RESEARCH PAPER

The protective effect of nano-curcumin in experimental model of acute pancreatitis: The involvement of TLR4/NF-kB pathway

Amir Rashidian [1], Homanaz Ghafari [1], Mohsen Chamanara [1], Ahmad-Reza Dehpour [1,2], Ahad Muhammadnejad [3]
Reyhaneh Akbarian [1], Seyyedeh Elaheh Mousavi [1], Seyed-Mahdi Rezayat [1]*

[1] Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[2] Experimental Medicine Research Center, Tehran University of Medical Sciences, Tehran, Iran
[3] Cancer Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran

ABSTRACT

Objective(s): The objective of the present study is to explore whether Nanocurcumin improves pancreatic inflammation through the inhibition of the TLR4/NFkB signaling pathway in cerulein-induced acute pancreatitis.

Materials and Methods: Acute pancreatitis was induced by five intraperitoneal (i.p.) injection of cerulein (50 µg/kg) with 1h intervals. Vehicle and nanocurcumin (100mg/kg/day) were given to the animals by oral gavage six days before the induction of pancreatitis. The last dose was administered 1 hour before pancreatitis induction. The serum level of amylase and lipase and the tissue level of MPO enzymes were assessed by biochemical analysis. Microscopic lesions were examined. In addition, the expression level of TLR4, NF-kB p65 and TNF-α proteins were measured by western blotting analysis.

Results: Nanocurcumin reduced the microscopic lesions. In addition, the drug decreased the level of amylase, lipase and MPO enzymes. Furthermore, nanocurcumin inhibited the cerulein-induced expression of TLR4, NF-kB p65 and TNF-α proteins.

Conclusion: It is suggested that the anti-inflammatory effect of nanocurcumin on cerulein-induced acute pancreatitis may involve the inhibition of the TLR4/NFkB signaling pathway.

Keywords: *Acute pancreatitis, Cerulein, Nanocurcumin,TLR4/NF-kB pathway*

INTRODUCTION

Acute pancreatitis (AP) is a lethal inflammatory condition of pancreas with a wide clinical spectrum of severity ranging from a mild condition of upper abdomen pain to sever form of pancreatic necrosis and even mortality. AP is clinically characterized by increased of pancreaticenzymes, sudden onset, and multiple organ failure (MOF) particularly, pulmonary insufficiency, renal failure, shock, and gastrointestinal bleeding [1]. The majority of atients with AP have mild disease, but 20-30% of patients develop sever acute pancreatitis (SAP) with significant mortality up to 40% due to multiple organ dysfunction syndrome (MODS) [2]. Since there is no specific treatment for AP, understanding the possible molecular mechanisms and inflammatory mediators is important to control and manage the disease. In spite of recent advances in molecular biology and immunogenetics, the exact pathogenesis of AP is not completely clear yet. AP is shown to initiate in two steps; first in acinar cells by activation of the intracellular pancreatic proenzymes and nuclear factor-κB (NF-κB) resulted in acinar cell injury and local inflammatory reaction [3]. Then it leads to a systemic inflammatory response syndromes (SIRS) due to massive production of inflammatory mediators resulted in systemic acinar cell death and organ dysfunction [4]. Activation of NF-κB enhanced releasing of various pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-1β, and IL-6 which play an important role in induction and severity of

* Corresponding Author Email: : Rezayat@tums.ac.ir
Note. This manuscript was submitted on April 5, 2018; approved on May 30, 2018

AP [5]. Toll-like receptors (TLRs) are a group of transmembrane and signal transduction receptors showed to have a critical role in the pathogenesis of AP. They can activate major intracellular signaling pathways result in the transcription of pro-inflammatory genes through the activation of NF-κB and/or activating protein (AP-1) [6]. TLR4 as the first identified member of this group has been known as the main trigger in activation of NF-κB [7]. Curcumin is an organic, nontoxic yellow pigment obtained from the rhizomes of turmeric (curcuma longa). Curcumin has been shown to have potent anti-inflammatory, anti-carcinogenic and antioxidant, hypoglycemic, wound-healing, and antimicrobial activities [8]. Nano-curcumin is a curcumin product registered as (SinaCurcumin®) for oral use by Nanotechnology Research Center of Mashhad University of Medical Sciences, Mashhad, Iran (IRC:1228225765). Each nanocurcumin soft gel contains 80 mg of curcumin in the form of nano-micelle. Because of hydrophobic nature of curcumin, its oral absorbtion is very poor. In addition, One study has been shown significant higher bioavalibility of nano-curcumin compared to curcumin powder [9]. The present study was designed to investigate the possible anti-inflammatory effect of nano-curcumin through the inhibition of the TLR4-linked NF-kB signaling pathway in cerulein-induced acute pancreatitis in mice.

MATERIALS AND METHODS
Materials
Cerulein was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nano-curcumin (Sinacurcumin®) was received as a gift from Nanotechnology Research Center of Mashhad University of Medical Sciences, Mashhad, Iran. The percent of encapsulation of curcumin in this product is near to 100% and the sizes are around 10 nm [9]. Formalin solution 35 % w/w was purchased from Merck Company (Darmstadt, Germany). All other solvents and chemicals used were of analytical grade. Nano-curcumin was dissolved in 2% Tween80-normal saline solution as vehicle. Vehicle and nano-curcumin were administered at a volume of 10 ml/kg.

Animals
Adult male NMRI mice weighing 25–30 g were obtained from the central animal house of the School of Medicine at Tehran University of Medical

Sciences. They were kept in polypropylene cages at 20–23°C under controlled environmental conditions (12 h light/dark cycles and humidity 50–60 %) and had free access to standard laboratory chow and water ad libitum. All animals were fasted overnight before the test day. This study was approved by the Ethical Committee of Tehran University of Medical Sciences. All animal experiments were carried out according to the ''Principles of Laboratory Animal Care'' (NIH publication 82-23, revised in 1985 and further implemented in 1996).

Induction of pancreatitis
Acute pancreatitis was induced by five intraperitoneal (i.p.) injection of 50 µg/kg cerulein with 1 h intervals according to the method previously demonstrated by Mazzon et al [10].

Experimental design
Animals (n = 24) were randomly divided into three groups (n = 8) as below: Control group: (normal saline + vehicle 10 ml/kg/day) Cerulein group (cerulein + vehicle 10 ml/kg/day) Nano-curcumin group (cerulein + nano-curcumin 100 mg/kg/day). The dose of nano-curcumin was chosen according to the study reported by Ke Zhong [11]. All treatments were carried out six days before the induction of pancreatitis. The last dose was administered 1 h before pancreatitis induction. Vehicle and nano-curcumin were given to the animals by oral gavage. Animals were sacrificed 6 h after the last injection of cerulein by cervical dislocation. Blood samples were collected by direct intracardiac puncture and stored at −80°C for biochemical analysis. The pancreas samples were cut into two pieces, one half of the tissue fixed in formalin 10% for histological examinations and the other half was frozen in liquid nitrogen for western blot analysis.

Analysis of serum amylase and lipase levels
The serum lipase and amylase levels were determined by using a commercial kit for lipase and amylase (Pars-Azmoon Company, Tehran, Iran) and were expressed as U/L.

Determination of myeloperoxidase activity
Pancreatic Tissue MPO activity was determined according to the method described by Bradley et al [12]. A portion of pancreas was homogenized in 50 mM of potassium phosphate buffer (pH 6)

Nanomed. J. 5(3): 138-143, Summer 2018

139

with 0.5% HTAB (hexadecyltrimethylammonium bromide). Then, the samples were sonicated in an ice bath for 10 seconds following by freeze-thawed thrice with sonication between cycles. Afterward, the homogenates were centrifuged for 15 minutes at 15000 rpm at 4°C. Then, aliquots of the supernatant were mixed with 50 mM phosphate buffer (pH 6) containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% H_2O_2. The resulting change of the reaction mixture was measured at 460 nm using a UV/Vis spectrophotometer. Myeloperoxidase activity was expressed as units (U) per gram of weight of wet tissue.

Histological examination

Paraffin-embedded pancreas tissue samples cut into 5 μm-thick sections and stained with hematoxylin and eosin (H&E). These sections were examined by blinded pathologist as to the experimental protocol. The severity of pancreatic edema, leukocyte infiltration and acinar vacuolization was graded with scores ranging from 0 to 3 described by Dembinski et al [13].

Western blot analysis

Pancreatic tissue samples were homogenized in lysis buffer (137 mM NaCl, 20 mM Tris–HCl pH 8.0, 1% NP-40, 10 % glycerol, 1 mM phenylmethyl sulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mM sodium vanadate). The supernatant was removed by centrifugation at 12,500 g for 20 min at 4°C. Total protein concentration was determined using the Micro BCA procedure (Pierce, Rockford, IL, USA). Equal amounts (100 μg) of protein from each sample were resolved by SDS-PAGE electrophoresis under reducing conditions, transferred to polyvinylidene fluoride (PVDF) membranes and then blocked with 5 % non-fat dry milk and 0.1 % Tween-20 in Tris-buffered saline at room temperature for 1 h. Membranes were incubated overnight at 4°C with rabbit anti-TLR4 (1:100 dilution), anti-NF-κB p65 (1:200 dilution), TNF-α (1:200 dilution) and anti β-actin (1:200 dilution) antibodies (Santa Cruz Biotechnology, USA). After washing with Tris-buffered saline Tween-20 (TBST), membranes were incubated with a secondary goat anti-rabbit IgG horseradish peroxidase conjugated (1:10,000 dilution) antibody (Santa Cruz Biotechnology, USA) at room temperature for 1 hr. Membranes were detected by using western blot detection system (Intron Biotechnology, UK) according to the instruction of the manufacturer and then exposed

to X-ray film (Thermo Scientific, USA). The density of protein bands was quantified using Quantity One® 1-D analysis software (Bio-Rad, USA).

Statistical analysis

The results were expressed as mean ± SEM. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism 6 software. Values of $P < 0.05$ were considered to be statistically significant.
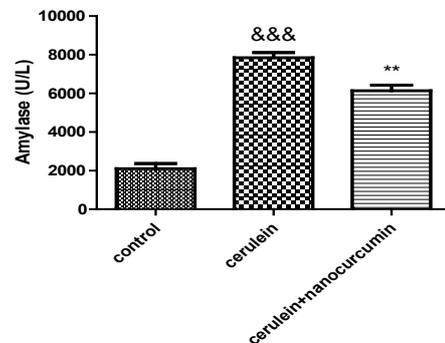
A
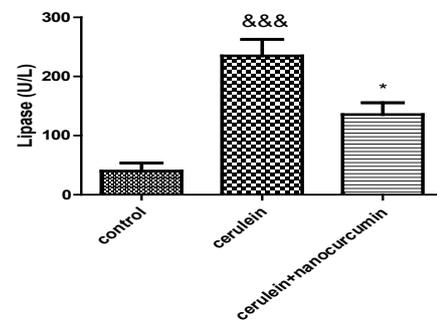
B

Fig 1. (A) Effect of Nanocurcumin on serum amylase level (U/L) of cerulein-induced acute pancreatitis in mice. (B) Effect of Nanocurcumin on serum lipase level (U/L) of cerulein-induced acute pancreatitis in mice. Data are expressed as means ± S.E.M (n=6). &&&$P < 0.001$ compared to control group and *$p < 0.05$, **$p < 0.01$ compared to cerulein group. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Tukey's post hoc test

RESULTS
Effect of nanocurcumin on serum amylase and lipase levels

The results of serum amylase and lipase levels were shown in (Fig 1 A and B) respectively. Both amylase and lipase enzymes were increased

in cerulein group compared to control group (*P*<0.001). The administration of nano-curcumin reduced serum amylase and lipase levels compared to cerulein group significantly (*P*<0.01 and p<0.05).
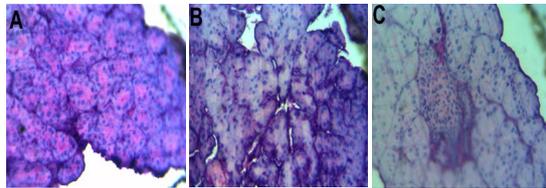

Fig 2. Histopathological features of normal pancreas and acute pancreatitis in mice. (A) Control: (B) Cerulein: (C) Nanocurcumin (100 mg/kg). Magnifications: ×200
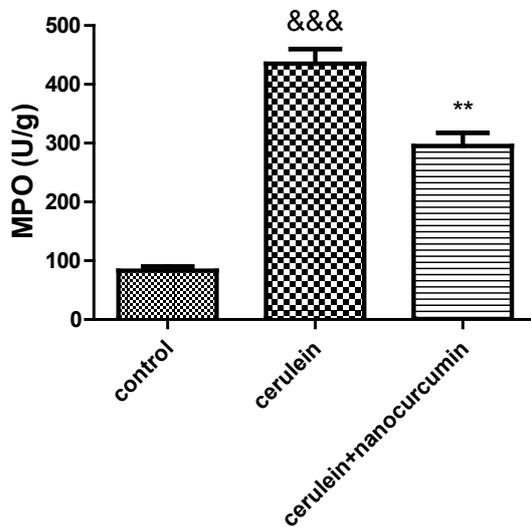

Fig 3. Effect of Nanocurcumin (100 mg/kg) on the level of myeloperoxidase (MPO) activity in the pancreas tissue. Results are expressed as mean ± S.E.M (n=6). &&&P<0.001 compared to control group. **p<0.01 compared to cerulein group. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Tukey's post hoc test

Effect of nanocurcumin on histological damage

All the histological features of pancreas were typically normal in the control group (Fig 2A). Histological examination of acute pancreatitis mice in cerulein group revealed pancreas damage characterized by acute inflammatory, leukocyte infiltration, intralobular and interlobular edema. But, no haemorrhage and pancreatic cells necrosis have been seen in this group (Fig 2B). The administration of nano-curcumin decreased edema and leukocyte infiltration in the pancreas tissue compared to cerulean group (Fig 2C).
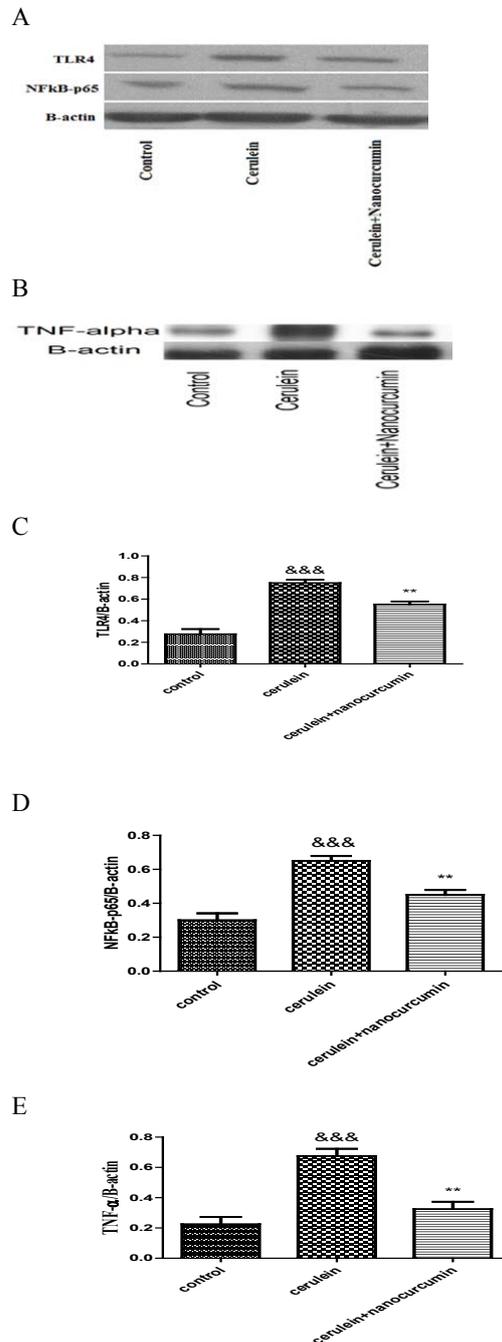

Fig 4. Expression of TLR4, NF-κB p65 and TNF-α proteins in the pancreas tissue of animals in three groups (A and B) Representative Western blot picture, showing the expression levels of TLR4, NF-κB p65, TNF-α and β-actin proteins in three groups. (C) Changes in the expression level of TLR4 protein. (D) Changes in the expression level of NF-kB p65 protein. (E) Changes in the expression level of TNF-α protein. Data are expressed as means ± S.E.M (n=6). &&&P<0.001 compared to control group and *p<0.05, **p<0.01 compared to cerulein group. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Tukey's post hoc test

Nanomed. J. 5(3): 138-143, Summer 2018

141

Effect of nanocurcumin on myeloperoxidase activity

As shown in Fig 3, the MPO activity was significantly higher in cerulein group compared to control group ($P<0.001$). Oral administration of nanocurcumin was significantly decrease the MPO activity compare to cerulein group ($P<0.01$).

Effect of nanocurcumin on TNF-α, TLR4 and NF-κB p65

The results of Western Blot analysis showed that the protein expression of TNF-α, TLR4 and NF-κB in the pancreatic tissue of the cerulein group were significantly increased compared to the control group ($P<0.001$). The treatment with nanocurcumin resulted in significant reduction in the expression of the three proteins in comparison with the cerulein group ($P<0.01$) (Fig 4A-E).

DISCUSSION

Curcumin has a long history of use as a traditional medicine since it is nontoxic and has a variety of therapeutic properties such as antioxidant, analgesic, anti-inflammatory, antiseptic and anticarcinogenic activity [14]. The present study shows that nanocurcumin decreases pancreatic damage induced by cerulein in the experimental model of acute pancreatitis in mice through the reduction of microscopic and biochemical (amylase, lipase and MPO) parameters as well as the inhibition of the overexpression of inflammatory mediator TNF-α, TLR4 receptor and nuclear transcription factor NF-kB p65. Also, the result of this study is in accordance with the previous investigation on the anti-inflammatory effect of curcumin against rat model of severe acute pancreatitis through the inhibition of TLR4/NF-kB signaling pathway [11]. Cerulein also known as Ceruletide, is a ten amino acid oligopeptide similar to cholecystokinin in its action and composition [15]. Cerulein induced acute pancreatitis is one of the most common model of experimental pancreatitis used in vivo. Intraperitoneal administration of cerulein produce interlobular and intralobular edema, leukocyte infiltration, cytoplasmic vacuolization and death of acinar cells [16]. In the present study, intraperitoneal administration of cerulein caused destruction of pancreatic tissue structure, inflammatory cells infiltration and tissue edema. On the other hand, the administration of nanocurcumin reduced the histological signs of inflammation such as edema, and infiltration of neutrophils.

Myeloperoxidase is a proteolytic enzyme with 140-kDa exist in Neutrophil granulocytes [17].

Following the induction of pancreatitis, the enzyme released from neutrophils leads to oxidative damage to the pancreatic tissue. The severity of tissue damage is correlated with the high activity of this enzyme [18]. The results of this study showed that treatment with nanocurcumin reduced the level of myeloperoxidase activity in the pancreatic tissue.

The key role of TNF-α as a pro-inflammatory mediator has been implicated in the pathogenesis of acute pancreatitis. In addition, there is direct evidence between block the action of TNF-α and delay in the onset of acute pancreatitis [19]. Furthermore, treatment with nanocurcumin significantly decreased cerulein-induced increase in the expression of TNF-α in the pancreatic tissue.

TLR4/NF-kB signaling pathway is one of the most important pathway that mediate inflammatory responses and involve in the pathogenesis of acute pancreatitis. TLR4 is activated by recognizing some ligands such as lipopolysaccharide (LPS), low-density lipoprotein, beta-defensins and heat shock protein, followed by the activation of myeloid differentiation protein (MyD88) as an important adaptor molecule in TLR signaling pathway. This in turn lead to the activation of transcription factor NF-kB followed by the induction of inflammatory cytokines such as TNF-α resulting in the initiation and/or progression of acute pancreatitis [7,11]. Recent studies have demonstrated that TLR4 expression is low in normal pancreas tissue, while its expression is relatively high in the experimental acute pancreatitis model [7, 20].

On the other hand, several studies revealed that the inhibition of TLR4/NF-kB signaling pathway reduced the damage to the pancreatic tissue in the animal model of acute pancreatitis [21, 22, 23]. In addition, some studies have revealed the protective effects of curcumin on experimental acute pancreatitis through the inhibition of various transcription factors such as NF-κB and AP-1 and inflammatory cytokines such as TNF-a, IL-1β and IL-6 [24, 25, 26].

CONCLUSION

In conclusion, to our knowledge, for the first time, the present study demonstrates that nanocurcumin ameliorates cerulein induced acute

pancreatitis in mice through the inhibition of TLR4/NF-kB signaling pathway. However, further evaluation is required on the precise mechanism of nanocurcumin as an anti-inflammatory agent in the management of acute pancreatitis.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

REFERENCES

1. Pandol SJ, Saluja AK, Imrie CW, Banks PA. Acute pancreatitis: bench to the bedside. Gastroenterology. 2007; 133(3): 1056.
2. Singh P, Garg PK. Pathophysiological mechanisms in acute pancreatitis: Current understanding. Indian J Gastroenterol. 2016; 35(3): 153-166.
3. Huang H, Liu Y, Daniluk J, Gaiser S, Chu J, Wang H, Li ZS, Logsdon CD, Ji B. Activation of nuclear factor-κB in acinar cells increases the severity of pancreatitis in mice. Gastroenterology. 2013; 144(1): 202-210.
4. Bhatia M, Wong FL, Cao Y, Lau HY, Huang J, Puneet P, Chevali L. Pathophysiology of acute pancreatitis. Pancreatology. 2005; 5(2-3): 132-144.
5. Watanabe T, Kudo M, Strober W. Immunopathogenesis of pancreatitis. Mucosal Immunol. 2017; 10(2): 283-298.
6. Vaz J, Akbarshahi H, Andersson R. Controversial role of toll-like receptors in acute pancreatitis. World J Gastroenterol. 2013; 19(5): 616-630.
7. Li Y, Zhou ZG, Xia QJ, Zhang J, Li HG, Cao GQ, Wang R, Lu YL, Hu TZ. Toll-like receptor 4 detected in exocrine pancreas and the change of expression in cerulein-induced pancreatitis. Pancreas. 2005; 30(4): 375-381.
8. Aggarwal BB, Sung B. Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets. Trends Pharmacol Sci. 2009; 30(2): 85-94.
9. Rahimi HR, Mohammadpour AH, Dastani M, Jaafari MR, Abnous K, Ghayour Mobarhan M, Kazemi Oskuee R. The effect of nano-curcumin on HbA1c, fasting blood glucose, and lipid profile in diabetic subjects: a randomized clinical trial. Avicenna J Phytomed. 2016; 6(5): 567-577.
10. Mazzon E, Genovese T, Di Paola R, Muià C, Crisafulli C, Malleo G, Esposito E, Meli R, Sessa E, Cuzzocrea S. Effects of 3-aminobenzamide, an inhibitor of poly (ADP-ribose) polymerase, in a mouse model of acute pancreatitis induced by cerulein. Eur J Pharmacol. 2006; 549(1-3): 149-156.
11. Zhong K. Curcumin Mediates a Protective Effect Via TLR-4/NF-κB Signaling Pathway in Rat Model of Severe Acute Pancreatitis. Cell Biochem Biophys. 2015; 73(1): 175-180.
12. Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Invest Dermatol. 1982; 78(3): 206-209.
13. Dembiński A, Warzecha Z, Ceranowicz P, Warzecha AM, Pawlik WW, Dembiński M, Rembiasz K, Sendur P, Kuśnierz-Cabala B, Tomaszewska R, Chowaniec E, Konturek PC. Dual, time-dependent deleterious and protective effect of anandamide on the course of cerulein-induced acute pancreatitis. Role of sensory nerves. Eur J Pharmacol. 2008; 591(1-3): 284-292
14. Amalraj A, Pius A, Gopi S, Gopi S. Biological activities of curcuminoids, other biomolecules from turmeric and their derivatives - A review. J Tradit Complement Med. 2016; 7(2): 205-233.
15. Anastasi A, Erspamer V, Endean R. Isolation and structure of caerulein, an active decapeptide from the skin of Hyla caerulea. Experientia. 1967; 23(9): 699-700.
16. Kim H. Cerulein pancreatitis: oxidative stress, inflammation, and apoptosis. Gut Liver. 2008; 2(2): 74-80.
17. Kannan N, Guruvayoorappan C. Protective effect of Bauhinia tomentosa on acetic acid induced ulcerative colitis by regulating antioxidant and inflammatory mediators. Int Immunopharmacol. 2013; 16(1): 57-66.
18. Klebanoff SJ, Kettle AJ, Rosen H, Winterbourn CC, Nauseef WM. Myeloperoxidase: a front-line defender against phagocytosed microorganisms. J Leukoc Biol. 2013; 93(2): 185-198.
19. Oruc N, Ozutemiz AO, Yukselen V, Nart D, Celik HA, Yuce G, Batur Y. Infliximab: a new therapeutic agent in acute pancreatitis? Pancreas. 2004; 28(1): e1-8.
20. Sharif R, Dawra R, Wasiluk K, Phillips P, Dudeja V, Kurt-Jones E, Finberg R, Saluja A. Impact of toll-like receptor 4 on the severity of acute pancreatitis and pancreatitis-associated lung injury in mice. Gut. 2009; 58(6): 813-819.
21. Pan LF, Yu L, Wang LM, He JT, Sun JL, Wang XB, Wang H, Bai ZH, Feng H, Pei HH. Augmenter of liver regeneration (ALR) regulates acute pancreatitis via inhibiting HMGB1/TLR4/NF-κB signaling pathway. Am J Transl Res. 2018; 10(2): 402-410.
22. Cen Y, Liu C, Li X, Yan Z, Kuang M, Su Y, Pan X, Qin R, Liu X, Zheng J, Zhou H. Artesunate ameliorates severe acute pancreatitis (SAP) in rats by inhibiting expression of pro-inflammatory cytokines and Toll-like receptor 4. Int Immunopharmacol. 2016; 38: 252-260.
23. Liu Z, Liu J, Zhao K, Shi Q, Zuo T, Wang G, Wang W. Role of Daphnetin in Rat Severe Acute Pancreatitis Through the Regulation of TLR4/NF-[Formula: see text]B Signaling Pathway Activation. Am J Chin Med. 2016; 44(1): 149-163.
24. Gulcubuk A, Altunatmaz K, Sonmez K, Haktanir-Yatkin D, Uzun H, Gurel A, Aydin S. Effects of curcumin on tumour necrosis factor-alpha and interleukin-6 in the late phase of experimental acute pancreatitis. J Vet Med A Physiol Pathol Clin Med. 2006; 53(1): 49-54.
25. Seo SW, Bae GS, Kim SG, Yun SW, Kim MS, Yun KJ, Park RK, Song HJ, Park SJ. Protective effects of Curcuma longa against cerulein-induced acute pancreatitis and pancreatitis-associated lung injury. Int J Mol Med. 2011; 27(1): 53-61.
26. Gulcubuk A, Haktanir D, Cakiris A, Ustek D, Guzel O, Erturk M, Karabagli M, Akyazi I, Cicekci H, Altunatmaz K, Uzun H, Ates K. Effects of curcumin on proinflammatory cytokines and tissue injury in the early and late phases of experimental acute pancreatitis. Pancreatology. 2013; 13(4): 347-354.

Nanomed. J. 5(3): 138-143, Summer 2018

143