-E-deficient mouse aorta. J Cell Biochem 2006; 98: 618-631.
[49] Lin SJ, Lee IT, Chen YH, Lin FY, Sheu LM, Ku HH, Shiao MS, Chen JW and Chen YL. Salvianolic acid B attenuates MMP-2 and MMP-9 expression in vivo in apolipoprotein-E-deficient mouse aorta and in vitro in LPS-treated human aortic smooth muscle cells. J Cell Biochem 2007; 100: 372-384.