Life Sciences 233 (2019) 116697

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Berberine induces ZIP14 expression and modulates zinc redistribution to protect intestinal mucosal barrier during polymicrobial sepsis

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ARTICLE INFO

Keywords:
Berberine
ZIP14
Zinc redistribution
Intestinal mucosal barrier
Sepsis
IGF-I
Tight junction

ABSTRACT

Aims: The present study investigated if berberine might induce Zrt-Irt-like protein 14 (ZIP14) and affect zinc redistribution to protect intestinal barrier in sepsis.
Main methods: Rodent model of sepsis was induced by cecal ligation and puncture (CLP). Plasma endotoxin was assayed by LAL test and plasma zinc was measured by flame atomic spectrophotometer. Gut mucosal permeability was determined by plasma FITC-dextran. Zinc content and ZIP14 mRNA in gut mucosa were assayed by spectrophotometer and qRT-PCR, respectively. Tight junction integrity of Caco-2 was evaluated by transepithelial electrical resistance (TEER). Tight junction (TJ) protein expression was detected by Western blotting.
Key findings: Berberine and zinc gluconate pretreatment to CLP rats improved survival rate, reduced plasma endotoxin level, alleviated hypozincemia, increased zinc accumulation and ZIP14 mRNA expression in the intestinal mucosa. Berberine and zinc gluconate pretreatment decreased CLP-elicited intestinal hyperpermeability to FITC-dextran. These effects of berberine in vivo were abolished by AG1024. In vitro, lipopolysaccharide (LPS) repressed zinc transfer into Caco-2 cells exposed to zinc gluconate. Berberine and IGF-I treatment increased ZIP14 protein expression and promoted zinc transfer into Caco-2 cells exposed to zinc gluconate plus LPS. Berberine treatment induced TJ protein (claudin-1 and occludin) and raised TEER in LPS-treated Caco-2 cells. These effects of berberine in vitro were partially inhibited by ZIP14 siRNA.
Significance: The present study reveals that berberine induces ZIP14 expression and affects zinc re- distribution to protect intestinal barrier in sepsis, which is partially linked with the activation of IGF-I signaling.

1. Introduction

The intestine is inhabited by a great number of microorganisms strictly prohibited from accessing to blood circulation. Physiologically, intestinal mucosal integrity contributes to homeostasis [1]. Pathologically, the harmful factors (e.g., severe burn, sepsis) dismantle intestinal mucosa and facilitate to bacterial translocation and toxin dissemination. Thus, the intestine is deemed as the 'motor' of sepsis and multiple organ failure [2].

Zinc primally functions as a cofactor for enzymes or a structural element for proteins. One important action of zinc is to modulate inflammatory as well as immune response. Zinc loss, malabsorption and redistribution in sepsis leads to zinc dyshomeostasis [3–5], as well as compromised immunity and gut barrier dysfunction [6,7]. It is well known that IGF-I plays an important role in the maintenance of intestinal barrier function [8,9]. Zinc deficiency in turn depresses the expression of IGF-I [10,11].

Zrt-Irt-like protein (ZIP) family consists of 14 members to control zinc uptake/transfer into cytoplasm. Among these, ZIP14 is particularly important for zinc homoeostasis in the gut [5,12]. ZIP14 is localized to the basolateral membrane of intestinal epithelial cells. Deletion of ZIP14 promotes the paracellular movement of bacteria and toxins into blood via the inhibition of TJ protein (e.g., claudin-1, claudin-3, occludin) expression as well as occludin phosphorylation [5,13]. LPS exerts a strong inhibition on ZIP14 expression in the gut [5]. Thus, ZIP14 is deemed as a potential target for gut mucosal barrier [5,14].

Berberine presents in several plant species including the *Coptis* sp. and *Berberis* sp. Berberine is widely used to treat infectious diarrhea, gastroenteritis, diabetus and other inflammatory diseases [15]. Berberine has the capability to suppress proinflammatory cytokines (e.g., TNF-α, IL-6, IL-1β) production and enzymes (e.g, myeloperoxidase, COX-2) expression. Berberine also induces mucins and TJ protein production to enhance intestinal mucosal barrier [16–18]. Importantly, berberine has recently been reported to induce IGF-I expression to

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https://doi.org/10.1016/j.lfs.2019.116697
Received 8 May 2019; Received in revised form 12 July 2019; Accepted 24 July 2019
Available online 25 July 2019

protect intestinal mucosa [8].

Cecal ligation and puncture in rodents has become the most widely used and the gold standard model for polymicrobial sepsis to study underlying mechanisms of sepsis [19]. Zinc gluconate is used to probe the significance of zinc in inflammatory response and sepsis [20]. AG1024, an inhibitor of IGF−IR, is used to block IGF-I signaling. So, in this present study we investigated whether berberine might elicit ZIP14 expression and affect zinc redistribution in the intestine via the activation of IGF-I pathway in sepsis.

2. Materials and methods

2.1. Animal experiments

2.1.1. Experimental animal and grouping

The male Wistar rats (270–310 g) were obtained from the Laboratory Animal Center of Nanjing Medical University, Jiangsu Province, China. They were housed at a controlled temperature with free access to food and water under a day/night cycle. Rats were acclimatized for one week prior to experiment and randomly assigned into sham ($n = 10$/group) and four experimental groups ($n = 15$/group): (I) CLP; (II) zinc gluconate plus CLP; (III) berberine plus CLP; (IV) berberine plus AG1024 plus CLP. All animal experiments complied with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

2.1.2. Pretreatment with zinc gluconate, berberine and AG1024

Zinc gluconate, berberine, and AG1024 were obtained from Sigma-Aldrich Corp. USA. These reagents were pretreated as previously described [8,21]. Zinc gluconate (2 mg/kg/day) was given via i.p. injection for 5 days prior to sepsis induction. Berberine (50 mg/kg/day, gavage) and AG1024 (3 mg/kg/day, i.p. injection) were also given for 5 days before sepsis induction. Rats in sham group were administered an equal volume of saline.

2.1.3. Induction of sepsis by cecal ligation and puncture (CLP)

The model of polymicrobial sepsis was induced by CLP [19]. In brief, anesthesia was induced by i.p. injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). A 3-cm midline abdominal incision was made, cecum was isolated and feces were pushed to distal cecum. The medium ligation was done with a surgical spool suture and cecum was then carried out a “through-and-through” puncture once with an 18-G needle. In sham rats, no manipulation was done before the closure of abdominal wall. Rats were sacrificed 48 h after operation.

2.1.4. Blood endotoxin determination

Blood samples drawn from vein at indicated time points were centrifuged at 6000 ×*g* for 5 min at 4 °C. Plasma samples were then diluted 1:10 with sterile water, mixed by vortex, placed in a 75 °C water bath for 10 min, cooled at room temperature for 10 min. Plasma endotoxin was assayed by limulus ameobocyte lysate kits (MP Biomedicals, USA) according to guideline. Standards and samples were incubated with LAL for 10 min, then with colorimetric substrate for 6 min at 37 °C. The absorbance at 405 nm was read. The concentration of plasma endotoxin was expressed as EU/ml.

2.1.5. Intestinal mucosal permeability measurement

Intestinal mucosal permeability was measured according to previous literatures [5]. In brief, rats were fasted overnight and FITC-dextran 4000 (Sigma-Aldrich, USA) was administered by gavage (0.4 mg/g) 1 h prior to sacrifice. FITC-dextran in plasma samples was measured by spectrofluorometry at Ex/Em 488/520 nm and calculated from a standard curve. The data were normalized to body weight and expressed as ng/ml/g.

2.1.6. Plasma zinc concentration measurement

Plasma samples were diluted 5 times in 0.1 M hydrochloric acid. The concentration of plasma zinc was assayed by flame atomic absorption spectrophotometer as previously described [21].

2.1.7. Intestinal mucosal zinc concentration measurement

Proximal jejunums were removed and rinsed with saline. Mucosal tissues were scraped with a glass slide on ice and collected in the Cryogenic vials and rapidly frozen in liquid nitrogen. The specimens then were stored at −80 °C prior to experiment. Wet mucosal scrapings were dried with a surgical gauze pad, sealed in a WHIRL-PAK bag, and then put into an 18-ml centrifuge tube. After 16-h lyophilization, dry tissue was weighed and digested in 3 ml of nitric acid overnight and then digested in a boiling water bath for 5 h the next day. After cooling, the remainder in the centrifuge tube was transferred to a 10-ml polystyrene test tube and was diluted up to 10 ml with double distilled water (DDW). 1 ml of this solution was diluted up to 10 ml with DDW. Mucosal zinc content was assayed by a spectrophotometer by comparison of the absorbance value to the standard curve and was expressed as μg/g dry tissue weight [22].

2.1.8. Quantification of mucosal ZIP14 mRNA expression

ZIP14 mRNA abundance in the intestinal mucosa was assayed by quantitative real-time PCR (qRT-PCR). Total RNA from mucosal tissues was extracted by using Trizol reagent (Invitrogen) according to guideline. RNA samples were treated with 10 U RNAse-free DNAse I (Promega Corporation), and 1 μg RNA was reverse transcribed into cDNA with 2.5 pmol oligo$(dT)_{18}$ primer and 5 U avian myeloblastosis virus reverse transcriptase XL (TaKaRa) in 25 μl reaction mixture at 42 °C for 40 min. The mRNA content was quantified by a Thermal Cycler Dice Real Time System. The forward and reverse primers for ZIP14 were 5′-AAGGAAAATGAGCAGACAGAGG-3′ and 5′-AGAAAAGG TAGAAACCCCCAAA-3′, respectively; and primers for GAPDH were 5′-GGCTCTCTGCTCCTCCCTGTTCTAG-3′ and 5′-CGTCCGATACGGCCA AATCCGT-3′, respectively. A SYBR Premix Ex Taq kit was used in this study.

2.2. Cell experiment

2.2.1. Cell culture and treatment

Caco-2, a human colorectal adenocarcinoma cell line, was widely used as a model of intestinal epithelial barrier [23]. Cells from Cell Culture Unit of Shanghai Science Academy, China, were used between passage 34 and 50. Cells were grown in DMEM with 10% FBS and 1% NEAA and incubated in an atmosphere with 5% CO_2 at 37 °C. Medium was refreshed every 3 days. All experiments were done on day 13–14 postseeding using fully differentiated cells [13].

2.2.2. Gene silencing by transfection of siRNA

The siRNAs for human ZIP14 gene and negative siRNA were purchased from Dharmacon (Thermo Scientific). The method for silencing ZIP14 gene has been described previously [24]. Briefly, 6 μl of Lipofectamine RNAiMAX (Invitrogen) and 100 pmol of RNAi duplex were mixed in 600 μl of Opti-MEM (Invitrogen) and added into each well of a 6-well plate. To confirm the efficiency of knockdown, ZIP14 protein were assayed by Western blotting according to [24].

2.2.3. Intracellular zinc measurement

Intracellular zinc was assayed by ZnAF-2DA, a cell-permeant dye with the high specificity to Zn^{2+}. After different treatments Caco-2 cells were incubated with 10 μM ZnAF-2DA (Sigma- Aldrich, USA) for 30 min before zinc assay. ZnAF-2DA fluorescence intensity was monitored at an emission wavelength of 535 nm and by the excitation wavelength at 485 nm [13]. The Zn^{2+} content was expressed as relative values against control monolayers.

2.2.4. Caco-2 monolayer barrier function analysis

Tight junction integrity was evaluated by measurement of transepithelial electrical resistance (TEER) in Caco-2 monolayers in Transwell filter supports with a Millicell-ERS voltohmmeter (Millipore). TEER value was recorded every 12 h (0, 12, 24, 36, 48, 60, 72 h points) after subtracting the resistance value of Transwell filters. The results of TEER are expressed as a percentage of initial value/baseline [7,13].

2.2.5. Determination of TJ proteins by Western blotting

TJ proteins in the cell monolayers were measured as described previously. Total protein was harvested from cells in each well using 100 μL of ice-cold lysis buffer supplemented with a protease inhibitor and phosphatase inhibitor. A bicinchoninic acid assay (BCA) protein assay kit was used to quantify protein content. Protein samples were separated by SDS-PAGE and then transferred to a PVDF membrane, which was blocked with 5% skim milk in TBS containing 0.05% Tween-20. The membrane was incubated with primary antibodies ZIP14 (1:1000), claudin-1 (1:1000), or occludin (1:1000) (Santa-Cruz, USA) overnight at 4 °C and incubated with secondary antibodies for 2-h at 37 °C. The gel bands were photographed and quantified with ChemiDoc XRS system. The target protein/beta-actin ratio was used to represent the relative expression of target protein.

2.3. Statistical analysis

The results are expressed as mean ± standard deviation. After analysis of homogeneity, the data of variance homogeneity or heterogeneity was tested by One-way ANOVA or Welch analysis. The LSD or Dunnett T3 test was used to determine the difference of means among different groups. Kaplan-Meier analysis was used for survival curves. $P < 0.05$ was considered statistically significant. Statistical analyses were done with the SPSS 19.0 statistical software package (SPSS Inc., Chicago, USA).

3. Results

3.1. Berberine and zinc gluconate improved while AG1024 reduced survival rates of septic rats

Survival rate is a sensitive index of different severity grades of sepsis induced by CLP [19]. In this study, we constructed a model of mid-grade sepsis. The alteration of survival rate was shown in Fig. 1. All rats in sham group were alive at postoperative 48 h, but in CLP group only 7 rats survived (survival rate of 46.7%). In zinc gluconate and berberine pre-treatment group 10 and 12 rats survived, corresponding to the survival rate of 66.7% and 80%, respectively. In CLP plus berberine plus AG1024 group, 9 rats survived and the survival rate dropped up to 60%.

3.2. Berberine and zinc gluconate reduced while AG1024 increased plasma endo-toxin levels of septic rats

CLP-induced sepsis leads to intestinal endotoxin accessing to blood [19]. The results of plasma endotoxin levels were shown in Fig. 2A. In sham rats, endotoxin was detectable but at very low levels at indicated time points (0, 12, 24, 36, 48 h). CLP induced a rapid increase in plasma endotoxin levels (0.07 ± 0.03, 0.82 ± 0.12, 1.40 ± 0.18, 1.78 ± 0.25, 1.98 ± 0.36 EU/ml, respectively). Zinc gluconate pre-treatment to CLP rats lowered endotoxin levels (0.07 ± 0.04, 0.65 ± 0.13, 1.09 ± 0.22, 1.28 ± 0.24, 1.49 ± 0.27 EU/ml, respectively). Berberine pretreatment to CLP rats also reduced endotoxin levels (0.08 ± 0.04, 0.63 ± 0.16, 1.03 ± 0.21, 1.22 ± 0.24, 1.37 ± 0.26 EU/ml, respectively). AG1024 pretreatment abolished the effect of berberine on plasma endotoxin (0.08 ± 0.03, 0.76 ± 0.13, 1.19 ± 0.17, 1.43 ± 0.25, 1.67 ± 0.33 EU/ml, respectively).

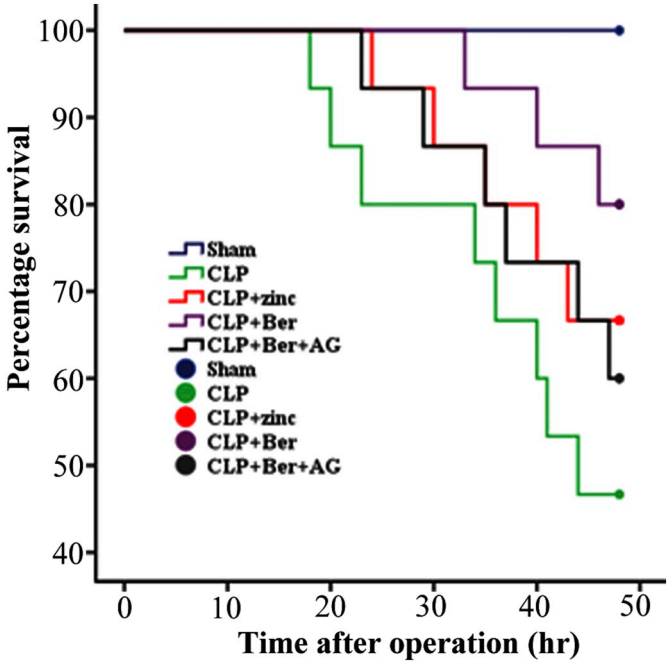

Fig. 1. The change of survival rate in postoperative 48 h in rats subjected to sham, CLP, CLP plus zinc gluconate (CLP + zinc), CLP plus berberine (CLP + Ber), and CLP plus berberine plus AG1024 (CLP + Ber + AG), respectively.

3.3. Berberine and zinc gluconate increased while AG1024 reduced plasma zinc levels of septic rats

Sepsis and systemic inflammation may induce hypozincemia [3]. The findings of plasma zinc levels were shown in Fig. 2B. In sham group plasma Zn^{2+} levels did not significantly shift. In CLP group Zn^{2+} concentration in plasma reduced in a time-dependent manner. Zinc gluconate and berberine pretreatment improved CLP-elicited hypozincemia, while AG1024 pretreatment abolished the action of berberine. Surprisingly, we found plasma Zn^{2+} level in all experimental groups rapidly declined in postoperative 24 h but slowly elevated in 24–48 h.

3.4. Berberine and zinc gluconate reduced while AG1024 increased intestinal mucosal permeability of septic rats

Zinc dyshomeostasis damages intestinal mucosal integrity leading to hyperpermeabity [6]. The levels of plasma FITC-dextran, a parameter to indicate a defect in intestinal mucosal permeability [5], were shown in Fig. 3A. FITC-dextran concentration in CLP-treated rats was more than 3-fold to that in sham rats (76.26 ± 11.16 vs. 24.08 ± 4.05 ng/ml/g). Zinc gluconate and berberine pretreatment reduced CLP-increased FITC-dextran in plasma (57.00 ± 9.35 and 54.25 ± 5.53 ng/ml/g, respectively). AG1024 pretreatment abrogated the effect of berberine giving rise to intestinal mucosal hyperpermeability to FITC-dextran (65.92 ± 9.10 ng/ml/g).

3.5. Berberine and zinc gluconate increased while AG1024 reduced Zn^{2+} accumulation in the intestinal mucosa of septic rats

Sepsis and systemic inflammation may induce zinc redistribution in different organs and tissues [5]. The results of Zn^{2+} accumulation in the intestinal mucosa was shown in Fig. 3B. Zn^{2+} accumulation in CLP group was lower than that in sham group (14.62 ± 2.38 vs. 28.05 ± 2.65 μg/g). Intraperitoneal injection of zinc gluconate to CLP rats increased Zn^{2+} accumulation in gut mucosa (19.37 ± 2.27 μg/g).

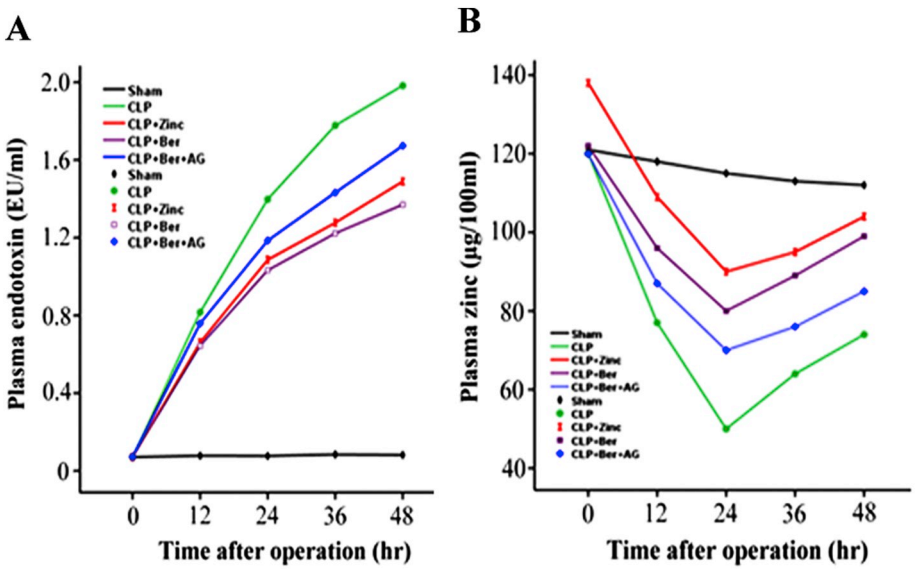

Fig. 2. The alteration of plasma endotoxin (A) and plasma zinc (B) level in postoperative 48 h in rats subjected to sham, CLP, CLP plus zinc gluconate, CLP plus berberine, and CLP plus berberine plus AG1024, respectively.

Gavage of berberine to CLP rats also increased the accumulation of Zn^{2+} in the intestinal mucosa (22.50 ± 2.51 μg/g). AG1024 pretreatment reduced berberine-induced Zn^{2+} accumulation (17.54 ± 2.58 μg/g).

3.6. Berberine, zinc gluconate and IGF-I increased intracellular zinc content in LPS-treated Caco-2 cells

Endotoxin may compromise zinc transport system [5]. As shown in Fig. 4, in comparison to saline, LPS did not significantly affect ZnAF-2DA fluorescence density in Caco-2 cells. Supplementation with zinc gluconate to Caco-2 cells elevated intracellular ZnAF-2DA density, suggesting Zn^{2+} was transferred into Caco-2 cells. However, cotreatment with zinc gluconate and LPS led to a marked reduction of intracellular ZnAF-2DA fluorescence density, indicating LPS disrupted Zn^{2+} transport system. In Caco-2 cells cotreated with zinc gluconate and LPS, provision with IGF-I or berberine markedly raised intracellular ZnAF-2DA fluorescence density, indicative of an improvement of zinc transport system.

3.7. Berberine and zinc gluconate increased while ZIP14 siRNA reduced TEER of Caco-2 cells exposed to LPS

TEER is a parameter of tight junction barrier function. The results of TEER of Caco-2 cells exposed to different treatments were demonstrated in Table 1 and the percentage from initial baseline were shown in Fig. 5. TEER did not notably alter in saline-treated Caco-2 cells at indicated time points. Conversely, LPS stimulus rapidly decreased TEER in a time-dependent manner, indicative of an impairment of TJ barrier function. Zinc gluconate and berberine treatment increased TEER in Caco-2 cells with exposure to LPS. ZIP14 siRNA abolished the effect of berberine giving rise to a reduction of TEER.

3.8. Berberine increased ZIP14 mRNA and protein expression in gut mucosa and Caco-2 cells in part via IGF-I pathway

The expressions of ZIP14 in gut mucosa and in Caco-2 cells were assayed, respectively. As indicated in Fig. 6, mucosal ZIP14 mRNA abundance in sham rats was higher than that in CLP rats. Berberine pretreatment increased ZIP14 mRNA production in CLP rats, suggesting

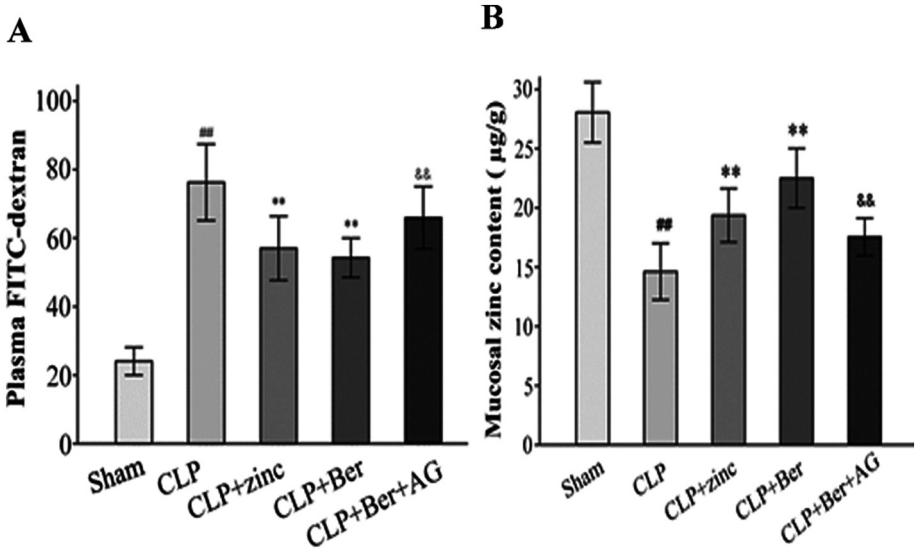

Fig. 3. The concentration of plasma FITC-dextran (A) and mucosal zinc (B) at postoperative 48 h in rats subjected to sham, CLP, CLP plus zinc gluconate, CLP plus berberine, and CLP plus berberine plus AG1024, respectively. The columns show means ± SD. $^{\#\#}P < 0.01$ vs. sham; $^{**}P < 0.01$ vs. CLP; $^{\&\&}P < 0.01$ vs. CLP + Ber, respectively.

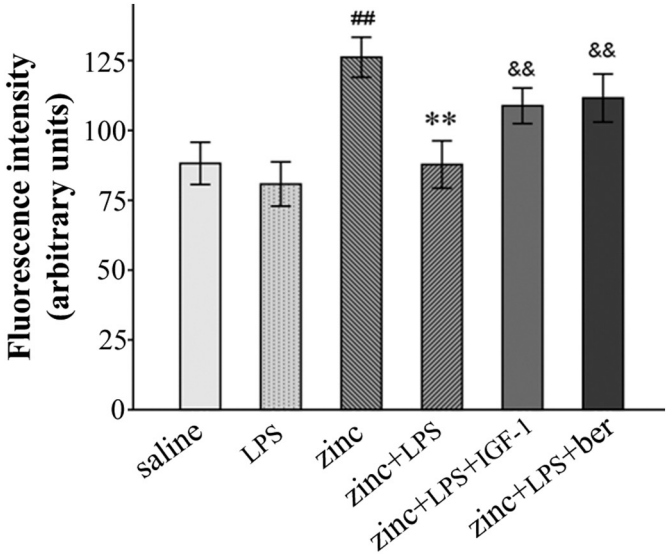

Fig. 4. The ZnAF-2DA fluorescence density in Caco-2 cells treated with saline, 500 ng/ml LPS (*Escherichia coli* O111:B4), 100 μM zinc gluconate, 100 μM zinc gluconate plus 500 ng/ml LPS (zinc + LPS), 100 μM zinc gluconate plus 500 ng/ml LPS plus 200 ng/ml IGF-I (zinc + LPS + IGF-I), 100 μM zinc gluconate plus 500 ng/ml LPS plus 20 μM berberine (zinc + LPS + ber), respectively. Columns show means ± SD. $^{\#\#}P < 0.01$ vs. control; $^{**}P < 0.01$ vs. zinc; $^{\&\&}P < 0.01$ vs. zinc + LPS, respectively.

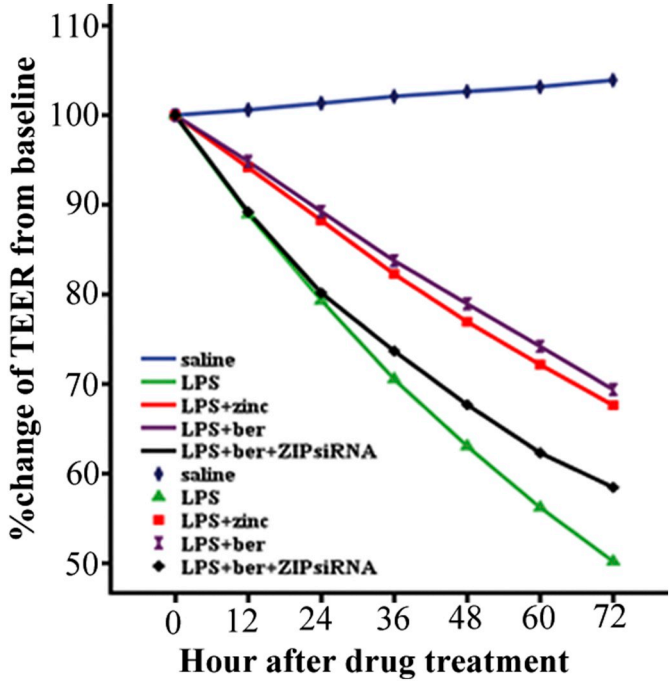

Fig. 5. The alteration of TEER of Caco-2 cells treated with saline, 500 ng/ml LPS, 500 ng/ml LPS plus 100 μM zinc gluconate, 500 ng/ml LPS plus 20 μM berberine, and 500 ng/ml LPS plus 20 μM berberine plus 100 pmol ZIP14 siRNA (LPS + ber + ZIPsiRNA), respectively.

berberine alleviated LPS-inhibited *Zip14* gene transcription. However, this action of berberine was abolished by AG1024. In vitro (Fig. 7), ZIP14 siRNA and LPS were found to reduce ZIP14 protein expression in Caco-2 cells. In contrast, IGF-I and berberine increased LPS-down-regulated in ZIP14 protein generation.

3.9. Berberine increased while ZIP14 siRNA reduced claudin-1 and occludin expression in Caco-2 cells

Intestinal mucosal hyperpermeability is related to decreased TJ protein expression. In Fig. 8, LPS significantly repressed claudin-1 and occludin protein synthesis in Caco-2 cells. Berberine treatment up-regulated LPS-decreased claudin-1 and occludin generation. Cotreatment with ZIP14 siRNA and berberine caused a downregulation of TJ proteins, suggesting ZIP14-zinc axis was involved in berberine-induced TJ protein expression.

4. Discussion

Zinc, the second most prevalent trace element in the body, participates in modulating host innate and adaptive immune response [3,25]. Alterated level of zinc in tissues might serve as one part of host's defense mechanism against pathogens. Besides, zinc is actively involved in regulating systemic inflammation [14]. In sepsis, however, zinc deficiency is prevalent [3] and one of the mechanisms is sepsis elicits zinc redistribution including zinc accumulation in the metabolically and immunologically active organs such as the liver and lymphatic nodes [3,26]. Zinc deficiency contributes to proinflammatory reaction of sepsis [14] because zinc is a component of pathogen-eliminating signal transduction pathways causing neutrophil extracellular traps formation, as well as inducing cell-mediated immunity over humoral immunity via the regulation of specific differentiation factors. Thus, zinc deficiency damages host tissue, increases organ damage and mortality [25,27]. In a model of endotoxemia, zinc is deficient in the gut [5], which plays a role in bacterial translocation and toxin dissemination [25]. Zinc supplementation reduces the severity of sepsis [27,28]. Therefore, the modulatin of zinc redistribution in the body might serve as a potential therapy target for sepsis [14].

Zinc deficiency inhibits the gene expression of IGF-I [10,29]. IGF-I immunoreactivities is moderate in enterocytes. IGF-I and IGF−IR are primarily localized on the surface of enterocytes [30,31]. IGF-I is positively involved in the maintenance of intestinal mucosal barrier [32,33]. Recently, berberine has been reported to stimulate enterocyte to express IGF-I [8]. So, we in this study speculated berberine might influence zinc redistribution to protect intestinal mucosal barrier via the activation of the IGF-I signaling during sepsis.

It is reported that repeated i.p. injections of 4 mg/kg/day zinc gluconate to adult rats causes clinical adverse effects in a few days. In

Table 1
Measurement of TEER of Caco-2 cells with different treatments.

Group	TEER ($\Omega \cdot cm^2$)						
	0 h	12 h	24 h	36 h	48 h	60 h	72 h
I	833.79 ± 31.15	838.67 ± 34.16	844.91 ± 33.46	851.27 ± 33.50	855.79 ± 33.54	860.19 ± 35.15	866.40 ± 33.16
II	837.07 ± 33.86	744.57 ± 31.01	664.05 ± 28.62	590.32 ± 31.10	527.80 ± 27.75	470.40 ± 28.70	420.19 ± 26.27
III	839.14 ± 38.54	790.15 ± 31.71	740.56 ± 27.65	690.32 ± 24.88	645.62 ± 26.69	605.34 ± 23.26	567.57 ± 25.93
IV	836.44 ± 36.54	793.16 ± 34.90	746.42 ± 28.69	700.49 ± 28.15	660.30 ± 27.60	620.61 ± 27.95	580.34 ± 24.55
V	835.78 ± 29.90	745.33 ± 33.70	670.00 ± 27.65	615.69 ± 25.18	565.70 ± 26.80	520.81 ± 25.31	488.64 ± 22.62

Note: I: saline; II: LPS; III: LPS plus zinc gluconate; IV: LPS plus berberine hydrochloride; V: LPS plus berberine hydrochloride plus ZIP14 siRNA.

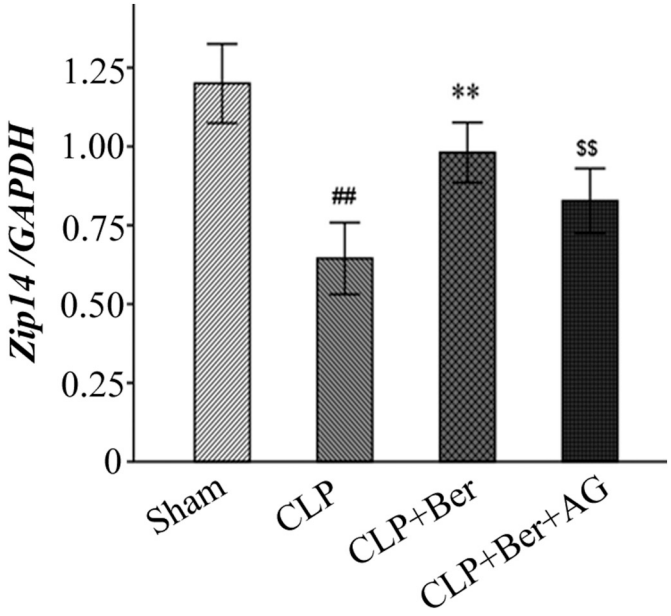

Fig. 6. The expression of *Zip14* mRNA in mucosa of rats subjected to sham, CLP, CLP plus berberine, and CLP plus berberine plus AG1024, respectively. Columns show means ± SD. $^{\#\#}P < 0.01$ vs. sham; $^{**}P < 0.01$ vs. CLP; $^{\$\$}P < 0.01$ vs. CLP + Ber, respectively.

contrast, no serious adverse effects in one week are observed in rats injected with 1 or 2 mg/kg/day of zinc gluconate [21]. So, in this experiment zinc gluconate was i.p. injected at the dose of 2 mg/kg/day. As expected, CLP induced mid-grade sepsis, as demonstrated by the marked reduction of survival rate, in consistent with previous findings [19]. Zinc gluconate pretreatment to CLP rats notably increased survival rate and potential mechanism is that zinc supplementation improves bacterial clearance [34]. Cotreatment with AG1024 and berberine lowered survival rate of septic CLP rats, indicating IGF-I signaling is activated by berberine [8]. Therefore, IGF-I contributing to bacterial clearance [35] is likely associated with zinc redistribution.

Intestinal mucosal disruption means mucosal hyperpermeability to enteric endotoxin. Our model of mid-grade sepsis induced gut hyperpermeability, as shown by increased plasma endotoxin concentration. Pretreatment with berberine or zinc gluconate to CLP rats reduced plasma endotoxin level in line with previous studies [27,36], indicative of the reduction of intestinal mucosal permeability and the possible mechanism is berberine and zinc increase epithelial tight junction barrier [37,38]. In contrast, AG1024 pre- treatment abrogated the effect of berberine on gut permeability, indicating this alkaloid decreases gut hyperpermeability in part via the activation of the IGF-I signaling.

In order to further confirm intestinal barrier dysfunction caused by CLP, we detected gut mucosal permeability to FITC-dextran [5]. The findings showed plasma FITC-dextran level in CLP rats was much higher than that in sham matches in consistent with previous studies [39]. Pretreatment with zinc gluconate or berberine to CLP rats reduced intestinal mucosal permeability to FITC-dextran. Cotreatment with berberine and AG1024 resulted in the increase in plasma FITC-dextran. In other words, IGF-I is involved in berberine-mediated intestinal barrier protection.

An internal redistribution of zinc can be induced by LPS and other inflammatory cytokines (e.g., TNFα, IL-6, IL-1β). For instance, in sepsis zinc accumulates in the lung, kidney and liver [40], while decreases in the plasma and gut [3,5]. Our study showed a transient zinc redistribution in the body, as demonstrated by the phenomenon that zinc level in plasma rapidly reduced in postoperative 24 h and then slowly increased in post-operative 24–48 h [5,41]. Berberine pretreatment also exerts a regulatory action on zinc redistribution in sepsis leading to raised plasma zinc content and zinc accumulation in the gut, but the effect of berberine on zinc redistribution could be abolished by AG1024. In vitro, LPS reduced Zn^{2+} transfer into Caco-2 exposed to zinc gluconate, while IGF-I and berberine facilitated to Zn^{2+} transfer into Caco-2. These in vivo and *vitro* studies indicated berberine affected zinc redistribution partially via activation of IGF-I signaling.

ZIP14 is actively involved in endosomal trafficking of zinc and hence is deemed as a target for sepsis therapy [14]. In sepsis, the reduction of ZIP14 expression in the gut is fully opposite from the increase in ZIP14 generation in the liver [5]. This phenomenon strongly hints different mechanisms by which to modulate ZIP14 in varied tissues and organs. We in vivo showed sepsis repressed ZIP14 mRNA production while berberine upregulated ZIP14 mRNA expression in the gut mucosa. AG1024 abolished the induction of berberine. In vitro, IGF-I and berberine also exhibit an inducible effect on ZIP14 protein synthesis in LPS-treated Caco-2. Some transcription factors such as AP-1, ATF4 and ATF6α are involved in modulating ZIP14 expression [12], while IGF-I influences the expressions of them [42,43].

Claudin-1, claudin-3, ZO-1 and occludin participate in the maintenance of intestinal mucosal integrity [5,6,13]. Studies have suggested zinc and berberine induce TJ protein production [8,13]. Our findings showed LPS strongly inhibited claudin-1 and occludin expression. In LPS-treated Cac-2 cells, berberine treatment upregulated claudin-1 and occludin. The upregulatory effect of berberine on TJ protein production

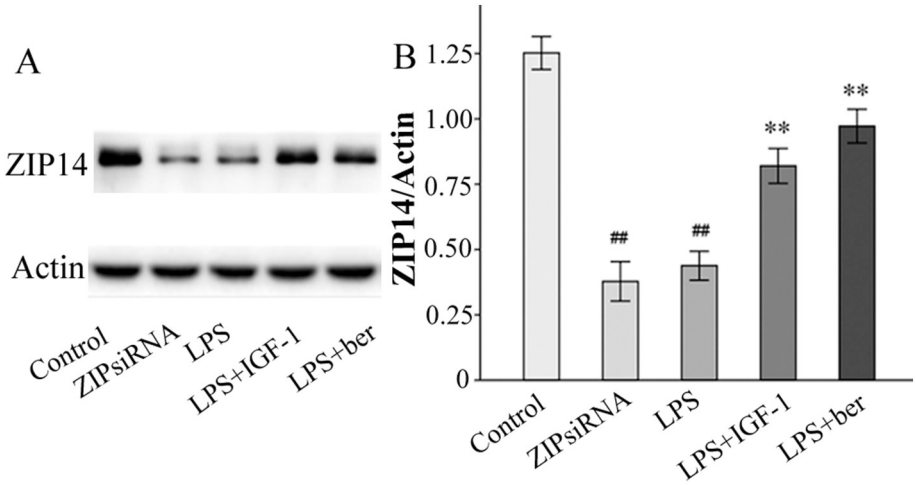

Fig. 7. The expression of ZIP14 protein in Caco-2 cells treated with saline, 100 pmol ZIP14 siRNA, 500 ng/ml LPS, 500 ng/ml LPS plus 200 ng/ml IGF-I, and 500 ng/ml LPS plus 20 μM berberine, respectively. Columns show means ± SD. $^{\#\#}P < 0.01$ vs. saline or LPS; $^{**}P < 0.01$ vs. LPS, respectively.

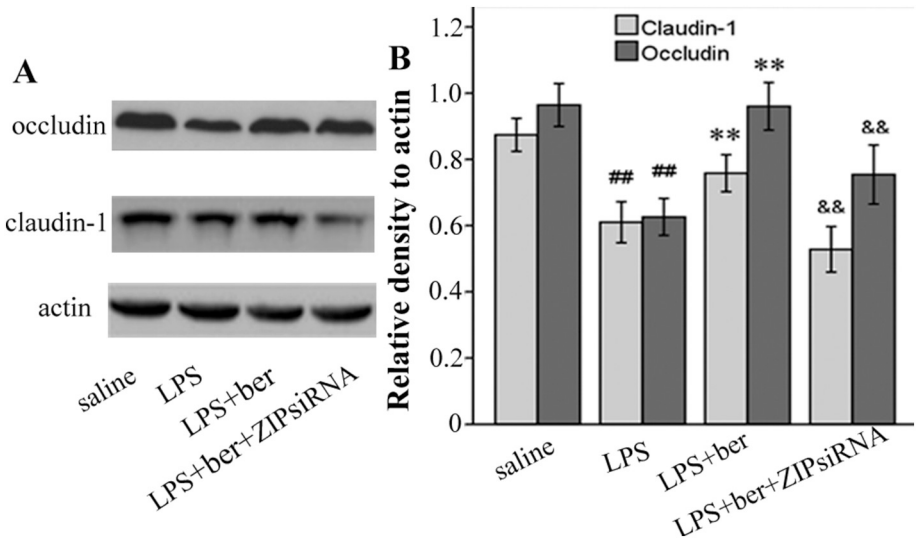

Fig. 8. The expression of occludin and claudin-1 protein in Caco-2 cells treated with saline, 500 ng/ml LPS, 500 ng/ml LPS plus 20 μM berberine, and 500 ng/ml LPS plus 20 μM berberine plus 100 pmol ZIP14 siRNA, respectively. Columns show means ± SD. $^{\#\#}P < 0.01$ vs. saline; $^{**}P < 0.01$ vs. LPS; $^{\&\&}P < 0.01$ vs. LPS + ber, respectively.

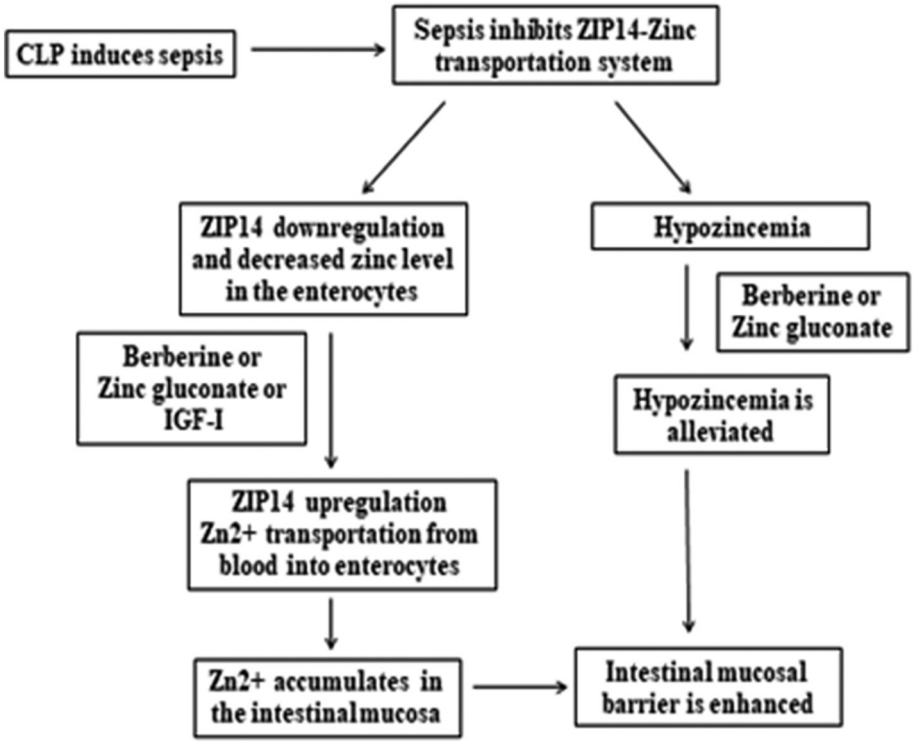

Fig. 9. An illustration is indicated to summarize the suggestive mechanisms of berberine on gut mucosal barrier protection.

was abolished by ZIP14 siRNA. Meanwhile, berberine-raised TEER, an important parameter of TJ integrity, was also decreased by ZIP14 siRNA. Accordingly, berberine activated ZIP14-zinc axis to increase intestinal tight junction structure.

Several animal models have shown supplemental zinc reduces severity of sepsis [14,27]. In neonate patients with sepsis, some trials show a beneficial effect of zinc in the form of a lower mortality rate and a better neurological development [44–46]. However, it was also reported zinc supplementation did not lead to any significant differences between zinc group and control group [47] or even showed a higher temperature to patient during acute phase response [48]. So, more clinical trials are needed to explore proper time and dose of zinc or berberine supplementation to patients who are in a state of sepsis.

5. Conclusions

The present data indicate in sepsis berberine may ameliorate hypozincemia and induce ZIP14 expression and then affect zinc redistribution in the intestinal mucosa to protect gut barrier in part via the activation of the IGF-I pathway (Fig. 9).

Declaration of Competing Interest

The authors of this manuscript declare that there are no conflicts of interest.

Acknowledgements

The authors thank Associated Professor Guangrong Zhou for his

revising this manuscript critically for intellectual content.

References

[1] R. Okumura, K. Takeda, Maintenance of intestinal homeostasis by mucosal barriers, Inflamm. Regen. 38 (2018) 5.
[2] M. Meng, N.J. Klingensmith, C.M. Coopersmith, New insights into the gut as the driver of critical illness and organ failure, Curr. Opin. Crit. Care 23 (2) (2017) 143–148.
[3] W. Alker, H. Haase, Zinc and sepsis, Nutrients 10 (8) (2018).
[4] J. Hoeger, T.P. Simon, T. Beeker, G. Marx, H. Haase, T. Schuerholz, Persistent low serum zinc is associated with recurrent sepsis in critically ill patients - a pilot study, PLoS One 12 (5) (2017) e0176069.
[5] G.J. Guthrie, T.B. Aydemir, C. Troche, A.B. Martin, S.M. Chang, R.J. Cousins, Influence of ZIP14 (slc39A14) on intestinal zinc processing and barrier function, Am. J. Physiol. Gastrointest. Liver Physiol. 308 (3) (2015) G171–G178.
[6] W. Zhong, Q. Li, Q. Sun, W. Zhang, J. Zhang, X. Sun, X. Yin, X. Zhang, Z. Zhou, Preventing gut leakiness and endotoxemia contributes to the protective effect of zinc on alcohol-induced steatohepatitis in rats, J. Nutr. 145 (12) (2015) 2690–2698.
[7] W. Zhong, C.J. McClain, M. Cave, Y.J. Kang, Z. Zhou, The role of zinc deficiency in alcohol-induced intestinal barrier dysfunction, Am. J. Physiol. Gastrointest. Liver Physiol. 298 (5) (2010) G625–G633.
[8] Y. He, X. Yuan, G. Zhou, A. Feng, Activation of IGF-1/IGFBP-3 signaling by berberine improves intestinal mucosal barrier of rats with acute endotoxemia, Fitoterapia 124 (2018) 200–205.
[9] K. Chen, T. Okuma, K. Okamura, Y. Tabira, H. Kaneko, Y. Miyauchi, Insulin-like growth factor-I prevents gut atrophy and maintains intestinal integrity in septic rats, JPEN J. Parenter. Enteral Nutr. 19 (2) (1995) 119–124.
[10] S. Muszynski, E. Tomaszewska, M. Kwiecien, P. Dobrowolski, A. Tomczyk-Warunek, Subsequent somatic axis and bone tissue metabolism responses to a low-zinc diet with or without phytase inclusion in broiler chickens, PLoS One 13 (1) (2018) e0191964.
[11] X. Kang, Z. Song, C.J. McClain, Y.J. Kang, Z. Zhou, Zinc supplementation enhances hepatic regeneration by preserving hepatocyte nuclear factor-4alpha in mice subjected to long-term ethanol administration, Am. J. Pathol. 172 (4) (2008) 916–925.
[12] T.B. Aydemir, R.J. Cousins, The multiple faces of the metal transporter ZIP14 (SLC39A14), J. Nutr. 148 (2) (2018) 174–184.
[13] Y. Miyoshi, S. Tanabe, T. Suzuki, Cellular zinc is required for intestinal epithelial barrier maintenance via the regulation of claudin-3 and occludin expression, Am. J. Physiol. Gastrointest. Liver Physiol. 311 (1) (2016) G105–G116.
[14] I. Wessels, R.J. Cousins, Zinc dyshomeostasis during polymicrobial sepsis in mice involves zinc transporter Zip14 and can be overcome by zinc supplementation, Am. J. Physiol. Gastrointest. Liver Physiol. 309 (9) (2015) G768–G778.
[15] C. Chen, Z. Yu, Y. Li, J. Fichna, M. Storr, Effects of berberine in the gastrointestinal tract - a review of actions and therapeutic implications, Am. J. Chin. Med. 42 (5) (2014) 1053–1070.
[16] A.W. Feng, W. Gao, G.R. Zhou, R. Yu, N. Li, X.L. Huang, Q.R. Li, J.S. Li, Berberine ameliorates COX-2 expression in rat small intestinal mucosa partially through PPARgamma pathway during acute endotoxemia, Int. Immunopharmacol. 12 (1) (2012) 182–188.
[17] A.W. Feng, C. Yu, Q. Mao, N. Li, Q.R. Li, J.S. Li, Berberine hydrochloride attenuates cyclooxygenase-2 expression in rat small intestinal mucosa during acute endotoxemia, Fitoterapia 82 (7) (2011) 976–982.
[18] X.T. Yu, Y.F. Xu, Y.F. Huang, C. Qu, L.Q. Xu, Z.R. Su, H.F. Zeng, L. Zheng, T.G. Yi, H.L. Li, et al., Berberrubine attenuates mucosal lesions and inflammation in dextran sodium sulfate-induced colitis in mice, PLoS One 13 (3) (2018) e0194069.
[19] D. Rittirsch, M.S. Huber-Lang, M.A. Flierl, P.A. Ward, Immunodesign of experimental sepsis by cecal ligation and puncture, Nat. Protoc. 4 (1) (2009) 31–36.
[20] S. Slinko, G. Piraino, P.W. Hake, J.R. Ledford, M. O'Connor, P. Lahni, P.D. Solan, H.R. Wong, B. Zingarelli, Combined zinc supplementation with proinsulin C-peptide treatment decreases the inflammatory response and mortality in murine polymicrobial sepsis, Shock 41 (4) (2014) 292–300.
[21] M. Andriollo-Sanchez, R. Claeyssen, J. Arnaud, L. Touvard, J. Denis, Y. Chancerelle, A.M. Roussel, D. Agay, Toxic effects of iterative intraperitoneal administration of zinc gluconate in rats, Basic Clin. Pharmacol. Toxicol. 103 (3) (2008) 267–272.
[22] R. Tocco-Bradley, M.J. Kluger, Zinc concentration and survival in rats infected with Salmonella typhimurium, Infect. Immun. 45 (2) (1984) 332–338.
[23] T. Lea, Caco-2 Cell Line, (2015), pp. 103–111.
[24] N. Zhao, J. Gao, C.A. Enns, M.D. Knutson, ZRT/IRT-like protein 14 (ZIP14) promotes the cellular assimilation of iron from transferrin, J. Biol. Chem. 285 (42) (2010) 32141–32150.
[25] N.Z. Gammoh, L. Rink, Zinc in infection and inflammation, Nutrients 9 (6) (2017).
[26] K.T. Crowell, B.E. Phillips, S.L. Kelleher, D.I. Soybel, C.H. Lang, Immune and metabolic responses in early and late sepsis during mild dietary zinc restriction, J. Surg. Res. 210 (2017) 47–58.
[27] D.L. Knoell, M.W. Julian, S. Bao, B. Besecker, J.E. Macre, G.D. Leikauf, R.A. DiSilvestro, E.D. Crouser, Zinc deficiency increases organ damage and mortality in a murine model of polymicrobial sepsis, Crit. Care Med. 37 (4) (2009) 1380–1388.
[28] S. Bao, M.J. Liu, B. Lee, B. Besecker, J.P. Lai, D.C. Guttridge, D.L. Knoell, Zinc modulates the innate immune response in vivo to polymicrobial sepsis through regulation of NF-kappaB, Am. J. Phys. Lung Cell. Mol. Phys. 298 (6) (2010) L744–L754.
[29] V.M. Blanco-Alvarez, G. Soto-Rodriguez, J.A. Gonzalez-Barrios, D. Martinez-Fong, E. Brambila, M. Torres-Soto, A.K. Aguilar-Peralta, A. Gonzalez-Vazquez, C. Tomas-Sanchez, I.D. Limon, et al., Prophylactic subacute administration of zinc increases CCL2, CCR2, FGF2, and IGF-1 expression and prevents the long-term memory loss in a rat model of cerebral hypoxia-ischemia, Neural Plast. 2015 (2015) 375391.
[30] S. Baghdiguian, B. Verrier, C. Gerard, J. Fantini, Insulin like growth factor I is an autocrine regulator of human colon cancer cell differentiation and growth, Cancer Lett. 62 (1) (1992) 23–33.
[31] A. Hakam, T.J. Yeatman, L. Lu, L. Mora, G. Marcet, S.V. Nicosia, R.C. Karl, D. Coppola, Expression of insulin-like growth factor-1 receptor in human colorectal cancer, Hum. Pathol. 30 (10) (1999) 1128–1133.
[32] C.D. Scopa, S. Koureleas, A.C. Tsamandas, I. Spiliopoulou, T. Alexandrides, K.S. Filos, C.E. Vagianos, Beneficial effects of growth hormone and insulin-like growth factor I on intestinal bacterial translocation, endotoxemia, and apoptosis in experimentally jaundiced rats, J. Am. Coll. Surg. 190 (4) (2000) 423–431.
[33] R. Fukushima, H. Saito, T. Inoue, K. Fukatsu, T. Inaba, I. Han, S. Furukawa, M.T. Lin, T. Muto, Prophylactic treatment with growth hormone and insulin-like growth factor I improve systemic bacterial clearance and survival in a murine model of burn-induced gut-derived sepsis, Burns 25 (5) (1999) 425–430.
[34] H.A. Ganatra, B.M. Varisco, K. Harmon, P. Lahni, A. Opoka, H.R. Wong, Zinc supplementation leads to immune modulation and improved survival in a juvenile model of murine sepsis, Innate Immun. 23 (1) (2017) 67–76.
[35] A. Ashare, A.B. Nymon, K.C. Doerschug, J.M. Morrison, M.M. Monick, G.W. Hunninghake, Insulin-like growth factor-1 improves survival in sepsis via enhanced hepatic bacterial clearance, Am. J. Respir. Crit. Care Med. 178 (2) (2008) 149–157.
[36] L. Niu, W. Qiao, Z. Hu, N. Li, Q. Huang, J. Gong, Q. Li, W. Zhu, J. Li, Berberine attenuates lipopolysaccharide-induced impairments of intestinal glutamine transport and glutaminase activity in rat, Fitoterapia 82 (3) (2011) 323–330.
[37] M. Chu, R. Ding, Z.Y. Chu, M.B. Zhang, X.Y. Liu, S.H. Xie, Y.J. Zhai, Y.D. Wang, Role of berberine in anti-bacterial as a high-affinity LPS antagonist binding to TLR4/MD-2 receptor, BMC Complement. Altern. Med. 14 (2014) 89.
[38] J.C. Lambert, Z. Zhou, L. Wang, Z. Song, C.J. McClain, Y.J. Kang, Preservation of intestinal structural integrity by zinc is independent of metallothionein in alcohol-intoxicated mice, Am. J. Pathol. 164 (6) (2004) 1959–1966.
[39] B.P. Yoseph, N.J. Klingensmith, Z. Liang, E.R. Breed, E.M. Burd, R. Mittal, J.A. Dominguez, B. Petrie, M.L. Ford, C.M. Coopersmith, Mechanisms of intestinal barrier dysfunction in sepsis, Shock 46 (1) (2016) 52–59.
[40] S. Abe, M. Matsumi, M. Tsukioki, S. Mizukawa, T. Takahashi, Y. Iijima, Y. Itano, F. Kosaka, Metallothionein and zinc metabolism in endotoxin shock rats, Experientia Suppl. 52 (1987) 587–594.
[41] R.S. Pekarek, W.R. Beisel, Effect of endotoxin on serum zinc concentrations in the rat, Appl. Microbiol. 18 (3) (1969) 482–484.
[42] S. Cascio, V. Bartella, C. Garofalo, A. Russo, A. Giordano, E. Surmacz, Insulin-like growth factor 1 differentially regulates estrogen receptor-dependent transcription at estrogen response element and AP-1 sites in breast cancer cells, J. Biol. Chem. 282 (6) (2007) 3498–3506.
[43] J.M. Rhoads, R.A. Argenzio, W. Chen, R.A. Rippe, J.K. Westwick, A.D. Cox, H.M. Berschneider, D.A. Brenner, L-glutamine stimulates intestinal cell proliferation and activates mitogen-activated protein kinases, Am. J. Phys. 272 (5) (1997) G943–G953 Pt 1.
[44] N. Banupriya, B. Vishnu Bhat, B.D. Benet, M.G. Sridhar, S.C. Parija, Efficacy of zinc supplementation on serum calprotectin, inflammatory cytokines and outcome in neonatal sepsis - a randomized controlled trial, J. Matern. Fetal Neonatal Med. 30 (13) (2017) 1627–1631.
[45] B. Newton, B.V. Bhat, B.B. Dhas, N. Mondal, S.M. Gopalakrishna, Effect of zinc supplementation on early outcome of neonatal sepsis—a randomized controlled trial, Indian J. Pediatr. 83 (4) (2016) 289–293.
[46] N. Banupriya, B.V. Bhat, B.D. Benet, C. Catherine, M.G. Sridhar, S.C. Parija, Short term oral zinc supplementation among babies with neonatal sepsis for reducing mortality and improving outcome - a double-blind randomized controlled trial, Indian J. Pediatr. 85 (1) (2018) 5–9.
[47] K. Mehta, N.K. Bhatta, S. Majhi, M.K. Shrivastava, R.R. Singh, Oral zinc supplementation for reducing mortality in probable neonatal sepsis: a double blind randomized placebo controlled trial, Indian Pediatr. 50 (4) (2013) 390–393.
[48] C.L. Braunschweig, M. Sowers, D.S. Kovacevich, G.M. Hill, D.A. August, Parenteral zinc supplementation in adult humans during the acute phase response increases the febrile response, J. Nutr. 127 (1) (1997) 70–74.