

## Original Article

# Glycyrrhizin down-regulates CCL2 and CXCL2 expression in cerulein-stimulated pancreatic acinar cells

Yaser Panahi<sup>1</sup>, Shohreh Fakhari<sup>1</sup>, Mehdi Mohammadi<sup>1</sup>, Mohammad Reza Rahmani<sup>1</sup>, Mohammad Saeid Hakhamaneshi<sup>2</sup>, Ali Jalili<sup>1,2</sup>

<sup>1</sup>Department of Immunology and Hematology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran; <sup>2</sup>Liver & Digestive Research Center, Kurdistan University of Medical Sciences, Sanandaj, Iran

Received December 29, 2014; Accepted May 3, 2015; Epub July 5, 2015; Published July 15, 2015

**Abstract:** Many inflammatory chemokines release from leukocytes and pancreatic acinar cells which play important roles in pathophysiology of acute pancreatitis (AP). Of interests, CXCL2 and CCL2 have been shown elevated in the plasma of patients with AP. We have recently found that Glycyrrhizin (GZ) attenuates AP in mice model. In this study, we aimed to investigate the direct effect of GZ on expression levels of CCL2 and CXCL2 in isolated pancreatic acinar cells. Isolated acinar cells were isolated from the pancreas of healthy C57BL/6mice, stimulated with cerulein ( $10^{-7}$  M) and then treated with either PBS or different doses of GZ. The levels of CCL2 and CXCL2 expression at mRNA were assessed by qRT-PCR. Conditioned media from supernatants of each cells culture condition were collected for detection of CCL2 and CXCL2 levels by ELISA. First, we observed that cerulein significantly upregulates both cytokines expression in acinar cells. Moreover, we treated the acinar cells with GZ and found that GZ significantly down-regulates CCL2 and CXCL2 expression at mRNA levels in a dose-dependent manner. Consistently, the conditioned media of GZ-treated cells contained a significant lower levels of CCL2 and CXCL2 ( $p < 0.05$ ). In conclusion, our data demonstrate for the first time that GZ directly downregulates CCL2 and CXCL2 levels in cerulein-stimulated acinar cells which may explain the mechanism of therapeutic effects of GZ in cerulein-induced AP in mice.

**Keywords:** Cerulein-induced acute pancreatitis, glycyrrhizin, CCL2, CXCL2

## Introduction

Acute pancreatitis (AP) is an inflammatory disease characterized by trypsinogen activation and infiltration of leukocytes into the pancreas which results in acinar cells damage, interstitial edema and hemorrhage [1]. Acute inflammation is the hallmark of AP and the data from animal studies have clearly shown that migration and accumulation of myeloid cells particularly monocytes and neutrophils and subsequently their activation play a crucial role in pathobiology of AP [2-4]. Mounting evidence indicate that pro-inflammatory mediators are involved in pathophysiology of AP, as many of them such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukins 6 (IL-6), IL-8, monocyte chemoattractant protein-1 (MCP-1/CCL2) and macrophage inhibitory protein-2 (MIP-2/CXCL2) have been shown elevated in the plasma of patients with AP [5-8].

In addition to inflammatory cells, recent studies demonstrated that pancreatic acinar cells release many inflammatory mediators including chemokines and oxygen free radicals. CCL2 and CXCL2 have been shown to be produced by acinar cells during early stage of AP. AS monocytes express the cognate receptor of CCL2, CCR2 and migrate towards a higher concentration of this chemokine, a high levels of secreted CCL2 in the inflamed pancreases tissue may chemoattract monocytes from circulation into pancreas [9, 10]. Moreover, it has been previously reported that CXCL2/CXCR2 axis may also be an important regulator of neutrophil trafficking in the pancreas [11]. Collectively, indicating that both CCL2 and CXCL2 may participate as early triggers for inducing inflammatory cascade in AP.

The root of *Glycyrrhiza glabra* (licorice) is widely used as a traditional medicine in Chinese and

## Glycyrrhizin reduces CCL2 and CXCL2

**Table 1.** Primer sequences for qRT-PCR

Product (bp)	Sequence	Primer
117	Sense: TCAGCCAGATGCAGTTAACG Antisense: TCTTTGGGACACCTGCTGC	CCL2
151	Sense: TCATAGCCACTCTCAAGGG Antisense: TTGGTTCTTCCGTTGAGGG	CXCL2
96	Sense: CTTGGGTATGGAATCCTGTG Antisense: ACTGTGTTGGCATAGAGGTC	$\beta$ -actin

Persian herbal medicine [12] Glycyrrhizin (GZ) is one of derivatives of licorice and has been used as sweetener agent in Europe and Asia for many years. GZ have been shown to exhibit many pharmacological effects such as anti-inflammatory, anti-oxidative and hepatoprotective effects [13, 14]. Accordingly, we have recently shown that GZ attenuates neutrophil and monocytes accumulation in pancreas and reduces tissue injury in an animal model of AP [4]. However, that its exact mechanism has not been yet elucidated. In the current study we aimed to examine the possible effect of GZ on CCL2 and CXCL2 expression in cerulein-stimulated acinar cells.

### Methods and material

#### *Isolation of pancreatic acinar cells*

Pancreatic acinar cells were isolated from mice by enzymatic digestion as previously reported [15, 16]. The acinar cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Lonza, Walkersville, MD, USA), 0.1 mg/ml soybean trypsin inhibitor (Sigma Chemical Company, St. Louis, Missouri), and 1% penicillin-streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. The cell viability was determined by the trypan blue dye exclusion test; more than 95% of the cells survived after 24 h culture.

#### *Cell culture*

Firstly acinar cells were stimulated with or without cerulein (10<sup>-7</sup> M) (Sigma Chemical, St. Louis, MO) and incubated at 37°C for three hours. To see the possible effect of GZ on expression of CCL2 and CXCL2, various concentrations of GZ (Sigma) were added to the cerulein-stimulated acinar cells. Following three and eight hours of incubations cells were harvested and used for and qRT-PCR and ELISA assays, respectively.

#### *RT-PCR and qRT-PCR*

Expression of mRNA for CCL2 and CXCL2 was evaluated in acinar cells. RNA was isolated using RNA extraction kit (Bioflux, Basel, Switzerland) and RNA was transcribed into cDNA using of Bioneer kit (Bioneer, Daejeon, South Korea). Reverse transcription-polymerase chain reaction (RT-PCR) procedures were carried out using primer (designed by Gene Runner software) sequences as listed in **Table 1**. Amplification was performed with a thermocycler (Mastercycler, Eppendorf, Westbury, NY) and the PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide. PCR products were visualized by gel document system.

Real Time quantitative Reverse Transcription PCR (Real-Time qRT-PCR) was performed in a sequence detection system (RotorGene™ 6000, Corbett Life Science, Sydney, Australia) and carried out using SYBR Green dye detection protocol. Relative quantitation of CCL2 and CXCL2 mRNA expression was calculated using the comparative CT method [17]. The relative quantitation value of mRNA expression for CXCL2 and CCL2 was normalized to an endogenous control  $\beta$ -actin gene.

#### *ELISA assay*

The concentration of CCL2 and CXCL2 levels were examined using ELISA kits according to the manufacturer instruction (R & D, Minneapolis, MN). Supernatants were collected 8h after incubating with various doses of GZ, centrifuged at 11000 rpm for 10 min to discard cell debris, and stored at -80°C until assayed.

#### *Statistical analysis*

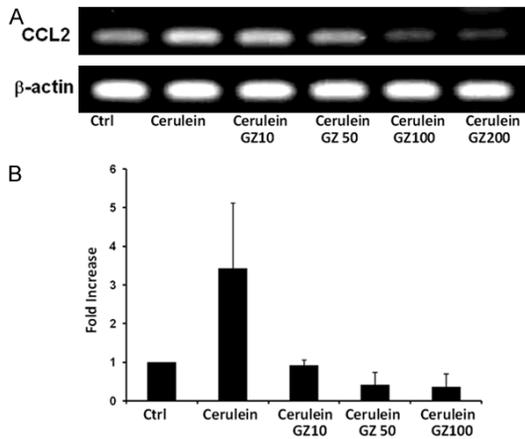
Arithmetic means and standard deviations were calculated and statistical significance was defined as  $p \leq 0.05$  using Student's *t* test.

### Results

#### *Effect of GZ on CCL2 expression in acinar cells*

First, to examine the effect of cerulein on CCL2 expression, isolated acinar cells were stimulated with 10<sup>-7</sup> M of cerulein for 3 hours and CCL2 expression in these was detected by conventional RT-PCR and qRT-PCR analysis. Our RT-PCR data show that CCL2 is expressed at a

## Glycyrrhizin reduces CCL2 and CXCL2

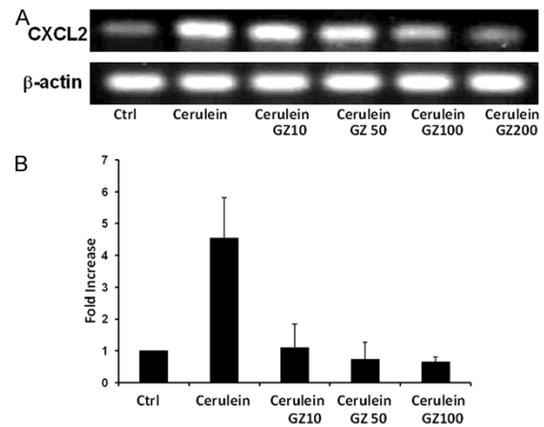


**Figure 1.** Effect of GZ on CCL2 expression in acinar cells. Acinar cells were isolated as described in “Material & Methods”. Cells were incubated with various concentrations of GZ for 3 h, then harvested and total mRNA was extracted, then mRNA was isolated and CCL2 expression was examined by qRT-PCR. A. RT-PCR analysis of mRNA transcripts for CCL2 in acinar cells.  $\beta$ -actin was used as internal loading control for the RT-PCR. B. qRT-PCR shows that CCL2 is significantly increased in cerulein-stimulated cells and GZ decreased it. CCL2 expression was normalized to an endogenous control  $\beta$ -actin gene. Data are represented of Mean  $\pm$  SEM of three independent experiments.  $P < 0.05$ .

low level in acinar cells, but it significantly upregulated by stimulating with cerulein. Interestingly, this stimulatory effect of cerulein on CCL2 expression was significantly decreased when acinar cells were incubated with various concentrations of GZ (**Figure 1A**). When we quantified the levels of CCL2, we observed that cerulein stimulation leads to  $3.43 \pm 1.6$  fold upregulation of CCL2 in these cells. In addition, GZ remarkably reduces CCL2 expression in a dose-dependent manner in cerulein-stimulated acinar cells (**Figure 1B**).

### Effect of GZ on CXCL2 expression in acinar cells

Next, acinar cells were incubated with cerulein and the level of CXCL2 was examined by both by conventional RT-PCR and qRT-PCR analysis. We observed that cerulein upregulates CXCL2 in acinar cells and GZ treatment downregulates it in a dose-dependent manner (**Figure 2A**). Our qRT-PCR data clearly show that cerulein enhances CXCL2 expression ( $4.5 \pm 1.27$ ) in acinar cells and that CXCL2 expression was significantly reduced when the cells were treated with



**Figure 2.** Effect of GZ on CXCL2 expression in acinar cells. A. RT-PCR analysis of mRNA transcripts for CXCL2 in acinar cells.  $\beta$ -actin was used as internal loading control for the RT-PCR. B. qRT-PCR shows that CXCL2 is significantly increased in cerulein-stimulated cells and GZ decreased it. CXCL2 expression was normalized to an endogenous control  $\beta$ -actin gene. Data are represented of Mean  $\pm$  SEM of three independent experiments.  $P < 0.05$ .

cerulein in the presence of various concentration of GZ (**Figure 2B**).

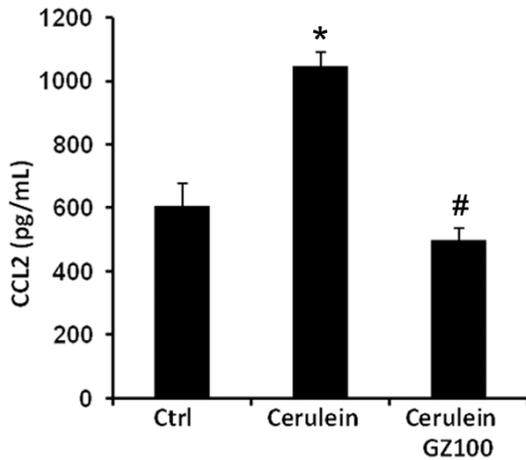
### GZ decreases CCL2 production by cerulein-stimulated acinar cells

In order to examine the effect of cerulein on CCL2 production by acinar cells, the cells were stimulated with cerulein for 8 h and then the conditioned media were collected and analyzed by Elisa. Our data clearly show that CCL2 is produced by acinar cells at a low levels ( $573 \text{ pg/mL} \pm 71$ ), but it significantly enhanced when the cells were stimulated with cerulein ( $1045 \text{ pg/mL} \pm 44$ ). However, when the cells were stimulated with cerulein in the presence of GZ, we found that GZ remarkably decreased CCL2 production by acinar cells ( $493 \text{ pg/mL} \pm 41$ ) (**Figure 3**), confirming our qRT-PCR data.

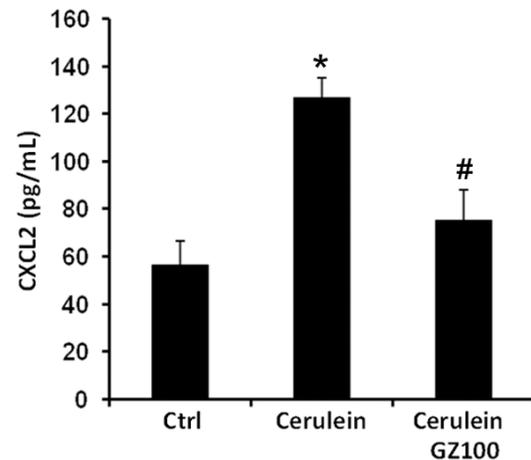
### Effect of GZ on secretion of CXCL2 from cerulein-stimulated acinar cells

Finally, as we exhibited that GZ reduces CXCL2 expression in cerulein-stimulated acinar cells at mRNA levels, we sought to determine its effect on CXCL2 expression at protein levels. As demonstrated in **Figure 4**, acinar cells produces a low level of CXCL2 ( $56 \text{ pg/mL} \pm 10$ ) and cerulein stimulation enhances it ( $126 \text{ pg/mL} \pm 8.4$ ). Consistently with our qRT-PCR, GZ treatment decreases CXCL2 secretion by ceru-

## Glycyrrhizin reduces CCL2 and CXCL2



**Figure 3.** GZ decreases CCL2 production by cerulein-stimulated acinar cells. Acinar cells were stimulated with cerulein either in the presence or absence of GZ for 8 h, then supernatants were then collected, centrifuged at 11000 rpm for 10 min to discard cell debris, and stored at  $-80^{\circ}\text{C}$  until assayed. CCL2 in the conditioned media of cells was detected according to manufacturer's instruction. Results correspond to the mean Mean  $\pm$  SEM of 3 independent experiments,  $P < 0.05$ . \* $P < 0.05$  compared with the ctrl; # $P < 0.05$  compared with the cerulein.



**Figure 4.** Effect of GZ on secretion of CXCL2 from cerulein-stimulated acinar cells. Acinar cells were stimulated with cerulein either in the presence or absence of GZ for 8 h, then supernatants were then collected, centrifuged at 11000 rpm for 10 min to discard cell debris, and stored at  $-80^{\circ}\text{C}$  until assayed. CXCL2 in the conditioned media of cells was detected according to manufacturer's instruction. Results correspond to the mean Mean  $\pm$  SEM of 3 independent experiments,  $P < 0.05$ . \* $P < 0.05$  compared with the ctrl; # $P < 0.05$  compared with the cerulein.

lein-stimulated acinar cells to the almost basal levels ( $75 \text{ pg/mL} \pm 13$ ).

### Discussion

AP has been regarded as an inflammatory disease and a numerous inflammatory cells and mediators have been documented to play crucial roles in the pathobiology of this disease. we have recently exhibited that GZ reduces the pathological alternations and the number of infiltrated myeloid cells into the pancreas tissue [4]. In the current study, we examined the effects of GZ on isolated pancreatic acinar cells and demonstrate that crulein upregulates both CXCL2 and CCL2 in acinar cells. However, GZ treatment downregulates them in a dose-dependent manner.

CCL2 has been shown to play in important role in migrating and trafficking of monocytes[10]. In addition, CCL2 is a key player in the pathogenesis of AP, and previous studies have comprehensively shown that inhibition of CCL2 by either a dominant-negative mutant CCL2 gene or its inhibitor reduces the histopathological features of AP [9, 18]. More importantly, clinical studies have revealed that serum levels of CCL2 is elevated in patients with AP [5].

Accordingly, we have recently reported that the serum levels of CCL2 is significantly increased in serum of mice with the cerulean-induced AP [4] indicating that CCL2 is an important player in pathobiology of AP. However, it precise mechanism is still not well understood. To seek the direct effect of GZ on CCL2 expression, acinar cells were stimulated with cerulein either in the presence or absence of GZ. Noteworthy, we observed that GZ reduces CCL2 at both mRNA and protein levels in acinar cells which may explain, at least partially, the mechanism of anti-inflammatory effects of GZ on AP. Consistently, previous in vitro studies have reported that GZ reduces CCL2 production by human monocytes [19] and granulocytes [20]. Since CCL2 is produced by other cells specially monocyte/macrophages [10], we have to consider that GZ might directly affect on monocyte/macrophages which need to be further elucidated.

Mounting evidence documented that neutrophils migrate and accumulate into pancreas tissue during AP leading to activating of many proenzymes and secreting of various inflammatory mediators [21, 22]. CXCL2 have been shown, through binding to its receptor CXCR2, to act as strong chemoattractant for neutrophils in mice

[23]. Consistently, migration of neutrophils into lung was significantly reduced in LPS-induced acute lung injury in CXCR2<sup>-/-</sup> mice [24] which is in the line with the previous findings from our laboratory showing that CXCL2 is significantly elevated in the serum of AP mice than the control [4]. Besides, we have recently reported that GZ administration leads to decreasing CXCL2 levels in mice with AP [4]. This intriguing observation promoted us to further study the direct role of GZ on CXCL2 expression in cerulein-stimulated acinar cells. Our data show that cerulein strongly upregulates CXCL2 at both mRNA and protein levels in acinar cells, indicating pancreatic acinar cells could be one of the main sources of CXCL2 secretion in AP. Hence, production of a high amount of CXCL2 may chemotactically attract neutrophils as the major inflammatory cells from circulation to inflamed tissue such as pancreas in AP [25, 26] which could at least partially explain our flow cytometry observations demonstrating that the number of neutrophils robustly increased in the pancreas of mice with AP in comparison with control group [4]. In the current study, we exhibit that GZ downregulates CXCL2 expression in cerulein-stimulated acinar cells. In line with our data, a recent study showed that GZ reduced IL-4 and IL-5 in the BAL of experimental allergic mice [27] indicating that GZ may possess an inhibitory activity on a wide range of inflammatory cytokines.

Taken together, we demonstrate cerulein stimulation leads to upregulation of both CCL2 and CXCL2 expression in pancreatic acinar cells and GZ inhibits those stimulatory effects of cerulein on CXCL2 and CCL2 expression. Thus, GZ might have a potential to inhibit inflammatory responses in patients with AP. However, further studies should be performed to investigate the precise mechanism by which GZ exerts anti-inflammatory effects in pancreatic acinar cells by determining the signaling pathways involved in downregulation of CXCL2 and CCL2.

### Acknowledgements

This work was supported by a grant for Thesis of YP who was an MSc student in Immunology at Kurdistan University of Medical Sciences.

### Disclosure of conflict of interest

The authors declare that they have no competing financial interest.

**Address correspondence to:** Dr. Ali Jalili, Department of Immunology & Hematology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanadaj, Iran. Tel: +98-871-6131290; E-mail: Ali130@gmail.com

### References

- [1] Sargent S. Pathophysiology, diagnosis and management of acute pancreatitis. *Br J Nurs* 2006; 15: 999-1005.
- [2] Sah RP, Garg P and Saluja AK. Pathogenic mechanisms of acute pancreatitis. *Curr Opin Gastroenterol* 2012; 28: 507-515.
- [3] Shanmugam MK and Bhatia M. The role of pro-inflammatory molecules and pharmacological agents in acute pancreatitis and sepsis. *Inflamm Allergy Drug Targets* 2010; 9: 20-31.
- [4] Fakhari S, Abdolmohammadi K, Panahi Y, Nikkhoo B, Peirmohammadi H, Rahmani MR, Moghadam AS and Jalili A. Glycyrrhizin attenuates tissue injury and reduces neutrophil accumulation in experimental acute pancreatitis. *Int J Clin Exp Pathol* 2013; 7: 101-109.
- [5] Rau B, Baumgart K, Kruger CM, Schilling M and Beger HG. CC-chemokine activation in acute pancreatitis: enhanced release of monocyte chemoattractant protein-1 in patients with local and systemic complications. *Intensive Care Med* 2003; 29: 622-629.
- [6] Gunjaca I, Zunic J, Gunjaca M and Kovac Z. Circulating cytokine levels in acute pancreatitis-model of SIRS/CARS can help in the clinical assessment of disease severity. *Inflammation* 2012; 35: 758-763.
- [7] Tu XH, Song JX, Xue XJ, Guo XW, Ma YX, Chen ZY, Zou ZD and Wang L. Role of bone marrow-derived mesenchymal stem cells in a rat model of severe acute pancreatitis. *World J Gastroenterol* 2012; 18: 2270-2279.
- [8] Makhija R and Kingsnorth AN. Cytokine storm in acute pancreatitis. *Journal of hepato-biliary-pancreatic surgery* 2002; 9: 401-410.
- [9] Bhatia M, Ramnath RD, Chevali L and Guglielmotti A. Treatment with bindarit, a blocker of MCP-1 synthesis, protects mice against acute pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2005; 288: 3.
- [10] Deshmane SL, Kremlev S, Amini S and Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res* 2009; 29: 313-326.
- [11] Awla D, Zetterqvist AV, Abdulla A, Camello C, Berglund LM, Spegel P, Pozo MJ, Camello PJ, Regner S, Gomez MF and Thorlacius H. NFATc3 regulates trypsinogen activation, neutrophil recruitment, and tissue damage in acute pancreatitis in mice. *Gastroenterology* 2012; 143: 1352-1360.

## Glycyrrhizin reduces CCL2 and CXCL2

- [12] Shibata S. A drug over the millennia: pharmacognosy, chemistry, and pharmacology of licorice. *Yakugaku Zasshi* 2000; 120: 849-862.
- [13] Ming LJ and Yin AC. Therapeutic effects of glycyrrhizic acid. *Nat Prod Commun* 2013; 8: 415-418.
- [14] Asl MN and Hosseinzadeh H. Review of pharmacological effects of *Glycyrrhiza* sp. and its bioactive compounds. *Phytother Res* 2008; 22: 709-724.
- [15] Kang M, Park KS, Seo JY and Kim H. Lycopene inhibits IL-6 expression in cerulein-stimulated pancreatic acinar cells. *Genes Nutr* 2011; 6: 117-123.
- [16] Yu JH, Lim JW, Namkung W, Kim H and Kim KH. Suppression of cerulein-induced cytokine expression by antioxidants in pancreatic acinar cells. *Lab Invest* 2002; 82: 1359-1368.
- [17] Schmittgen TD and Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008; 3: 1101-1108.
- [18] Ishibashi T, Zhao H, Kawabe K, Oono T, Egashira K, Suzuki K, Nawata H, Takayanagi R and Ito T. Blocking of monocyte chemoattractant protein-1 (MCP-1) activity attenuates the severity of acute pancreatitis in rats. *J Gastroenterol* 2008; 43: 79-85.
- [19] Takei M, Kobayashi M, Li XD, Pollard RB and Suzuki F. Glycyrrhizin inhibits R5 HIV replication in peripheral blood monocytes treated with 1-methyladenosine. *Pathobiology* 2005; 72: 117-123.
- [20] Takei M, Kobayashi M, Herndon DN, Pollard RB and Suzuki F. Glycyrrhizin inhibits the manifestations of anti-inflammatory responses that appear in association with systemic inflammatory response syndrome (SIRS)-like reactions. *Cytokine* 2006; 35: 295-301.
- [21] Abdulla A, Awla D, Thorlacius H and Regner S. Role of neutrophils in the activation of trypsinogen in severe acute pancreatitis. *J Leukoc Biol* 2011; 90: 975-982.
- [22] Awla D, Abdulla A, Syk I, Jeppsson B, Regner S and Thorlacius H. Neutrophil-derived matrix metalloproteinase-9 is a potent activator of trypsinogen in acinar cells in acute pancreatitis. *J Leukoc Biol* 2012; 91: 711-719.
- [23] Kielian T, Barry B and Hickey WF. CXC chemokine receptor-2 ligands are required for neutrophil-mediated host defense in experimental brain abscesses. *J Immunol* 2001; 166: 4634-4643.
- [24] Reutershan J, Morris MA, Burcin TL, Smith DF, Chang D, Saprito MS and Ley K. Critical role of endothelial CXCR2 in LPS-induced neutrophil migration into the lung. *J Clin Invest* 2006; 116: 695-702.
- [25] Day RB and Link DC. Regulation of neutrophil trafficking from the bone marrow. *Cell Mol Life Sci* 2012; 69: 1415-1423.
- [26] Kobayashi Y. The role of chemokines in neutrophil biology. *Front Biosci* 2008; 13: 2400-2407.
- [27] Ram A, Mabalirajan U, Das M, Bhattacharya I, Dinda AK, Gangal SV and Ghosh B. Glycyrrhizin alleviates experimental allergic asthma in mice. *Int Immunopharmacol* 2006; 6: 1468-1477.