Journal of Ethnopharmacology 145 (2013) 573–580

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

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

Shikonin ameliorates cerulein-induced acute pancreatitis in mice

Jie Xiong [a,1], Jianbo Ni [a,1], Guoyong Hu [a], Jie Shen [a], Yan Zhao [a], Lijuan Yang [b], Jiaqing Shen [b], Guojian Yin [a], Congying Chen [a], Ge Yu [a], Yanling Hu [a], Miao Xing [a], Rong Wan [a,**], Xingpeng Wang [a,b,*]

[a] Department of Gastroenterology, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200072, China
[b] Department of Gastroenterology, Shanghai First People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200080, China

ARTICLE INFO

Article history:
Received 14 August 2012
Received in revised form
14 October 2012
Accepted 12 November 2012
Available online 28 November 2012

Keywords:
Acute pancreatitis
Shikonin
Inflammation
NF-κB
Cytokine

ABSTRACT

Ethnopharmacological relevance: Shikonin, a highly liposoluble naphthoquinone pigment isolated from the traditional medical herbs *Lithospermum erythrorhizon* (*LE*), was considered to exhibit an anti-inflammatory property. While the potential of shikonin to ameliorate acute pancreatitis (AP) is unknown. Our aim was to investigate the effects of shikonin in a murine model of cerulein-induced pancreatitis.

Materials and methods: AP was induced in mice by six intraperitoneal injection of cerulein (50 μg/kg) at hourly intervals. Vehicle or shikonin (50 mg/kg) was pretreated 2 h before the first cerulein injection. After 6 h, 9 h and 12 h of the first cerulein injection, the severity of acute pancreatitis was assessed by biochemistry, myeloperoxidase activity, histological grading, proinflammatory cytokines levels and nuclear factor kappa B (NF-κB) activity.

Results: Shikonin administration significantly reduced serum amylase and lipase activities, pancreatic histological scores, TNF-α, IL-1β, IL-6 levels, MPO activity and NF-κB activity.

Conclusion: Taken together, these results suggest that shikonin might protect against experimental pancreatitis by reducing release of inflammatory cytokines via inhibition of NF-κB activity. The therapeutic role of shikonin in AP needs further investigation.

1. Introduction

Acute pancreatitis (AP) is a common clinical condition, whose incidence has been increasing over recent years. Most patients with AP suffer from the mild edematous form, which is considered to be a self-limiting disease with a low complication rate (Whitcomb, 2006; Kim et al., 2011). However, upto 25% of patient with AP experience a severe attack and about 30% of them will die (Pandol et al., 2007; Hu et al., 2011). The complete mechanism of AP has not been established so far. The initial events occur in pancreatic acinar cell including activation of zymogens in acinar cell and release of proinflammatory cytokines such as factor TNF-α, interleukin (IL)-1β, and IL-6 (Yu et al., 2009; Zhang et al., 2010). Considering the central importance of the inflammatory response in pancreatitis, therapeutic strategies should be aimed at the key steps leading to this response. Among the multitude inflammatory molecules involved in this disease, it has been established that inhibition of nuclear factor kappa B (NF-κB) activation which is the upstream "master regulators" of many inflammatory molecules can attenuate inflammatory responses and the severity of AP (Zhang et al., 2007; Rakonczay et al., 2008; Wang et al., 2009). Shikonin, a highly liposoluble naphthoquinone pigment isolated from the traditional medical herbs *Lithospermum erythrorhizon*, has long been used in traditional Asian medicine for the treatment of burns, anal ulcers, hemorrhoids, infected crusts, bedsores, external wounds and oozing dermatitis (Chen et al., 2002; Kourounakis et al., 2002). It has been established that shikonin possesses different medicinal properties such as antibacterial, improving wound healing, anti-inflammatory, anti-thrombotic and anti-tumor effects (Staniforth et al., 2004; Wu et al., 2004; Kundakovic et al., 2006; Singh et al., 2006; Yang et al., 2009). In studies of the anti-inflammatory potential of shikonin and shikonin derivatives, these compounds have been shown to be effective in inhibiting the activation of NF-κB (Cheng et al., 2008; Andujar et al., 2010). Cerulein-induced pancreatitis is an experimental mouse model of human acute pancreatitis characterized by early activation of NF-κB, releasing of proinflammatory cytokines, and histological alterations (Gukovsky et al., 1998). Here, we investigated the effects of shikonin in a murine model of cerulein-induced pancreatitis.

* Corresponding author at: Department of Gastroenterology, Shanghai First People's Hospital, Shanghai Jiaotong University School of Medicine, 100 Haining Road, Hongkou District, Shanghai 200080, China. Tel.: +86 1880 173 6057.
** Corresponding author at: Department of Gastroenterology, Shanghai Tenth People's Hospital, Tongji University School of Medicine, 301 Yanchang Road, Shanghai 200072, China.
E-mail addresses: wanrong1970@163.com (R. Wan), wangxingpeng@yahoo.com.cn (X. Wang).
[1] These authors contributed equally to this work.

0378-8741/$ - see front matter Crown Copyright © 2012 Published by Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jep.2012.11.032

2. Materials and methods

2.1. Animals and materials

Male BALB/c mice (age 8 week, weight 20–21 g) were purchased from Shanghai Laboratory Animal Co Ltd (SLAC, Shanghai, China). Animals were maintained on a 12 h light/12 h dark cycle at 22 °C, given water ad libitum, fed standard laboratory chow and allowed to acclimatize for a minimum of 1 week. Mice were randomly assigned to control or experimental groups. All the animal-related procedures were approved by the Animal Animal Ethical Committee of Tongji University. This study was also under the permission of Science and Technology Commission of Shanghai Municipality (ID: SYXK 2007-0006) with the permit number 2011-RES1. All animals were fasted for 12 h before the induction of AP. Purified shikonin (> 98%) was purchased from the National Institute for the Control Pharmaceutical and Biological Products (Beijing, China). Cerulein, dimethyl sulfoxide (DMSO), eosin and hematoxylin were purchased from Sigma Chemical (Sigma-Aldrich, St. Louis, MO). Antibodies against NF-κB p65, inhibitory κB α(IκB α), inhibitory κB β(IκB β), Histone-H1, β-actin and peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology Company (Santa Cruz, CA, USA).

2.2. Experimental design

Shikonin was dissolved in vehicle (2% DMSO). To examine the biochemical toxicity of shikonin in vivo and obtain the optimal dose of shikonin for preventing cerulein-induced pancreatitis, we performed a preliminary study. 16 mice were divided into four groups randomly ($n=4$ for each group): group1, vehicle-treated; group 2, 3 and 4, shikonin-treated (50, 100 and 200 mg/kg, *per os* (*p.o*)), respectively. Mice were sacrificed at 6 h after the vehicle or shikonin administered. Blood samples were taken and the fresh serum was used for assaying biochemical parameters by the department of clinical laboratory in shanghai tenth people's hospital. After the evaluation of the biochemical parameter result, a suitable dose (50 mg/kg) which had no observed adverse effect (NOAE) to mice was found. Acute oral toxicity study of shikonin was also performed by estimating the LD_{50} value (520 mg/kg) and observing the toxicity signs of shikonin at gradually increasing dose. The dose of 50 mg/kg showed no sign of toxicity (see supplementary material for detail).

Then three doses (12.5 mg/kg, 25 mg/kg and 50 mg/kg) which were all below the dose of NOAE were used to treat cerulein-induced pancreatitis. Acute pancreatitis was induced by six injections of cerulein (50 μg/kg, i.p. at intervals of 1 h) as described previously (Babu et al., 2012). The normal control mice were given saline (0.9% NaCl) solution intraperitoneally instead cerulein ($n=4$ for each group). Vehicle or shikonin was administered 2 h before the first cerulein injection (*p.o*). All animals were sacrificed at 12 h after the first injection of cerulein, a time point at which pancreatic damage had already peaked. The effect of shikonin was evaluated by the levels of serum amylase, an indicator which was usually considered to be closely related to pancreatic damage, to get an optimal dose. The optimal dose of shikonin (50 mg/kg) was used for the next series of experiments. Then 36 mice were divided into three groups randomly ($n=12$ for each group): group 1, normal control; group 2, cerulein+vehicle-treated; group 3, cerulein+shikonin-treated. The induction of acute pancreatitis and the administration of shikonin or vehicle were performed the same as the preliminary study. Mice were sacrificed at 6 h, 9 h and 12 h after the first cerulein injection, four mice at every time point in each group. Blood samples were taken to determine the serum amylase, lipase and cytokine levels. The pancreas were rapidly removed from each mouse and fixed in formalin for morphologic examination and scoring. A portion of each pancreas was stored at −80 °C for the further investigation.

2.3. Histological analysis

A portion of the head of the pancreas was fixed in 4% neutral formaldehyde solution for 12 h, embedded in paraffin, and cut into 5-μm thick sections which were stained with hematoxylin and eosin to observe the morphological changes under a light microscope. The assessment of edema, vacuolization, inflammatory cell infiltration and acinar cell necrosis as previously described (Dembinski et al., 2008). Ten microscopic fields were randomly chosen to observe them in each mouse. Histological scoring of pancreatic tissue was performed to grade the extent of

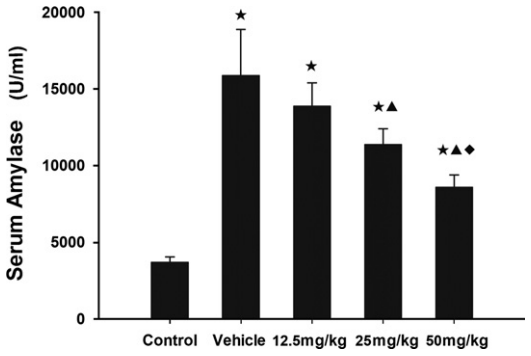

Fig. 1. Preliminary study results. Mice ($n=4$ for each group) were given 6 hourly injection of cerulein (50 μg/kg). Shikonin (12.5 mg/kg, 25 mg/kg and 50 mg/kg) or vehicle was administrated 2 h before the first cerulein injection. The control group were given saline (0.9% NaCl) solution intraperitoneally instead cerulein. Mice were sacrificed 12 h after the first injection of cerulein. *$P<0.05$ vs control group, ▲$P<0.05$ vs vehicle-treated group and ◆$P<0.05$ vs 25 mg/kg shikonin-treated group.

Table 1
The biochemical toxicity of shikonin in vivo.

Group	TP	AlB	BUN	CREA	ALP	GPT	GOT	AMY
	(g/L)	(g/L)	(mmol/L)	(μmol/L)	(U/L)	(U/L)	(U/L)	(U/mL)
Vehicle	47.2 ± 1.2	30.7 ± 0.9	6.5 ± 1.8	45.9 ± 4.6	142.4 ± 18.2	58.7 ± 22.8	142.6 ± 24.3	3740 ± 182
SK (50 mg/kg)	46.8 ± 1.4	29.8 ± 1.2	7.2 ± 1.4	48.2 ± 3.2	150.6 ± 15.6	69.4 ± 16.8	150.1 ± 18.0	3686 ± 224
SK (100 mg/kg)	44.7 ± 2.0*	27.0 ± 1.4*	7.9 ± 1.3	52.8 ± 5.8*	158.8 ± 12.4	100.8 ± 15.9*	168.1 ± 25.3	3786 ± 168
SK (200 mg/kg)	42.1 ± 2.7*	25.2 ± 2.1*	9.5 ± 1.9*	58.1 ± 6.7*	162.2 ± 14.8	142.3 ± 26.6*	232.3 ± 21.0*	3752 ± 289

Data are represented as the mean ± SD ($n=4$ for each group). SK, shikonin; TP, total protein; ALB, albumin; BUN, blood urea nitrogen; CREA, creatinine; GPT, glutamic-pyruvic transaminase; GOT, glutamic-oxalacetic transaminase; ALP, alkaline phosphatase; AMY, amylase.

* $P<0.05$ vs vehicle-treated group. The results were similar in 3 independent experiments.