Therapeutic Effect of Crocin on the NASH Model by Targeting the Fas Receptor Signaling Pathway

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ABSTRACT

Background: *The role of hepatocyte apoptosis and inflammation has been implicated in the progression of nonalcoholic steatohepatitis (NASH). Overproduction of reactive oxygen species (ROS) appears to accelerate these pathways through the activation of Fas receptor signaling. Therefore, we explored the hepatoprotective effects of crocin as a strong free radical scavenger against oxidative damages leading to NASH development.*

Methods: *Thirty-two male mice were randomly divided into control, NASH, NASH + crocin, and crocin groups. They received an intraperitoneal injection of crocin twice a week, for 3 weeks. For NASH model induction, the animals were fed with a Western diet and exposed to cigarette smoke for 8 weeks. At the end of the experiment, liver histology, biochemical, and biomolecular analyses were done to evaluate the antioxidant, anti-inflammatory, and anti-apoptotic activities of crocin in the NASH model.*

Results: *Evaluation of the features of the NASH model revealed steatosis, inflammatory infiltrate, and ballooning degeneration. Metabolic dysfunction was associated with elevated serum levels of the lipid profile and decreased hepatic liver enzymes. The increased content of malondialdehyde (MDA) and reduced antioxidant activities confirmed hepatotoxicity induction. There was a significant increase in expression level of Fas, caspase 3, and NF-κB genes that was also associated with elevation in hepatic TNF-α content. Moreover, expression the of Fas receptor protein was significantly detected on the hepatocyte membrane.*

Treatment with crocin effectively improved NASH-related parameters, and the histopathological findings were also parallel with the resulting changes.

Conclusion: *Crocin can be introduced as a candidate hepatoprotective agent against NASH by virtue of its anti-inflammatory, antioxidant, and anti-apoptotic properties, possibly through regulation of the Fas death receptor pathway.*

Keywords: *Apoptosis, cigarette smoke, Fas receptor, inflammation, nonalcoholic steatohepatitis, Western diet*

INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is now regarded as a severe medical condition of nonalcoholic fatty liver disease (NAFLD), with an unpleasant progression to late stage of liver failure.[1]

According to the current and accepted theory for pathogenesis of NAFLD, lipid deposition in hepatocytes leads to development of hepatic steatosis. Subsequently, steatotic hepatocytes are more vulnerable to oxidative stress as a key contributor, increasing the probability of disease progression and NASH development.[2,3]

Liver cell death and hepatic inflammation have been represented as the main features of NASH.[4] Hepatocellular apoptosis, as the most common cause of cell death, is typically considered to occur in the liver of the patients with NASH.[5] The Fas/CD95 receptor is one of the death receptors belonging to the tumor necrosis factor (TNF)-receptor superfamily[6] and is widely expressed by hepatocytes, especially in disease processes.[7] It mainly mediates apoptotic signaling in the liver. The downstream consequence of its activation is recruitment of several intracellular caspases including caspase-3, caspase-6, and caspase-7.[4]

In addition to the role of Fas/CD95 as a main mediator of apoptosis, it may also contribute to the induction of inflammation initiated by upregulation of nuclear factor kappa B (NF-κB) activity.[8]

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As mentioned above, oxidative stress resulting from excessive intracellular reactive oxygen species (ROS) can trigger hepatocellular injury associated with development of NASH. In this context, ROS have been previously implicated in a key role in cell death through Fas-mediated apoptotic pathways.[9]

Overexpression of the Fas receptor, which has been reported to be increased in liver disease, plays an important role in the progression of NASH.[5] Moreover, apoptosis as a main contributor to liver injury in NASH appears to be promoted by oxidative stress and ROS production that can activate Fas-mediated cell death signaling pathways.[10] Therefore, it is hypothesized that oxidative injury by elevated ROS can be a trigger for apoptotic and non-apoptotic Fas-related signaling pathways.

In spite of growing attempts for development of novel prognostic and therapeutic interventions, no effective drug therapy has been approved for this disorder.[11]

It seems that the modulating strategy against ROS-induced hepatocellular injury through Fas receptor activation is worthy of consideration. Considering the free radical regulatory function of antioxidants[12] and the capability of ROS to induce oxidative stress as a main apoptosis-driving factor, antioxidant therapy can be a promising approach to protect against cytotoxicity.

The antioxidant activity of crocin has been well understood previously.[13] The cytoprotective effects of crocin have also been reported, and it has achieved a special place in pharmaceutical science based on its anti-apoptotic, anti-inflammatory, and anti-tumor functions.[14,15] However, the mechanisms involved are not fully understood.

So far, no study has investigated the hepatoprotective effect of crocin against apoptosis and inflammation in a mouse model of NASH. Therefore, our study was designed to evaluate the possible anti-apoptotic and anti-inflammatory effects of crocin in the NASH model, focusing on activation of the Fas receptor as an oxidative stress-mediating factor.

MATERIALS AND METHODS

Animal Grouping and Experimental Procedures

A total of 32 adult male NMRI mice (25-30 g) were housed in a controlled environment (temperature of 22 ± 2°C, 12 h cycles of light and dark), with free access to their desired diet. However, they were exposed to fasting and only had free access to tap water 12 h prior to beginning the experiments. All the mice were treated according to the guidelines of Care and Use of Laboratory Animals.

For induction of the NASH model, the mice were fed *ad libitum* with a Western diet[11] containing 30% of fat (tallow), 30% of carbohydrates, and 0.4% of cholesterol for 8 weeks, and were exposed to cigarette smoke during the last 4 weeks of diet feeding (4 cigarettes daily, 5 days a week, for 4 weeks).[16,17]

Crocin (Sigma-Aldrich Co., USA) at a dose of 100 mg/kg was used according to the previous report, to confirm its hepatoprotective (antioxidant, anti-fibrotic, and anti-inflammatory) effects in mice.[18]

Crocin was administered through intraperitoneal injection at the end of the NASH induction period, twice a week for 3 weeks (Figure 1).

The control group of animals remained on standard chow and were exposed to air while the NASH groups were fed with a Western diet[11] and exposed to cigarette smoke until the 11th week. It should be noted that mice received saline injections during the last 3 weeks.

Therefore, the mice were randomly and equally assigned to one of the following 4 groups, each consisting of 8 animals: Control (Ctrl), NASH, NASH + crocin 100 mg/kg (NASH+Cr), and crocin 100 mg/kg (Cr).

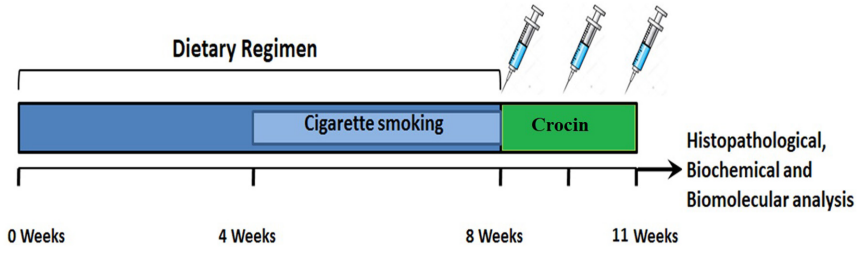

Figure 1. Schematic of study design.

Finally, the sera and all the harvested livers were separated for histopathological, biochemical, and molecular evaluations.

Determination of the Biochemical Parameters

Blood samples were collected under deep anesthesia produced by ketamine and xylazine injection (80 + 6 mg kg^{-1}, i.p., respectively) and were immediately centrifuged. The sera were separated to biochemically determine levels of aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) as biomarkers of liver injury, and concentrations of triglyceride (TG), total cholesterol,[12] and high-density lipoprotein (HDL) were specified using the appropriate commercial kits (Pars Azmoon; IR Iran), by a serum autoanalyzer (BT-1500-A-A, Rome, Italy). The level of low-density lipoprotein-cholesterol (LDL-C) was calculated as total cholesterol – (HDL-C + triglyceride/5).

Determination of the Antioxidant Activity

Liver samples were rapidly removed and homogenized to measure the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) using standard commercial kits (Zellbio GmbH, Germany).

Determination of MDA Concentration

The concentration of hepatic malondialdehyde (MDA), as the most widely used marker of lipid peroxidation, was assessed after centrifuging (10 000 g, 15 min, 4°C) liver homogenate according to reaction of MDA with thiobarbituric acid. The result was measured colorimetrically at 532 nm according to the manufacturer's instructions, and expressed on the diagnostic ZellBio kit (Zell Bio, Germany).

Determination of TNF-α Level

The hepatic concentration of tumor necrosis factor alpha (TNF)-α from the liver tissue of mice was measured by a commercial enzyme-linked immunosorbent assay kit, according to the manufactures' instructions based on the biotin double-antibody sandwich technology (BT, China).

Liver Histology

Liver tissue slices were obtained and fixed in 10% formalin before staining with hematoxylin-eosin (H&E) and Oil Red O dyes. Hepatic tissue histology was evaluated to blindly score according to semi-quantitative NAFLD activity score (NAS) by an experienced pathologist. The sum of scores from steatosis (0-3), lobular inflammation (0-3), and hepatocellular ballooning degeneration (0-2) was considered for confirmation of NASH (score of 0-2: absence of NASH; 3-4: borderline; and 5-8: NASH).[19,20] Oil Red O staining was used for evaluation of the abundantly deposited lipid droplets in cytoplasm.

Real-time PCR

Total RNA was extracted from frozen tissue samples using the RNeasy Plus Mini Kit (Qiagen; Qiagen GmBH, Hilden, Germany). The concentration and purity of RNA were estimated spectrophotometrically at wavelengths of 260 nm and 280 nm using the NanoDrop device (Thermo Scientific S.N: D015). Reverse transcription of RNA was performed for synthesis of cDNA using a QuantiTect Reverse Transcription Kit (Qiagen; Qiagen GmBH, Hilden, Germany) according to the manufacturer's instructions. The mRNA expression levels of Fas (CD95), caspase-3, and NF-κB were measured based on quantitative real-time polymerase chain reaction (PCR) protocol on a light Cycler (Roche Diagnostics Indianapolis, Ind, USA) using the SYBR Green Master Mix and Applied Biosystems StepOne Real-Time PCR System. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), representing the standard housekeeping gene, was used for normalization of expression level of target genes. The primer sequences used for each gene were as follows: mouse Fas: TCTCATGGGAAGAGTGATGC (forward) and TGTCTGGGGTTGATTTTCCA (reverse); caspase: GCTTGGAACGGTACGCTAA (forward) and CTTGCTCCCATGTATGGTCT (reverse); NF-κB: ACC CGAAACTCAACTTCTGT (forward) and TAACAG CATGGGGGAAAACT (reverse); and GAPDH: CAGTGGCAAAGTGGAGATTG (forward) and TTGATGTTAGTGGGGTCTCG (reverse).

Immunostaining Analysis

Paraffin-embedded formaldehyde-fixed liver sections were stained immunohistochemically for detection of the Fas (CD95) death receptor-related antigen. According to the immunohistochemical method, sections of tissue blocks, 5 μm thick, were deparaffinized and rehydrated in graded ethanol. Antigens were retrieved by microwaving in 10 mmol citrate buffer solution (pH 6.0). Hydrogen peroxide was used for blocking endogenous peroxidase activity with 1:10 dilution in methanol. Phosphate-buffered saline (PBS) was applied for prevention of non-specific binding. The sections were incubated with Fas monoclonal antibody as the primary antibody, and the rabbit polyclonal antibody was applied as the secondary antibody. 3,3′-diaminobenzidine[18] was used as a chromogen to

show antibody. Finally, the sections were counterstained in hematoxylin and fast green staining dyes. They were then cover-slipped. Positive and negative controls were stained at the same setting, treating with PBS as primary antibodies.

Statistical Analysis

All data were expressed as means ± standard error of means (SEM). One-way analysis of variance along with Tukey's post-hoc test was used for identification of significant differences between experimental groups. The Kruskal–Wallis test was used to analyze histopathological scoring.

RESULTS

Histological Evaluations

Histopathological examination of liver sections in the control group showed normal architecture. However, there was no considerable difference with respect to liver parenchyma alterations between control (Ctrl) and crocin -treated groups (Cr) ($P > .05$) (Figure 2). The Western diet and the exposure to cigarette smoke caused the development of NASH features, with significant micro- and macro-vesicular steatosis scored as grade 3, obvious ballooning degeneration, and the increased inflammatory infiltration (grade 3) in liver sections of the NASH group mice. Inflammatory infiltrate was prominently seen in the periportal area, conforming to grade 2. There was a significant increase in NAS compared to the control group ($P < .01$). As shown in Figure 2D, treatment with crocin substantially reduced both steatosis (grade 2) and liver injuries and the crocin-treated groups revealed significantly less NAS than the control group ($P < .05$).

Oil Red O staining indicated the marked deposition of lipid droplets in hepatocytes, as shown in Figure 2C.

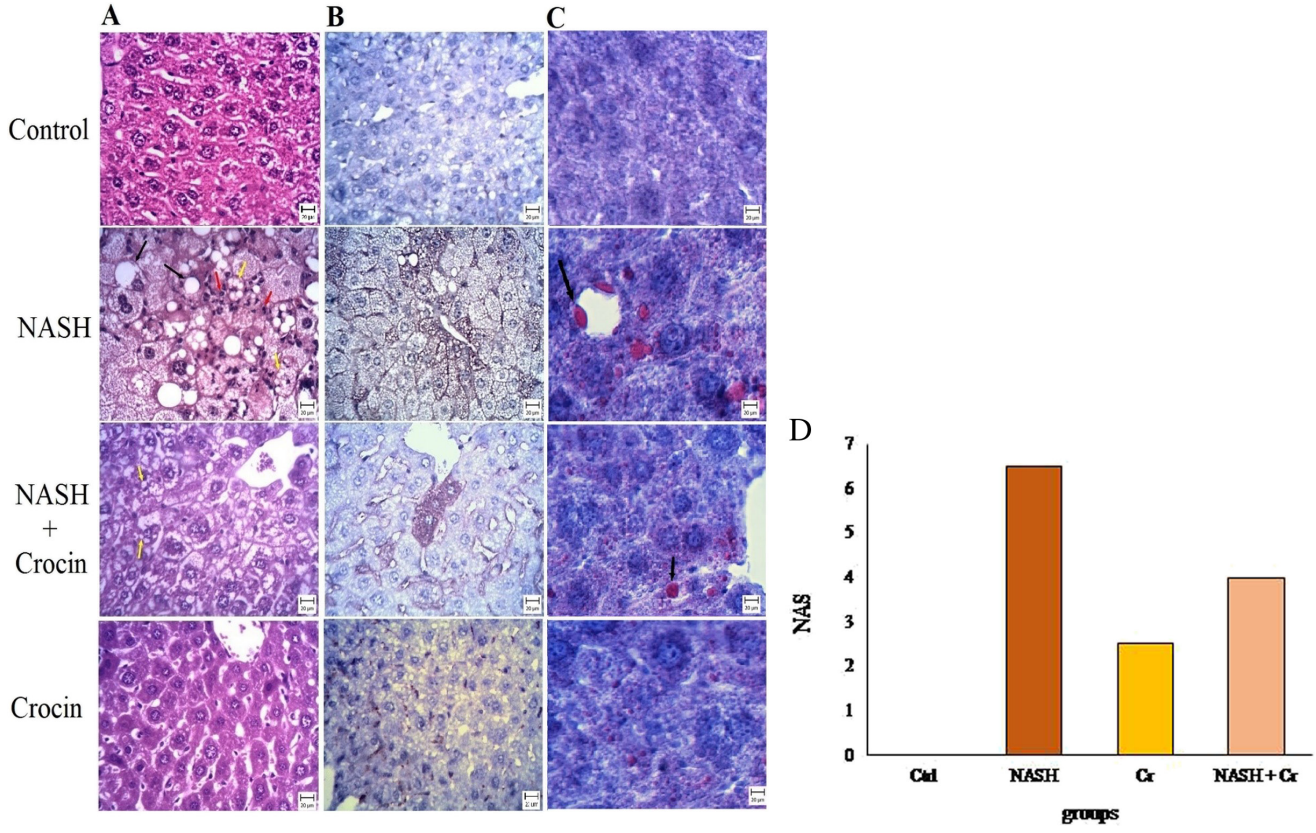

Figure 2. The effect of crocin on the histopathological features of liver. (A) Representative microscopic images of liver sections stained with hematoxylin and eosin. Black and yellow arrows represent macro- and micro-vesicular lipid droplets respectively. Red arrows represent inflammatory infiltration. (B) Immunohistochemical staining specific for Fas receptor. Positive hepatocytes for Fas were detected mainly stained on the cell surface. (C) Oil Red O staining for lipid droplet accumulation in hepatocytes. Black arrows represent deposition of lipid. (D) The effect of crocin on NAS score with hematoxylin and eosin staining using the semi-quantitative NAS System. Data are analyzed as mean rank of hepatic histopathological scores in different groups. Ctrl, control; Cr, crocin; NAS, NAFLD activity scoring.

Table 1. The Effect of Crocin on Serum Activity Levels of ALT, AST, and ALP

	Ctrl	NASH	Cr	NASH + Cr
ALT (U/L)	64.9 ± 3.4	303.6 ± 7.1**	85 ± 4.5	123.5 ± 7.7
AST (U/L)	133 ± 2.9	611.7 ± 17.8***	202.3 ± 9.3	247.6 ± 17.9
ALP (U/L)	188 ± 6.2	666 ± 37.1***	213 ± 6.3	208.1 ± 6.8

Data are expressed as means ± SEM, n = 8 in each group. **$P < .01$, ***$P < .001$ significant difference compared to mice subjected to NASH model and treated with crocin. Ctrl, control; Cr, crocin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

Table 2. The Effect of Fas Receptor Silencing on Serum Concentration of TG, TC, HDL, and LDL

	Ctrl	NASH	Cr	NASH + Cr
TC (mmol/L)	83.8 ± 4.2	328.2 ± 8.9***	79.5 ± 4.1	117.7 ± 17.7
TG (mmol/L)	72.1 ± 3.5	258.6 ± 7.8**	74 ± 4.6	76.7 ± 6.9
LDL (mmol/L)	23.3 ± 0.8	54.5 ± 2.8*	22.5 ± 1.3	34.7 ± 4.1
HDL (mmol/L)	49.7 ± 1.3	17.8 ± 0.9***	48.3 ± 1.9	42.8 ± 1.8

Data are expressed as means ± SEM, n = 8 in each group. ***$P < .001$ significant difference compared to mice subjected to NASH model and treated with crocin. Ctrl, control; Cr, crocin; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

According to our immunohistochemical evaluations, the Fas receptor was expressed mainly on the hepatocyte membranes in the NASH group. Semi-quantitative analyses showed that hepatic expression of Fas was strongly decreased in crocin-treated mice (Figure 2B).

Biochemical Assessments

Table 1 shows serum activity levels of the liver function test (ALP, ALT, and AST).

Compared to the control group, the marked elevation of serum ALT, AST, and ALP activities after 8 weeks confirmed hepatocellular damage in the NASH group ($P < .01$, $P < .001$). As shown in Table 2, induction of the NASH model led to a significant increase in the serum level, as seen in the lipid profile. Conversely, high activity levels of ALT, AST, and ALP were decreased to near normal levels ($P > .05$) in the Cr group. The lipid profile was also decreased in serum of the Cr group compared to the NASH group ($P > .05$).

The activity levels of GPx and SOD were significantly lower than those of the control group, after NASH induction ($P < .01$, $P < .001$) (Figure 3), but MDA concentration was enhanced significantly (Figure 4) ($P < .001$). Compared to the NASH group, there was a significant decrease in the lipid peroxidation biomarker level and significant elevation in antioxidant activity in the Cr group.

Molecular Assessments

Activation of apoptosis signaling pathways (especially the death receptor) by ROS has been established.[9] Hence, herein, it was attempted to investigate whether crocin administration can control Fas activation and its downstream effectors. Compared to the control group (Figure 5), induction of NASH resulted in a strong

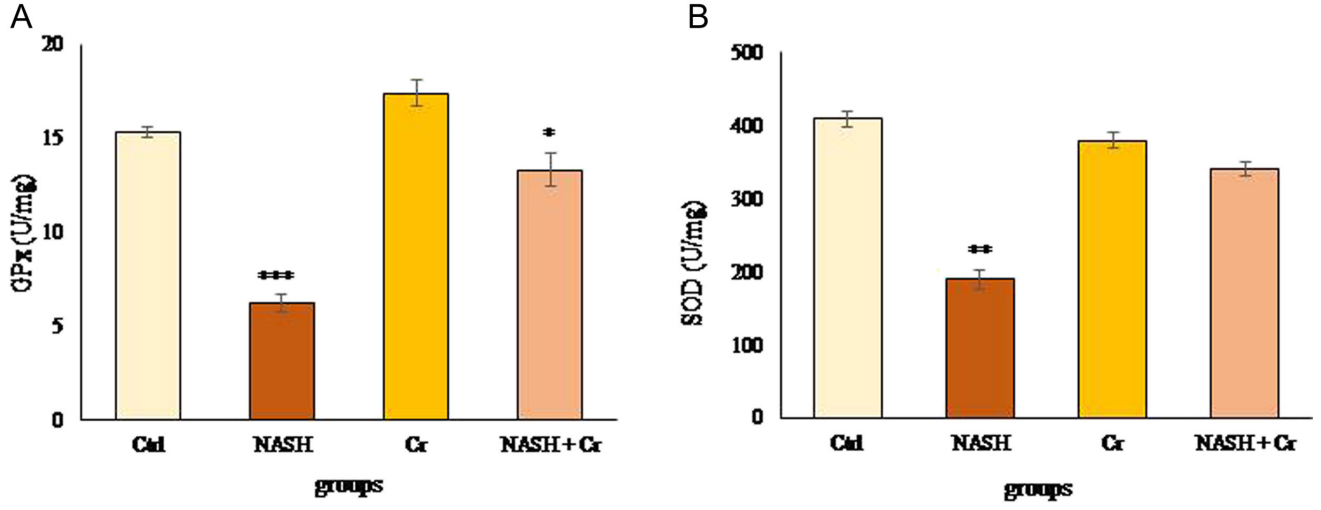

Figure 3. The effect of crocin on antioxidant activity of GPx (A), and SOD (B). Data are expressed as means ± SEM, n = 8 in each group. ***$P < .001$ significant difference compared to mice subjected to NASH model. Ctrl, control; Cr, crocin; GPx, glutathione peroxidase; SOD, superoxide dismutase.

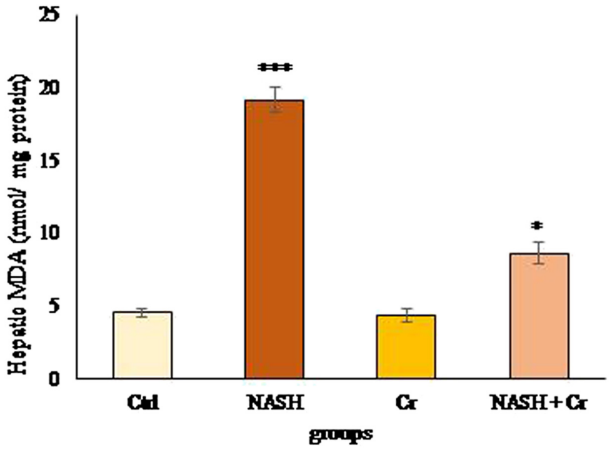

Figure 4. The effect of crocin on antioxidant concentration of lipid peroxidation marker, MDA. Data are expressed as means ± SEM, n = 8 in each group. ***$P < .001$ significant difference compared to mice subjected to NASH model. Ctrl, control; Cr, crocin; MDA, malondialdehyde.

increase in expression levels of Fas receptor and caspase -3 gene in the liver ($P < .001$), and crocin significantly attenuated these alterations compared to the NASH group ($P < .05$).

As an oxidative stress-responsive transcription factor, NF-κB appears to be involved in non-apoptotic Fas-induced inflammatory pathways. Our findings revealed a significant increase in the level of NF-κB gene expression following NASH induction ($P < .001$). According to Figure 5C, crocin administration could downregulate the hepatic level of NF-κB gene expression, compared to the results in the NASH group ($P < .01$).

In analyzing inflammatory response, TNF-α hepatic level was considered, which may be regulated by NF-κB to promote liver injury. As expected, considerable elevation was achieved in TNF-α concentration in the liver of mice in the NASH groups' ($P < .001$). However, animals treated

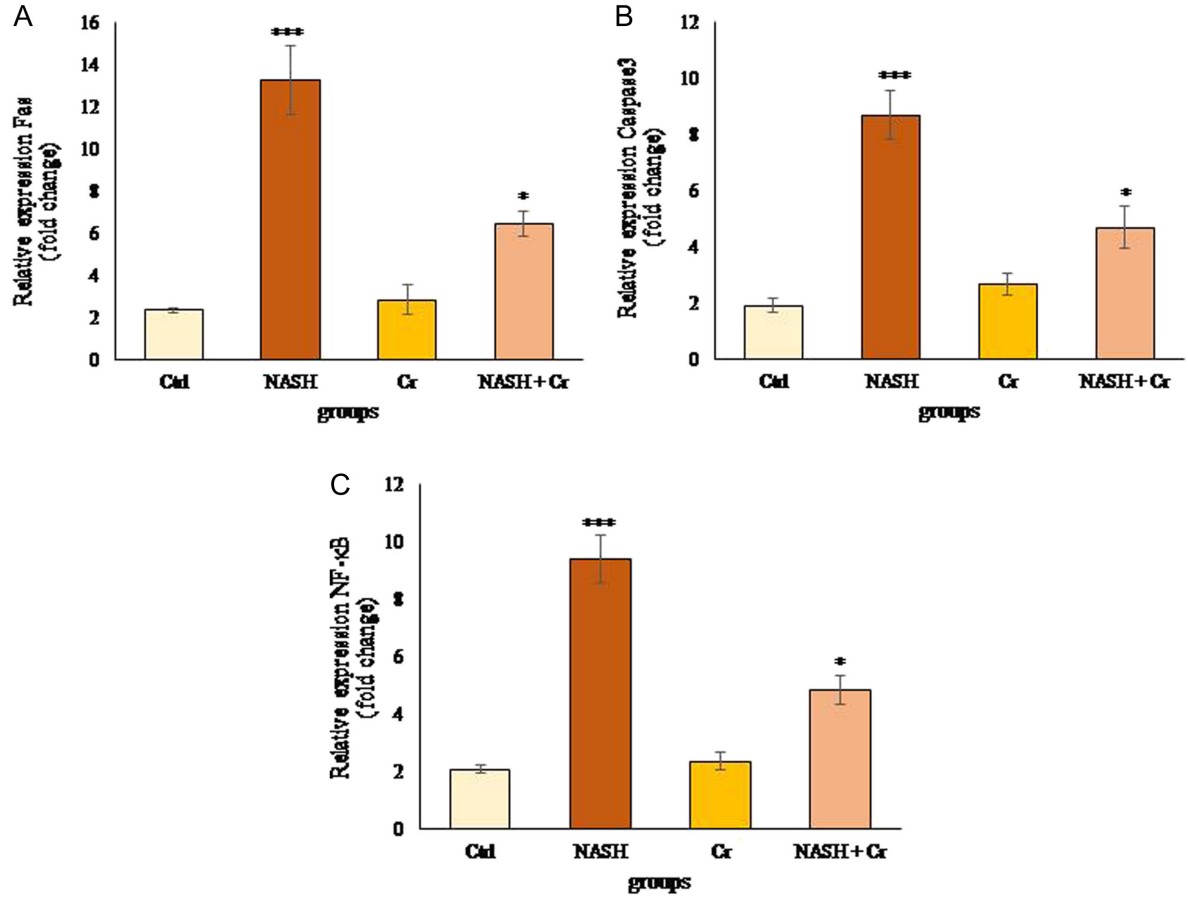

Figure 5. The effect of crocin on gene expression levels of Fas (A), caspase3 (B) and NF-κB (C). Data are expressed as means ± SEM, n = 8 in each group. **$P < .01$ and ***$P < .001$ significant difference compared to mice subjected to NASH model. Ctrl, control; Cr, crocin; NF-κB, nuclear factor kappa B.

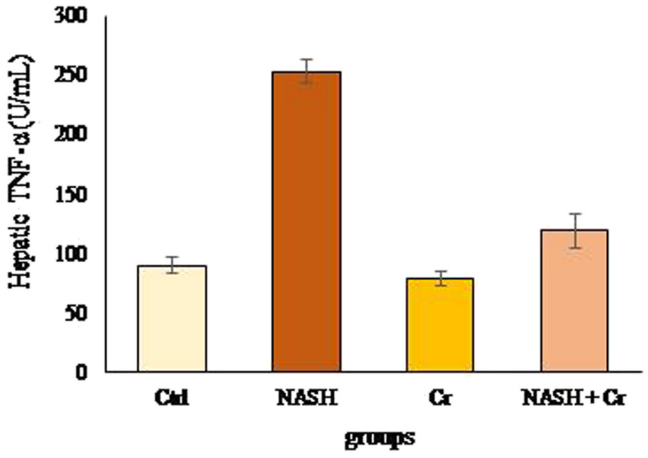

Figure 6. The effect of crocin on hepatic inflammatory cytokine levels. TNF-α. Data are expressed as means ± SEM, n = 8 in each group. $^{**}P < .01$ and $^{***}P < .001$ significant difference compared to mice subjected to NASH model. Ctrl, control; Cr, crocin; TNF-α, tumor necrosis factor-α.

with crocin showed a significant suppression in hepatic TNF-α level, as confirmed by a decrease in hepatocellular degeneration and inflammatory infiltrate in comparison with the NASH group ($P < .05$) (Figure 6).

DISCUSSION

Due to the rising trend of the disproportionate intake of a fat-rich diet along with consumption of carbohydrate-rich foods known as "fast food" or "cafeteria-style" food that has increased over the past decades, NAFLD/NASH has to be considered as the most common liver disease globally.[21] In this context, cigarette smoke has been introduced as an exacerbating cofactor contributing to disease aggravation.

Despite the growing efforts to achieve an effective treatment for NASH, the current therapeutic strategies are mostly based on lifestyle management of modifiable risk factors.[22,23]

Besides, due to the complex mechanisms involved, identifying the molecular role of agents driving the pathogenesis of NASH as therapeutic targets would be essential in effective drug discovery.[24]

Cell death, as the critical mechanism of NASH progression, results in hepatocyte injury and inflammation, and oxidative stress plays a central role in mediating these cellular damages.

Crocin is well known for its medical properties, especially as antioxidant in different tissues.[25] Therefore, here, crocin was chosen based on its activity.

To the best of our knowledge, this is the first study focusing on the effects of crocin on liver injury in experimental mouse model of NASH.

The relationship between activation of Fas receptor, oxidative stress, hepatic cell injury, and inflammation signaling, all of which have been implicated to be involved in NASH progression, was evaluated. According to our promising results, crocin can be introduced as a potential agent against hepatic damage based on its regulatory function that counteracts the expression of NASH-related genes and biochemical changes.

The results of this study indicated that sub-chronic feeding of mice with a Western diet and exposure to cigarette smoke, similar to those habits currently found in our human lifestyle, could lead to induction of steatosis, ballooning degeneration, and an inflammation infiltrate that histologically confirmed the development of NASH.

In our study, crocin treatment was found to reduce NASH-related histopathological features, and was associated with improvement of lipid profile and hepatic enzyme serum levels.

The liver, as a major organ, is mostly exposed to ROS which mediate tissue damage.[26] On the other hand, oxidative stress, as a factor that affects liver disease, is an important cause of the severity and progression of the diseases.[26,27]

Increased hepatic MDA concentration, as an indicator of lipid peroxidation, and decreased SOD and GPx activity levels have all been shown in NASH group of mice, which are commonly referred to as oxidative stress. As expected, crocin was able to reverse these alterations, thereby confirming its antioxidant role in the NASH model.

These results are in accordance with the previous findings reporting the central role of oxidative stress in pathogenesis of NAFLD. In addition to the decreased activity levels of hepatic antioxidant enzymes, MDA level has been also found to increase in patients with NAFLD.[28,29] In a preclinical model of NASH, a significant decrease was also shown in gene expression levels or activity of SOD, GPx, and catalase (CAT).[30] Thus, several studies have

suggested that higher levels of oxidative stress biomarkers are associated with the increased odds for disease exacerbation.[31,32] Accordingly, oxidative stress could be considered as a major factor contributing to the progression of NAFLD.[33,34]

Oxidative stress can also trigger steatohepatitis, promoting cell death.[10,34] Therefore, in the next step of the study, the effect of crocin on expression of Fas (CD95/Apo-1) receptor was confirmed, which is considered to be an important inducer of apoptosis, known as the major mechanism of cell death.[4]

This receptor is normally expressed on hepatocytes and has been implicated to be involved in NASH development according to its enhanced expression level in patients.[7] It has been found to make the liver more vulnerable to damages mediated by the activation of apoptotic and non-apoptotic signaling pathways.[35] Hence, herein, it was attempted to investigate the probable effect of crocin on alterations in the Fas signaling pathways involved in development of NASH. In this regard, several studies have demonstrated that the Fas receptor can activate downstream caspase 3 gene expression in the extrinsic apoptotic pathway that can be triggered with oxidative stress.[9] Compared to the controls, NASH mice showed pronounced expression of Fas receptor protein, as determined by IHC staining on hepatocytes. It was also accompanied by the elevated expression levels of Fas and caspase-3 genes, indicating Fas signaling involvement. Together with the decrease in expression of Fas and caspase-3 mRNAs, low levels of Fas-positive hepatocytes in our IHC staining were achieved after crocin administration.

Although one of the main roles of ROS during NASH progression is induction of the Fas receptor ligand and consequently, activation of Fas signaling, it has been reported that ligation of Fas, in turn, results in more ROS production and pronounced cell death.

On this basis, it can be concluded that the protective effect of crocin is probably due to inhibition of excessive ROS production.

Accordingly, our results, showing that treatment with crocin was able to attenuate expression of the Fas and caspase-3 genes, raised the probability of its therapeutic potential as an anti-apoptotic candidate in the context of NASH therapy, as previously demonstrated in several models of tissue injury.[36]

In addition to caspase-related apoptotic signaling, activation of the Fas receptor may also contribute to non-apoptotic signaling including inflammatory responses, thereby leading to activation of NF-κB and enhancement of pro-inflammatory functions.[6]

As expected, crocin was effectively able to attenuate NASH-induced increase in hepatic TNF-α content and its anti-inflammatory capacity was shown to be mediated by downregulation of NF-κB expression. This was in accordance with the previous reports indicating crocin's ability to alleviate inflammation-driven tissue injuries in part by suppression of NF-κB expression.[37]

In our previous study, we provided the evidence regarding the importance of suppression of Fas signaling involved in development of NASH.[17]

Therefore, in this research, we attempted to assess a hypothesis based on the possible role of crocin in modulation of Fas receptor signaling for NASH therapy.

Altogether, based on these observations, it can be said that crocin might be able to improve the deficient hepatic functions induced in the mouse model of NASH.

CONCLUSION

Overexpression of the Fas (CD95) receptor has been shown in liver disease, and oxidative stress has been introduced as the main contributor to hepatic damage in NASH, which appears to activate the Fas signaling pathway. Therefore, in the present study, the potential therapeutic effect of crocin on NASH features including inflammation and hepatocyte injury in the setting of hepatic steatosis was evaluated. Our findings indicated that crocin effectively controlled the NASH-induced hepatocellular damage by suppression of oxidative stress and activation of Fas death receptor.

Ethics Committee Approval: The study was approved by the experimental animals ethics committee of Ahvaz Jundishapur University of Medical Science (IR.AJUMS.ABHC.REC.1398.043).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Design – M.S.A.; Supervision – M.S.A.; Resources – K.M.; Materials – K.M.; Data Collection and/or Processing

– S.F.; Analysis and/or Interpretation – S.F., R.A.; Writing Manuscript – S.F.; Critical Review – M.S.A.

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Declaration of Interest: The authors have no conflict of interest to declare.

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Availability of Data: The data that support the findings of this study are available and can be provided by Feryal Savari who is writing her thesis based on the current study. Unfortunately the data is not publicly available yet.

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