

A preliminary study of the anti-inflammatory and anti-apoptotic effects of crocin against gastric ischemia-reperfusion injury in rats

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The aim of the present study was to investigate the protective effect of crocin on gastric mucosal lesions caused by ischemia-reperfusion (I/R) injury in rats. Thirty-two male rats were randomly divided into sham, I/R, I/R + crocin pretreatment and crocin alone groups. To induce I/R lesions, the celiac artery was clamped for 30 min, and the clamp was then removed to allow reperfusion for 3 h. Crocin-pretreated rats received crocin (15 mg/kg, i.p.) 30 min prior to the induction of I/R injury. Samples of gastric mucosa were collected to quantify the protein expression of caspase-3, an apoptotic factor, and inducible nitric oxide synthase (iNOS), a pro-inflammatory protein, by Western blot. Pretreatment with crocin decreased the total area of gastric lesions and decreased the protein expression levels of caspase-3 and iNOS induced by I/R injury. Our findings showed a protective effect of crocin in gastric mucosa against I/R injury. This effect of crocin was mainly mediated by reducing the protein expression of iNOS and caspase-3.

Uniterms: Crocin/protective effect. Gastric mucosal/lesions/ ischemia-reperfusion. Gastric lesions/treatment/experimental study. Caspase-3. Inducible nitric oxide synthase.

O objetivo do presente estudo foi investigar o efeito protetor da crocina em lesões da mucosa gástrica causadas por isquemia-reperfusão (I/R) em ratos. Trinta e dois ratos machos aleatoriamente divididos em grupos de ratos normais, operados como controle, I/R, I/R + pré-tratamento com crocina e crocina sozinha. Para induzir lesões I/R, a artéria celiaca foi grampeada durante 30 minutos e, em seguida, o grampo foi removido para permitir a reperfusão por 3 h. Ratos com pré-tratamento com crocina receberam crocina (15 mg/kg, ip) 30 minutos antes da indução do dano I/R. Amostras de mucosa gástrica foram coletadas para quantificar a expressão da proteína da caspase-3, o fator apoptótico, e óxido nítrico sintase induzível (iNOS), uma proteína anti-inflamatória, pela técnica de Western Blot. O pré-tratamento com crocina diminuiu a área total de lesões gástricas e a expressão de níveis de caspase-3 e iNOS induzidas pelo dano I/R. Nossos resultados mostraram o efeito protetor da crocina na mucosa gástrica contra o dano I/R. Este efeito foi mediado, principalmente, por diminuição da expressão das proteínas iNOS e caspase-3.

Unitermos: Crocina/efeito protetor. Mucosa gástrica/lesão/isquemia-reperfusão. Lesões gástricas/tratamento/estudo experimental. Caspase-3. Óxido nítrico sintase induzível.

INTRODUCTION

It is well established that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in the development of gastric ischemia-reperfusion (I/R) injury (Yoshikawa *et al.*, 1989, Ishii *et al.*, 2000). Also,

the contribution of nitric oxide (NO) in I/R-induced gastric lesions has been documented (Wada *et al.*, 1998). It has been shown that NO reacts with ROS and produces highly toxic substances such as peroxynitrite and singlet oxygen (Beckman *et al.*, 1990, Noronha-Dutra *et al.*, 1993). Naturally, NO is produced from L-arginine in mammalian tissues, catalyzed by three isoforms of NO synthase, two constitutive (eNOS and nNOS) and one inducible (iNOS) form. In oxidative stress, iNOS gene transcription and promoter activity are increased, regulating chromatin

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modification and leading to cellular injury (Huang *et al.*, 2011, Yang *et al.*, 2012).

Crocin is the most important and abundant antioxidant constituent of *Crocus sativus* stigma (Abdullaev, 1993). Beneficial effects of crocin have been reported on the nervous (Zheng *et al.*, 2007), cardiac (Goyal *et al.*, 2010) and renal (Hosseinzadeh *et al.*, 2005) systems. Moreover, crocin has been shown to exert anti-inflammatory property by reducing edema in acetic acid-induced edema in the rat paw model (Hosseinzadeh, Younesi, 2002). Moreover, saffron and its active constituents, crocin and safranal, have been reported to attenuate the development of gastric mucosal lesions in different rat models of experimentally induced gastric ulcers, including water immersion restraint stress-, histamine-, non-steroidal anti-inflammatory drugs-, and pylorus-ligated-induced gastric lesions (Inoue *et al.*, 2005, Al-mofleh *et al.*, 2006, Xu *et al.*, 2009). The mechanism of this protection was proposed to be mediated by a decrease in gastric non-protein sulfhydryl content (Al-mofleh *et al.*, 2006).

NOS inhibitors such as L-NIO [*N*-(1-iminoethyl)-L-ornithine], L-NMA (*N*-methyl-L-arginine), L-NIL [*N*-(1-iminoethyl)-L-lysine], and L-NNA (*N*-nitro-L-arginine) have been shown to inhibit gene expression of iNOS, NO production, and caspase-3 activity (Kiang, 2004). These results imply that iNOS activates apoptotic pathways, which in turn damage tissues. To our knowledge, there is no report about the anti-inflammatory and anti-apoptosis effects of crocin on gastric mucosa following I/R injury in the rat. Therefore, the aim of the present study was to evaluate the protective effect of crocin on gastric mucosal lesions induced by I/R injury in the rat by evaluating the changes in the protein expression levels of active caspase-3, an apoptotic factor, and inducible nitric oxide synthase (iNOS), a pro-inflammatory protein, in gastric mucosa.

MATERIAL AND METHODS

Material

Chemicals: Crocin was purchased from Sigma. All antibodies were purchased from Abcam (USA).

Animals

Male Wistar rats weighing 150-200 g were purchased from the animal house of Ahvaz Jundishapur University of Medical Sciences. The animals were fed conventional diet and had free access to tap water. They were maintained under standard conditions of humidity, temperature (22±2 °C) and 12-h light/dark cycle. The animals were deprived of food but not water overnight before intervention. All experiments

were carried out in accordance with the regulations set by the ethics committee of Ahvaz Jundishapur University of Medical Sciences (RDC9311).

Methods

Animal grouping and experimental procedures

The rats were randomly assigned to 4 groups (n=8): sham, I/R, I/R + crocin pretreatment and crocin alone groups. Gastric I/R injury was induced according to Wada *et al.*, (1996). Briefly, under anesthesia with a mixture of ketamine and xylazine (60+15 mg/kg, i.p.), the rats underwent a midline laparotomy and the celiac artery was carefully isolated from its adjacent tissues. The celiac artery was then clamped by a ligature for 30 min to induce ischemia, and the ligature was removed to allow reperfusion for 3 h. The I/R injury group received a single i.p. injection of vehicle (normal saline) 30 min prior to ischemia. Sham-operated rats underwent laparotomy without inducing I/R injury. To investigate the gastroprotective effect of crocin against mucosal damage induced by I/R injury, one group of animals (I/R + crocin pretreatment) received a single intraperitoneal injection of crocin at a dose of 15 mg/kg, 30 min prior to I/R injury. In a preliminary study, we evaluated the gastroprotective effect of crocin at 3 doses (7.5, 15, and 30 mg/kg) against I/R injury and showed that the optimal dose was 15 mg/kg (Mard, Azad, 2014). Therefore, this dose of crocin was used in the present work. The crocin alone group received crocin (15 mg/kg, i.p.) without any surgical operation. At the end of the experiment, animals were killed by cardiac puncture. To measure the gastric mucosal lesions, the stomach of animals was removed, opened along the greater curvature, rinsed with physiological saline and pinned out in ice-cold saline. To calculate the degree of gastric lesions, the total area of mucosal lesions were measured by Image J software. The lesion area was expressed as the percentage of ulcerated area/the total area of the glandular stomach except for the fundus using the following formula: UI (%) = [Ulcerated area/total stomach area except fundus]×100 (Yonezawa *et al.*, 2007). Immediately after taking a photograph of the stomach for measurement of the surface area of gastric lesions, samples of gastric mucosal tissue (50 mg each) including the lesion area and the surrounding ulcer margin were quickly excised, snap-frozen and stored in liquid nitrogen for molecular analysis.

Microscopic evaluation of mucosal damage

For histological evaluation, stomachs from all groups were fixed in 10% formalin, dehydrated in a graded ethanol series, and embedded in paraffin. Afterwards,

5- μ m sections of tissue were cut, stained with hematoxylin and eosin and assessed microscopically (Olympus IX50).

Protein extraction

The Tripure Isolation Reagent (Roche Diagnostics, Indianapolis, USA) was used to extract the total proteins from gastric mucosal tissue, according to the manufacturer's instructions. The protein pellets obtained from the gastric mucosal samples were dissolved in 1% SDS; protein concentration was determined by the Bradford assay, and mucosal proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Western blot analysis

Mucosal proteins were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk dissolved in Tris-buffered saline with 0.1% Tween 20 (TBST, pH 7.6) for 6 h and then incubated overnight at 4 °C with anti-iNOS antibody (rabbit polyclonal, 1:400; Abcam [ab95441], USA), anti-caspase-3 antibody (rabbit polyclonal, 1:1000; Abcam [ab90437], USA) or anti-beta actin antibody (mouse monoclonal, 1:5000; Abcam [ab20272], USA). After 5 washes with TBST, membranes were incubated with a HRP-conjugated rabbit polyclonal anti-mouse IgG antibody; 1:7000) for 2 h at room temperature. Labeled proteins were detected using a chemiluminescence Western blotting system. The expression of the proteins studied was semiquantified by Image J analysis software and the values were normalized to β -actin.

Statistical analysis

Data are shown as mean \pm S.E.M. Statistical analysis was performed by one-way ANOVA and followed by a post hoc Turkey test. Significance was set at the $P < 0.05$ level.

RESULTS

Effect of crocin pretreatment on gastric mucosal lesions induced by I/R injury

Microscopic examination showed gastric lesions with multiple erosions, exfoliation and necrosis of superficial cells, hemorrhage in the mucosal layer, marginal inflammation and neutrophil aggregation and severe alterations in the architecture of glandular parts of the gastric mucosa 3 h after ischemia. Pretreatment with crocin significantly attenuated the gastric lesions induced by I/R injury. No gastric damage was observed in the gastric mucosa of crocin alone and sham-operated rats (Figure 1). As shown in Figure 2, crocin pretreatment

significantly decreased the total area of mucosal lesions as compared with the I/R injury rats ($P < 0.001$).

Effect of crocin pretreatment on mucosal protein expressions of active caspase-3 and iNOS

The level of iNOS protein expression in I/R + crocin pretreatment animals was significantly lower than in control I/R injury rats ($P < 0.01$) (Figure 3A). Figure 3A also shows that iNOS protein was not expressed in

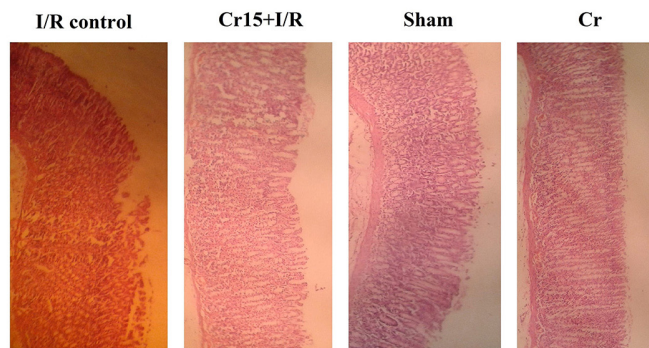


FIGURE 1 - Histological evaluation of the gastric mucosa. Representative gastric sections were obtained 3.5 h after I/R injury. Crocin alone (Cr) and sham-operated groups depict no disruption in the surface epithelium. The I/R injury animals show multiple erosions (A-B), exfoliation and necrosis of superficial cells (arrow shows necrosis in Figure 1A), hemorrhage in the mucosal layer (arrows show hemorrhage in Figure 1B) and severe alterations in the architecture of glandular parts of the gastric mucosa. Cr15+I/R: rats pretreated with crocin (15 mg/kg 30 min before intervention) demonstrate mild disruption of the surface epithelium. All sections stained with hematoxylin and eosin; $\times 100$ magnification.

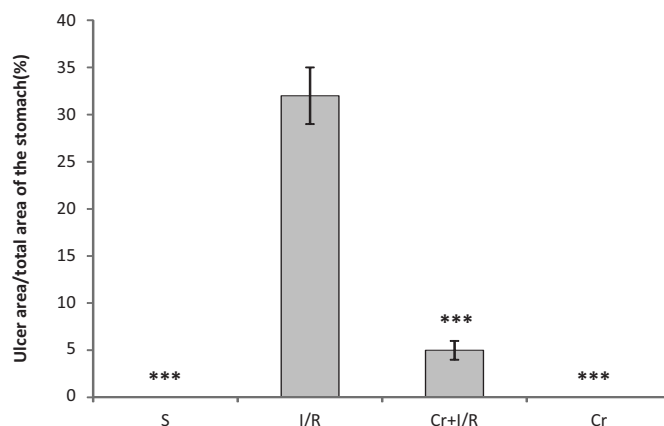


FIGURE 2 - The calculated ulcer index in S: sham-operated group, I/R: I/R injury animals, Cr+I/R: rats pretreated with crocin (15 mg/kg, i.p.) 30 min before intervention, Cr: crocin alone group (intact animals that received crocin at 15 mg/kg, i.p.). *** $P < 0.01$ relative to I/R injury group. Data are expressed as mean \pm S.E.M.

the crocin-alone and sham-operated groups. As shown in Figure 3B, 30 min gastric ischemia followed by 3 h reperfusion significantly increased the basal level of active caspase-3 expression as compared with the sham-operated animals ($P<0.01$). This level was significantly decreased by crocin pretreatment ($P<0.01$). There was no difference in the level of protein expression of caspase-3 between crocin-alone and sham-operated rats.

DISCUSSION

The results of this study showed that: (1) pretreatment with crocin decreased the total surface area of the acute gastric mucosal lesions induced by I/R; (2) the protein expression level of iNOS was lower in crocin-pretreated rats than in the I/R injury rats; and (3) the protein expression level of active caspase-3 was higher in the I/R injury rats than in I/R+crocin-pretreated and sham-operated rats.

It has been shown that mRNA and protein expression of iNOS is upregulated following gastric I/R injury in mice and rats (Kobata *et al.*, 2007; Yi *et al.*, 2012). Consistent with these findings, the present study demonstrated that iNOS protein expression drastically increased after I/R injury in the rat stomach. The present results also showed that pretreatment with crocin significantly reduced mucosal lesions and the level of iNOS expression. Our

findings showed that crocin, when administered alone, did not produce any change in the protein expression level of iNOS. This finding is in agreement with a previous study showing that crocin, when administered alone, caused no change in basal NO level in microglial cells but markedly protected these cells against LPS-induced inflammation (Nam *et al.*, 2010). These findings suggest that the beneficial gastroprotective effect exerted by crocin is partly mediated by inhibition of iNOS protein expression.

To gain insight into other possible mechanisms that mediate the beneficial effects of crocin against I/R-induced gastric mucosal injury, a molecular marker for apoptosis (expression of active caspase-3) was also investigated. It has been shown that Yangsan flavonoid protected the heart against myocardial I/R injury through the down-regulation of the expression of apoptosis genes including caspase-3 and adenine nucleotide translocator-1 in rats (Zhang *et al.*, 2014). Moreover, *N*-acetylcysteine has been reported to protect the gastric mucosa against I/R injury in rats by reducing mRNA expression of caspase-3 in gastric mucosa (Zhou *et al.*, 2010). Therefore, these cited findings show that antioxidants protect the gastric mucosa against I/R injury through their anti-apoptotic activity. The results of the present study showed that 3.5 hours following I/R injury, the level of caspase-3 protein expression increased. As shown in Figure 3B, the

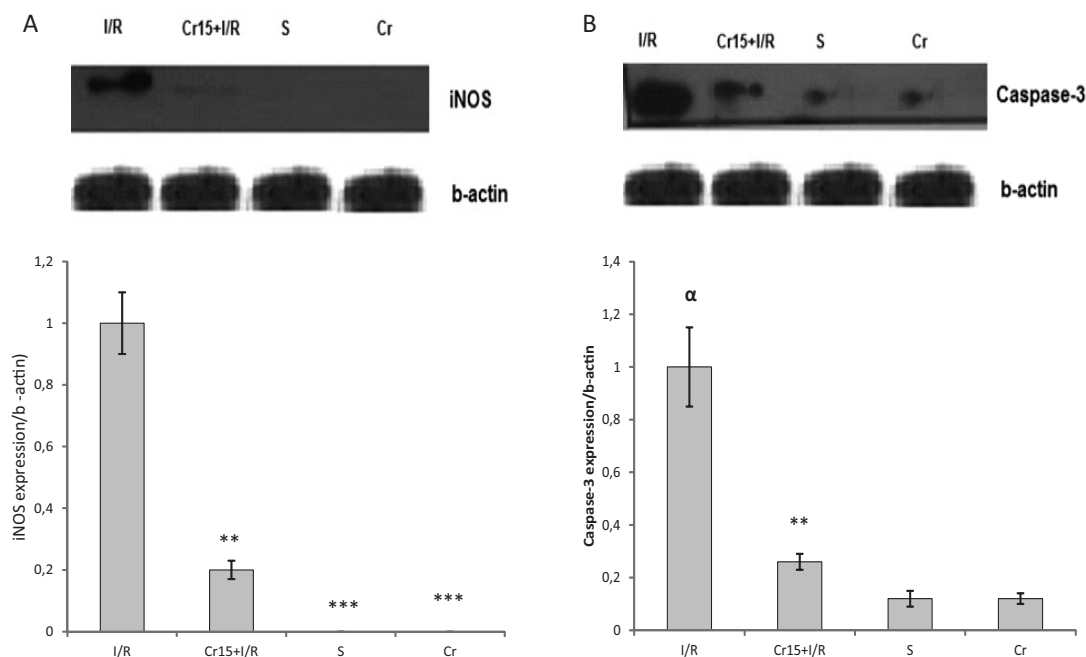


FIGURE 3 - Western blot analysis of iNOS (A) and caspase-3 (B) protein expression in the rat gastric mucosa following I/R injury. I/R: I/R injury animals, Cr15+I/R: rats pretreated with crocin (15 mg/kg, i.p.) 30 min before intervention, S: sham-operated group, Cr: crocin alone group (intact animals that received crocin at 15 mg/kg, i.p.). Crocin pretreatment caused a significant decrease in iNOS and caspase-3 protein expression. $^{\alpha}P<0.001$ indicates a significant increase versus sham-operated and crocin alone groups; $^{**}P<0.01$ and $^{***}P<0.01$ versus control I/R injury group. Data are expressed as mean \pm SEM. The protein levels were normalized to β -actin.

protein expression level of active caspase-3 significantly decreased with crocin pretreatment. Consistent with this finding, crocetin [another active constituent of saffron] has been shown to inhibit apoptosis at early stages of the brain injury following cerebral contusion, through up-regulation of Bcl-2 expression (Bie *et al.*, 2011). Moreover, crocin has been reported to prevent retinal ischemia/reperfusion-induced apoptosis in retinal ganglion cells in rats (Qi *et al.*, 2013). The protein expression level of caspase-3 with crocin alone was not different from sham-operated baseline values. Therefore, it can be concluded that another gastroprotective effect of crocin against I/R injury is partly mediated by decreasing caspase-3 protein expression.

In addition to crocin having a direct effect of on caspase-3 expression, it might have indirectly decreased caspase-3 expression by inhibiting iNOS expression in crocin-pretreated rats. As mentioned earlier, NOS inhibitors, in addition to down-regulating the gene expression of iNOS, inhibit caspase-3 activity (Kiang, 2004). Moreover, iNOS has been demonstrated to enhance intestinal apoptosis following I/R injury in rats (Wu *et al.*, 2002). Consistent with previous reports, the present findings showed that the basal levels of protein expression of caspase-3 and iNOS increase in I/R injury rats. Therefore, these results together suggest that the decrease in caspase-3 protein expression in crocin-pretreated rats may be secondary to the down-regulation of iNOS protein expression by crocin pretreatment.

CONCLUSION

In this *in vivo* study, an acute I/R-induced gastric lesion model was used to identify the role of iNOS and caspase-3 in the mechanisms of the gastroprotective effect of crocin. The results showed that gastric I/R injury caused a significant increase in iNOS and caspase-3 protein expression, which was significantly attenuated by pretreatment with crocin.

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