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Crocinn attenuates inflammation of lung tissue in ovalbumin-sensitized mice by altering the expression of endoplasmic reticulum stress markers

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Abstract

Endoplasmic reticulum (ER) stress plays a pivotal role in the pathogenesis of asthma. The present study aimed to investigate the reducing or suppressing effects of crocin in ovalbumin (OVA)-sensitized mice on ER stress markers. Mice were divided into six groups ($n = 5$ per group) including control, OVA-sensitized (OVA), OVA-treated crocin (OVA-Cr25, OVA-Cr50, and OVA-Cr100 mg/kg), and OVA-treated dexamethasone (1 mg/kg), (OVA-Dexa) groups. Animals 5 later groups were sensitized to OVA and the treatment groups received intraperitoneally crocin/dexamethasone in the last 5 days of the model. At the end of the study, lung tissue was evaluated for airway inflammation, caspase 12 and CHOP protein levels, and expression of ER stress markers using real-time-PCR. Sensitization with OVA significantly caused airway inflammation and induction of ER stress in mice compared to the control group based on the elevated inflammatory cells and ER stress markers in the lung tissue. Treatment with crocin and dexamethasone reduced airway inflammation and suppressed ER stress markers. Interestingly, in the OVA-Cr100 group, the suppressive effects on ER stress apoptotic markers were comparable to the OVA-Dexa group. The results suggest that crocin mediates maladaptive ER stress conditions possibly by creating adaptive ER stress status and driving protein folding correctly.

Abbreviations

AP1

activator protein 1

CAT

catalase

CHOP

C/EBP homologous protein

COPD

chronic obstructive pulmonary diseases

eIF2 α

eukaryotic translation initiation factor 2 α

ER

endoplasmic reticulum

GPx

glutathione peroxidase

GSH

glutathione

GST

glutathione-S-transferase

I/R

ischemia/reperfusion

iNOS

inducible nitric oxide synthase

IRE1 α

inositol-requiring protein 1 α

LP

lipid peroxidation

MDA

malondialdehyde

MPO

myeloperoxidase

NO

nitric oxide

OVA

ovalbumin

PC

protein carbonyls

PERK

protein kinase RNA-like ER kinase

ROS

reactive oxygen species

SOD

superoxide dismutase

UPR

unfolded protein response

XBP1

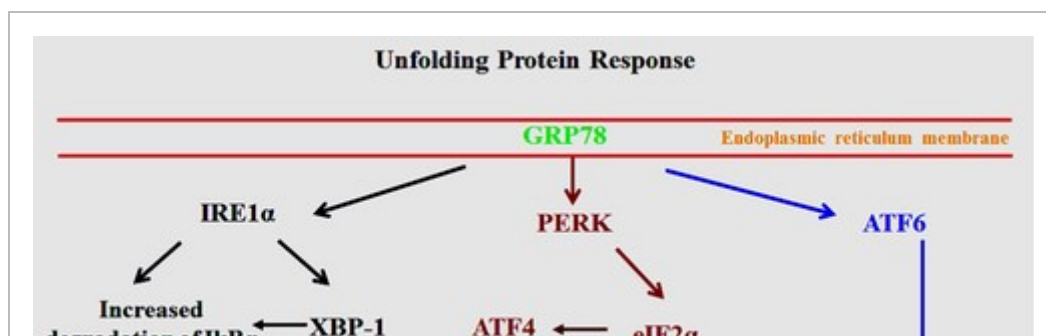
X-box-binding protein 1

XO

xanthine oxidase

1 | INTRODUCTION

The endoplasmic reticulum (ER) is the organelle that is primarily responsible for folding and modifying the proteins synthesized by ribosomes, but also includes other functions such as calcium homeostasis, lipid biosynthesis, and cellular stress.¹ ER stress is caused by the accumulation of misfolded/unfolded proteins within the ER lumen as a result of certain factors such as viral infection, reduction/oxidation imbalance, calcium imbalance, and increase in loaded of processed proteins.² Under ER stress conditions, various pathways known as unfolded protein response (UPR) are activated to restore normal organelle function³ (Figure 1). Activation of the UPR pathways results in one of the following two conditions: either ER homeostasis is provided (adaptive UPR), or as a result of prolonged and sustained activation of the UPR, the apoptotic process is designed (maladaptive UPR).⁴ Once ER stress is induced, a chaperone in the ER membrane, GRP78/BiP, binds to misfolded/unfolded proteins and disassociates from the membrane, initiating the three classical UPR pathways.^{5, 6} The first pathway is mediated by protein kinase RNA-like ER kinase (PERK) which leads to upregulation of ATF4 by phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α).^{5, 6} The second pathway is mediated by inositol-requiring protein 1 α (IRE1 α). By activating IRE1 α , X-box-binding protein 1, a nuclear transcription factor, is activated and it increases the expression of genes associated with UPR. Finally, the third pathway is dependent on ATF6, which when activated is transported to the Golgi and produces cleaved ATF6. Cleaved ATF6 translocates into the nucleus and induces several UPR-related genes.^{5, 6} On the other hand, in maladaptive UPR, a transcription factor regulating cell death called C/EBP homologous protein (CHOP/GADD153) is activated, which by upregulation of proapoptotic genes (Bax/Bak) and downregulation of antiapoptotic genes (Bcl-2), leads to the activation of caspase cascades, caspase 12 and caspase 3, cause the initiation of apoptosis process.^{7, 8} ER stress has been shown to be involved in the pathogenesis of some inflammatory diseases such as diabetes, obesity, asthma, chronic obstructive pulmonary diseases (COPD), cardiovascular disease, pulmonary fibrosis, and neurodegenerative diseases⁹⁻¹³ (Figure 1).



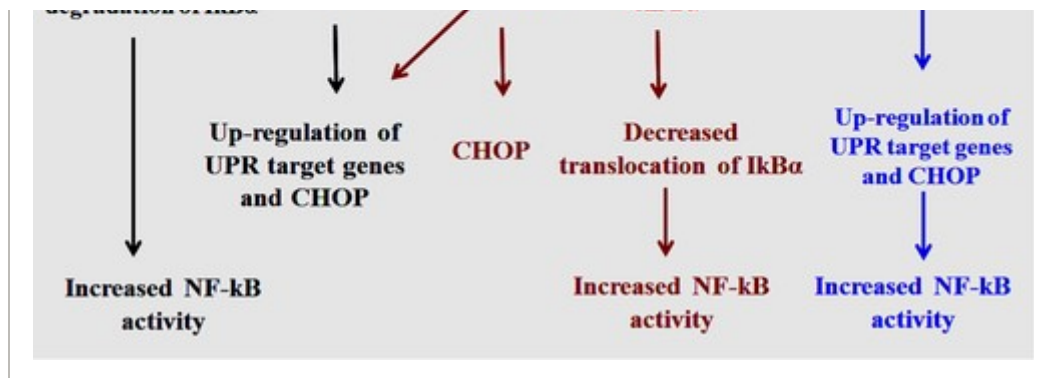


FIGURE 1

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Unfolding protein response (UPR) pathways. GRP78 is a chaperone that senses the accumulation of unfolded/misfolded proteins within the ER lumen and initiates the UPR process. There are three classic pathways for UPR, including PERK, IRE1α, and ATF6. Under prolonged and sustained ER stress condition, all three of these pathways can activate the apoptosis process through CHOP. In the IRE1α pathway through XBP1, in the PERK pathway through ATF4, and finally the ATF6 pathway itself activates CHOP-induced apoptosis

Pharmacologically, crocin is one of the active ingredients of saffron (*Crocus sativus* L.). Various animal and human studies have reported different therapeutic effects for crocin, including anti-inflammatory, antioxidant, anticancer, anti-depressant, analgesic, antiatherosclerosis, and smooth muscle relaxant.¹⁴⁻¹⁶ On the other hand, much evidence has shown the protective and therapeutic effects of crocin in asthma,¹⁷⁻¹⁹ although its exact mechanism is not fully understood. The suppressive effects of crocin on ER stress have been demonstrated in some studies such as myocardial ischemia/reperfusion (I/R) injury, Parkinson's disease model, and streptozotocin (STZ)-induced diabetic model.²⁰⁻²² To date, a study showing the role of crocin in asthma-induced ER stress is unclear. Based on previous studies, we hypothesized that crocin could reduce/suppress ER stress in ovalbumin (OVA)-sensitized mice, especially its suppressive role in the ER stress-induced apoptosis. For this purpose, we tested the gene expression of possible molecular chaperones involved in the ER stress-induced apoptosis, such as ATF4, ATF6, and XBP1 as well as caspase 12 and CHOP protein levels.

2 | EXPERIMENTAL PROCEDURES

2.1 | Chemicals

Quantitative enzyme-linked immunosorbent assay (ELISA) kits for IL-6, IL-1β, TNF-α, CHOP, caspase12, and IgE were obtained from Crystal Day. The crocin standard (>98%) was obtained from Sigma-Aldrich. OVA was obtained from Sigma-Aldrich. Aluminum hydroxide gel was obtained from Thermo Fisher. All RT-PCR chemicals was obtained from Yektatajhiz Co. All other chemicals used in the study were of analytical grade.

2.2 | Experimental design

Thirty adult male mice (20–25 g) were prepared by the Animal House of Tabriz University of Medical Sciences. The animals were adapted to the environment for a week in the Animal House of Ardabil University of Medical Sciences, Ardabil, Iran. Standard controlled conditions such as 22 ± 2°C and 12 h: 12 h light–dark cycle were applied to all animals. Throughout the study period, the animals had free access to food and drinking water. All animal procedures and tests were performed according to ethical guidelines approved by the Committee on Animal Research of ARUMS (IR.ARUMS.RE.1398.001). The animals were divided into six designed groups and treated according to Table 1 and Figure 2. In order to induce experimental asthma, 10 µg of OVA and 2 mg of Al(OH)₃ were injected intraperitoneally on days 0, 7, and 14. The animal was then exposed to 1% OVA aerosol for 15 min through the nose for 5 days from the 28th day. In crocin groups, 1 h before the challenge with OVA, intraperitoneal injection was performed based on the designed doses. In the OVA group, saline was injected intraperitoneally instead of crocin.

TABLE 1. Sensitization of animals were done according to previously described paper²³ and treatment with saline, crocin or dexamethasone were done intraperitoneally. The dose of crocine and dexamethasone were chosen according to previous studies.²⁴

Groups	Intraperitoneal saline or OVA administration, days 0, 7, and 14	Nebulized saline or OVA 1%- for 30 min on days 28–32	Treatment 1 h before nebulization on days 28–32	Group abbreviation
Control	Saline	Saline	Saline	Control
OVA-administration	OVA 10 µg + Al(OH) ₃ 2 mg/kg	OVA	Saline,	OVA
OVA+ Crocin 25 mg/kg	“	“	Crocin 25 mg	OVA-Cr25
OVA+ Crocin 50 mg/kg	“	“	Crocin 50 mg	OVA-Cr50
OVA+ Crocin 100 mg/kg	“	“	Crocin 100 mg	OVA-Cr100
OVA+ Dexamethasone 1 mg/kg	“	“	Dexamethasone	OVA-Dexa

Procedure	OVA (IP)	OVA (IP)	OVA (IP)	OVA (Nebulized)
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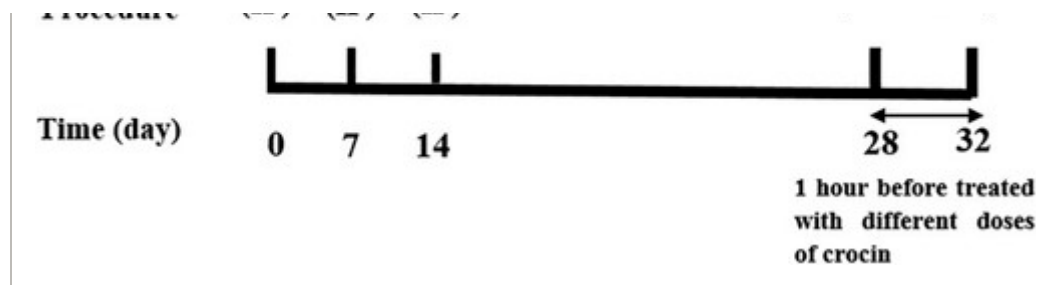


FIGURE 2

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Experimental design flow chart and treatment with saline, crocin or dexamethasone (last 5 days of the model). OVA; ovalbumin, IP; Intraperitoneally

2.3 | Bronchoalveolar lavage fluid collection

Animals were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) at the end of the study. Bronchoalveolar lavage fluid (BALF) sample was collected by injecting and aspirating 0.5 ml phosphate buffer saline (PBS) three to five times after exposing the trachea and cannulating it with a catheter. The BALF sample was centrifuged for 10 min at 4°C at 2500 rpm and its supernatant was used for total and differential cell counting.²⁵

2.4 | Total and differential white blood cell count

Total white blood cell (WBC) count was performed by hemocytometer and Wright-Giemsa staining, and differential cell count was performed by standard protocols of differentiating 200 cells per slide under magnification of ×400 light microscope.²⁵

2.5 | Tissue sampling and protein measurement

After induction of anesthesia, the lung tissues of the animals were isolated and after freezing with liquid nitrogen, the tissues were rapidly transferred to −70°C until OVA-sensitive IgE, CHOP, and caspase-12 protein levels were assayed. After weighing the tissue samples, homogenization was performed in PBS (pH 7.2–7.4) and centrifuged at 4°C at 3000 rpm for 20 min. The collected supernatant was used to measure OVA-specific IgE, CHOP, and caspase-12 proteins using a mouse ELISA kit by the manufacturer's instructions (Crystal Day).

2.6 | Real-time polymerase chain reaction

For measurement of ER-stress markers (GRP78, ATF4, ATF6, XBP1, and CHOP) mRNA expression levels, we used the method of the previous study.²⁶ Table 2 shows the locked nucleic acid (LNA) forward and reverse primer sets (Exiqon) for mRNA. The PCR products were normalized with GAPDH genes for mRNA samples. Results were expressed as fold change versus the relevant controls.²⁶

TABLE 2. Primer set list for mRNAs

Gene name	Primer sequence
ATF4	Forward primer: TGACCTGGAAACCATGCCAGA
	Reverse primer: GGGGAAAGGCTGCAAGAATGT
ATF6	Forward primer: ATGCATAAAACCCAGCAGCC
	Reverse primer: CTTCCCTAGCCTCCCCAACAC
GRP78	Forward primer: TGAGAGGTCACCCGAAGACAG
	Reverse primer: GGTCATTCTTAACGGCCACCA
XBP-1	Forward primer: AGCACTCAGACTATGTGCACC
	Reverse primer: TCCAATTGTCCAGAATGCCC
CHOP	Forward primer: CCTCGCTCTCCAGATTCCAGT
	Reverse primer: CTCCTTCATGCGTTGCTTCCC
GAPDH	Forward primer: ACTCCACTCACGGCAAATTC
	Reverse primer: TCTCCATGGTGGTGAAGACA

2.7 | Pathological assessment

The animals' lungs were fixed in 10% neutral buffered formalin (37%, Merck) and embedded in paraffin blocks, cut into 4-μm thick sections, stained with hematoxylin–eosin, and finally examined by light microscopy. Evaluated criteria for lung pathological changes in different groups were air-filling, interstitial hyperemia, interstitial pneumonia, and epithelial detachment. Scoring for each lung pathological changes was identified from 0 to 3 as follows; 0 = normal; 1 = patchy injury, 2 = local injuries, and 3 = scattered injuries.

2.8 | Statistical analysis

In the current study, results are reported as mean ± SEM. Comparisons among different groups were performed using variance (ANOVA test) with Tukey–Kramer post hoc test. In addition, Kruskal–Wallis statistical test was used to analyze the pathological results. *p* < 0.05 was considered the significance level.

3 | RESULTS

3.1 | Effects of crocin on the BALF cell infiltration

The total WBCs in the OVA group showed a significant increase compared to the control group ($p < 0.001$). Instead, results showed that the total WBC count in the OVA-crocin and OVA-Dexa treated groups was significantly reduced compared to the OVA group ($p < 0.001$ for all) (Figure 3A).

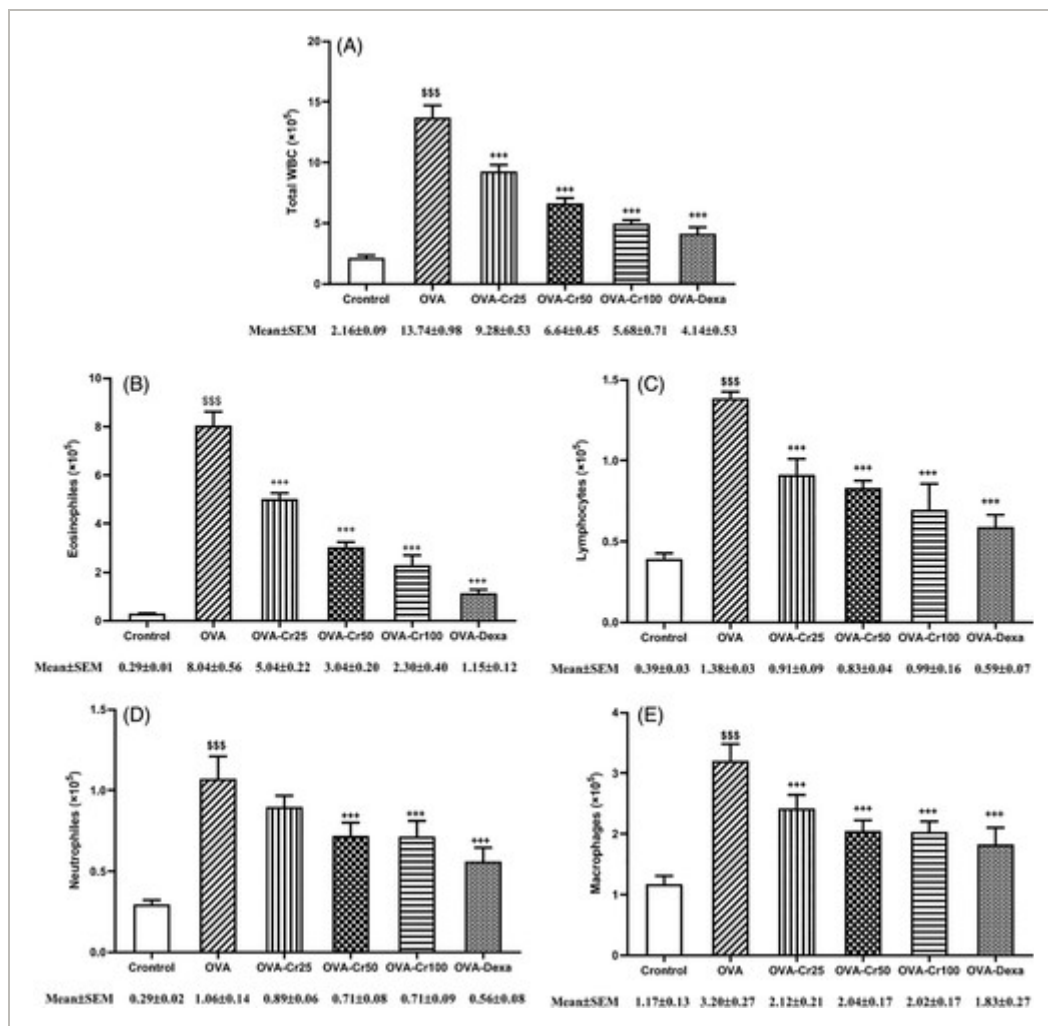


FIGURE 3

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Total WBC (A), eosinophil (B), lymphocyte (C), neutrophil (D), and macrophage (E) counts in the BALF of the control, asthmatic (OVA), OVA-treated crocin 25 mg/kg (OVA-Cr25), OVA-treated crocin 50 mg/kg (OVA-Cr50), OVA-treated crocin 100 mg/kg (OVA-Cr100), and OVA-treated dexamethasone 1 mg/kg (OVA-Dexa) groups. Data are shown as mean ± SEM. \$\$\$: $p < 0.001$ control versus OVA group. +++: $p < 0.001$ OVA group vs. crocin and dexamethasone treated groups. For each group, $n = 5$. Comparisons between groups were made using analysis of variance test

The results of differential cell count revealed that in the OVA group there were increased levels of all inflammatory cells compared to the control group, including eosinophils,

lymphocytes, neutrophils, and macrophages ($p < 0.001$ for all cases, Figure 3D,E).

Interestingly, the increase of eosinophil cells in BAL samples of the OVA group was higher compared to other inflammatory cells. On the other hand, the levels of all inflammatory cells in the treated groups (OVA-Cr25, OVA-Cr50, OVA-cr100, and OVA-Dexa) showed significant improvement compared to the OVA group ($p < 0.001$ for all cases, Figure 3B–E). However, the mean value of inflammatory cell types in the BAL sample of all treated groups was still higher than the control group.

3.2 | Effect of crocin on ER-stress gene expression levels in the lung tissue

Increased expressions of GRP78 and ATF4 were observed in the lung tissue of OVA-sensitized mice compared to the control group ($p < 0.001$ for both cases, Figure 4A,B).

Treatment with different concentrations of crocin (25, 50, and 100 mg/kg) and dexamethasone significantly corrected the increased GRP78 expression levels compared to the OVA-untreated group ($p < 0.001$ for all cases) (Figure 4A). The expression level of ATF4 in lung tissue was also significantly decreased in the treated groups, OVA-Cr25, OVA-Cr50, OVA-Cr100, and OVA-Dexa ($p < 0.05$ for OVA-Cr25 and $p < 0.001$ for other cases, Figure 4B).

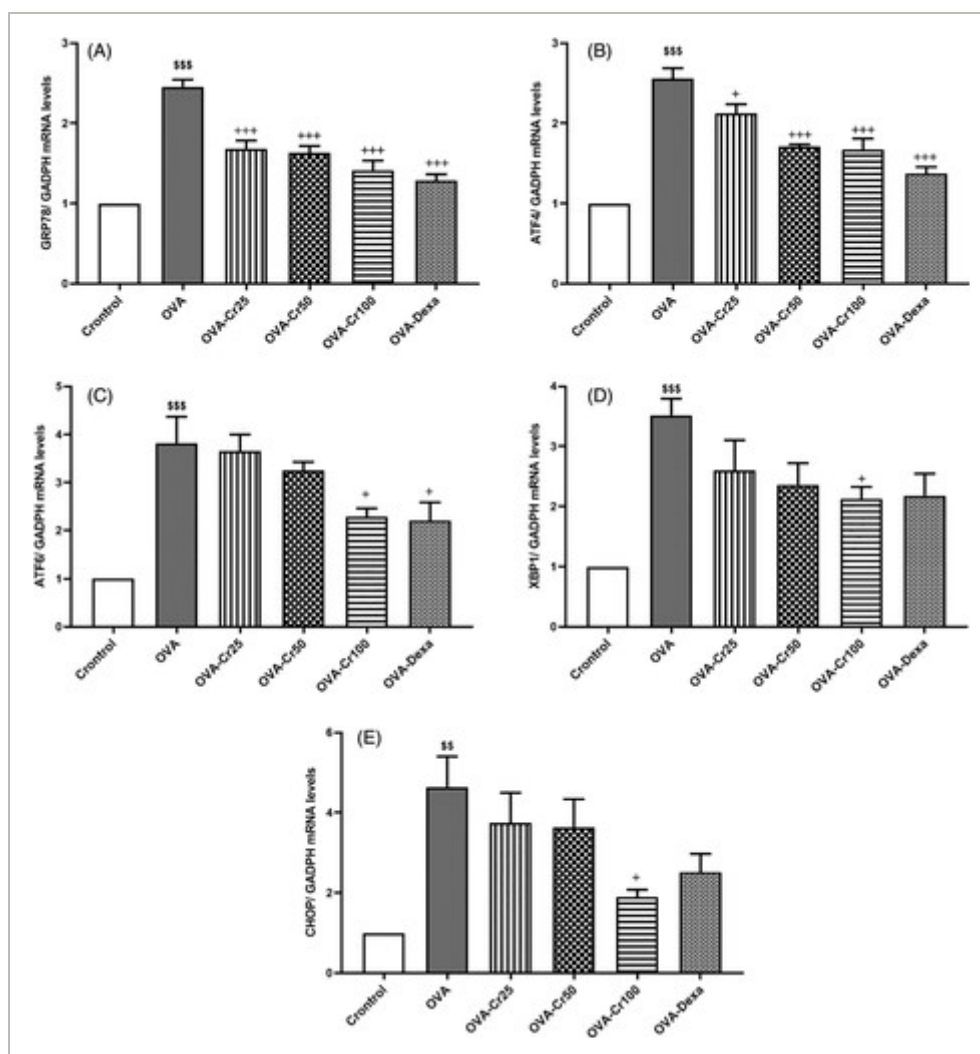


FIGURE 4
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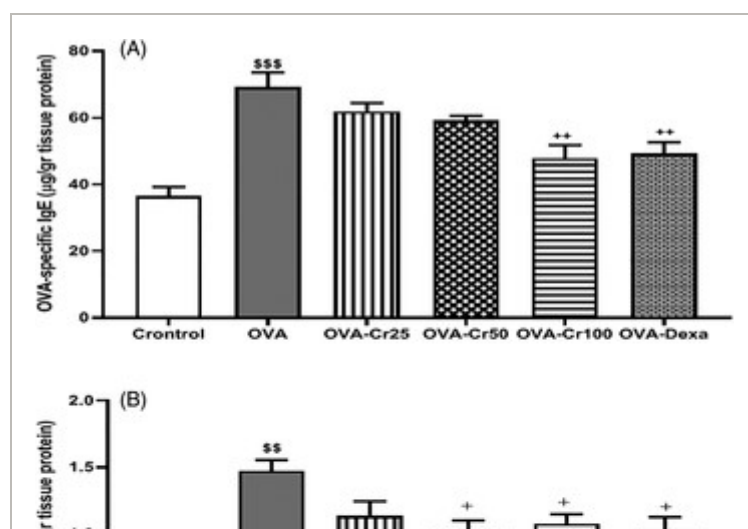
Lug tissue ER stress markers gene expression level. Mean + SEM of (A) GRP78, (B) ATF4, (C) ATF6, (D) XBP1, and (E) CHOP expression levels. Abbreviations are the same as Figure 2. \$\$: $p < 0.01$, \$\$\$: $p < 0.001$ control versus OVA group. +: $p < 0.05$, +++: $p < 0.001$ OVA group vs. crocin and dexamethasone treated groups. For each group, $n = 5$. Comparisons between groups were made using analysis of variance test

Elevated expression of ATF6 was also seen in the lung tissue of OVA-sensitized mice compared to the control group ($p < 0.001$), which was significantly reduced in the OVA-Cr100 and OVA-Dexa groups (for both $p < 0.05$, Figure 4C). Also, XBP1 expression in lung tissue of the OVA group was significantly increased compared to the control group ($p < 0.001$). On the other hand, treatment with crocin showed that only the OVA-Cr100 group had a significant reduction in XBP1 expression level ($p < 0.05$, Figure 4D). Although suppression effect on XBP1 expression was observed in OVA-Dexa group but was not statistically significant.

The results also showed that CHOP expression level was higher in the OVA group than in the control group ($p < 0.01$). Crocin treatment in OVA-Cr100 group significantly reduced CHOP expression level compared OVA group ($p < 0.05$, Figure 4E) but the inhibitory effect on CHOP expression was not significant in the OVA-Dexa group.

3.3 | Effects of crocin on the OVA-specific IgE, CHOP, and caspase-12 protein levels in the lung tissue

Elevated levels of OVA-specific IgE protein were detected in the lung tissue of the OVA group compared to the control group ($p < 0.001$) (Figure 5A). Treatment with crocin in the OVA-Cr100 group as well as the OVA-Dexa group showed a significant decrease in OVA-specific IgE protein concentration compared to the OVA group ($p < 0.01$ for both). However, no significant difference was observed with other concentrations of crocin (25 and 50) compared to the OVA group despite the decrease in the concentration of OVA-specific IgE protein in lung tissue (Figure 5A).



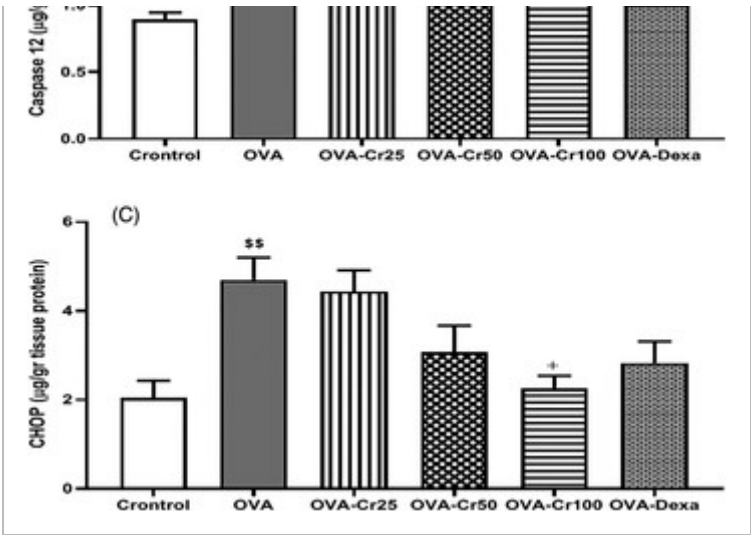


FIGURE 5

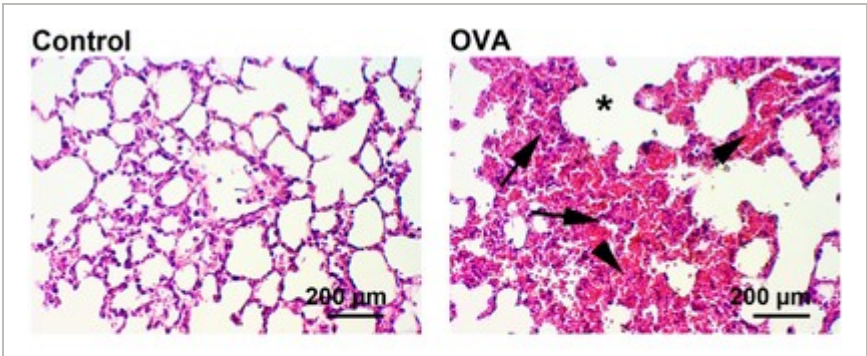
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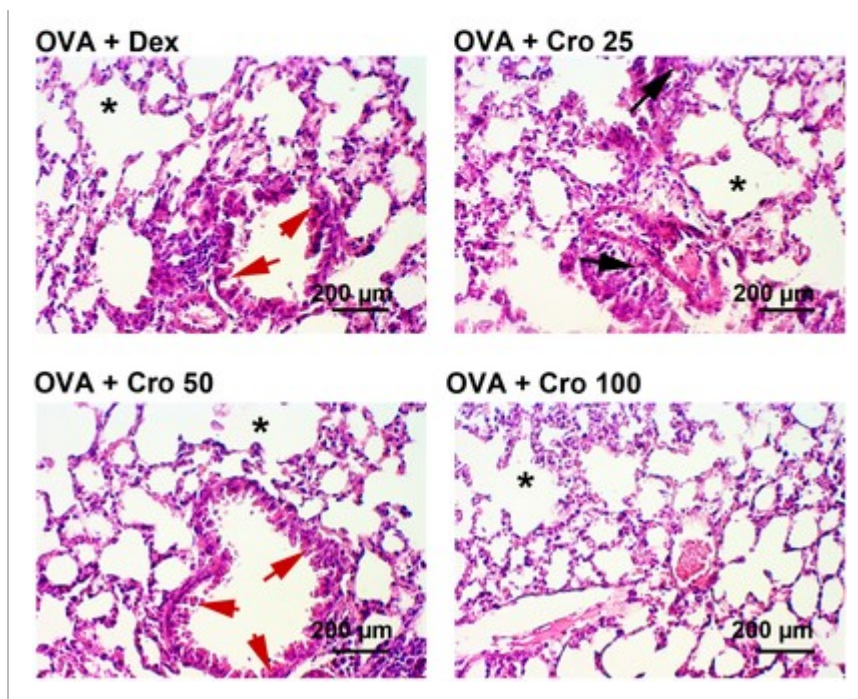
Lug tissue protein levels of (A) OVA-specific IgE, (B) caspase 12, and (C) CHOP. Values are expressed as mean + SEM. Abbreviations are the same as Figure 2. \$\$: $p < 0.01$, \$\$\$: $p < 0.001$ control versus OVA group. +: $p < 0.05$, ++: $p < 0.01$ OVA group versus crocin and dexamethasone treated groups. For each group, $n = 5$. Comparisons between groups were made using analysis of variance test

The results showed increased caspase 12 protein levels in the lung tissue of OVA-sensitized mice compared with controls ($p < 0.01$). Treatment with Cr50, Cr100, and dexamethasone significantly reduced the mean value of caspase 12 protein levels ($p < 0.05$ for all cases, Figure 5B). In addition, increased levels of CHOP protein as a result of sensitization with OVA compared to the control group ($p < 0.01$) were observed that treatment with Cr100 led to a decrease in CHOP protein levels in the lung tissue ($p < 0.05$, Figure 5C).

3.4 | Effects of crocin on lung tissue pathological changes

Pathological results showed that in ovalbumin sensitization significantly led to the development of pathological changes such as hyperemia, epithelial detachment, air filling, and interstitial pneumonia compared with the control group ($p < 0.001$ for all) (Figure 6). Treatment with different concentration of crocin (50 and 100 mg/kg) as well as dexamethasone significantly reduced all pulmonary pathological changes ($p < 0.05$ to $p < 0.001$) (Table 3).



**FIGURE 6**
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Pathological changes of pulmonary tissue (magnification = X80) in control group, OVA-sensitized group (OVA), crocin-treated groups (OVA-Cr25, OVA-Cr50, and OVA-Cr100), and dexamethasone-treated group (OVA-Dex). The asterisk indicates interstitial air-filling, the headache sign indicates hyperemia, arrow sign indicates interstitial pneumonia, and the red arrow sign indicates the detachment of the epithelium

TABLE 3. Lung tissues pathological scales

Pathological results	Scores in groups (for each group, $n = 5$) (minimum-maximum)					
	Control	OVA	OVA-Cr25	OVA-Cr50	OVA-Cr100	OVA-Dex
Air-filling	(0–1)	(2, 3)\$\$\$	(1–3)\$\$\$	(1, 2)\$\$	(0–2)\$	(1, 2)\$+
Hyperemia	(0–1)	(2, 3)\$\$\$	(2, 3)\$\$\$	(1–3)\$\$+	(1–3)\$\$+	(1, 2)++
Interstitial pneumonia	(0–1)	(2, 3)\$\$\$	(2, 3)\$\$\$	(1–3)\$\$\$+	(0–2)\$+	(0–2)++
Detachment of epithelium	(0–0)	(2, 3)\$\$\$	(1–3)\$\$\$	(1–3)\$\$\$	(0–2)++	(1, 2)++

Notes: Lung tissues pathological scales in the control animals, sensitized animals (OVA), sensitized animals received crocin (OVA-Cr25, OVA-Cr50, and OVA-Cr100), and sensitized animals received dexamethasone (OVA-Dex). \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ control versus OVA group. + $p < 0.05$, ++ $p < 0.01$ OVA group vs. crocin and dexamethasone treated groups. Comparisons between groups were made using Kruskal Wallis test. The numbers were placed inside the parentheses, implying the minimal and maximal pathological score in each group.

4 | DISCUSSION

The most important findings of the present study were: (1) elevated inflammatory cells in the respiratory airways of OVA-sensitized mice, (2) crocin administration reduced inflammation of the airways, (3) sensitization with OVA leading to the induction of ER stress with increased UPR components in the lung tissue in mice, which were inhibited after treatment with crocin and dexamethasone, (4) crocin reduced lung inflammation of OVA-sensitized mice possibly through inhibition of ATF4-CHOP, ATF6-CHOP, and XBP1-CHOP signaling, and (5) crocin (100 mg/kg) decreased protein levels of CHOP and caspase 12 in lung tissue of OVA-sensitized mice, which was comparable to the effects of dexamethasone.

The main feature of asthma is a chronic inflammation of the airway, which is clinically characterized by airway obstruction, inflammation of the airway, and airway hyperresponsiveness.^{27, 28} Increased inflammatory cells such as neutrophils, eosinophils, macrophages, activated mast cells, and T helper cell type 2 are evident in the airways of asthmatic patients.^{29, 30} Histopathological changes of the airways in asthmatic patients include increased airway smooth muscle, increased number of blood vessels and hyperemia, detachment of epithelial tissue, and lymphocyte infiltration.³¹ The results of the present study showed that in OVA-sensitized mice there was an increase in inflammatory cells in the BALF such as neutrophils, eosinophils, and macrophages. On the other hand, the results showed that in the OVA-sensitized groups, the serum level of OVA-specific IgE was significantly higher than control group. In addition, the promotion of interstitial pneumonia with massive immune cell recruitment and hyperemia indicates along with bronchiolar epithelial cells injury showed OVA-related changes. Indeed, results indicated that an asthma model induced in OVA-sensitized animals.

It has been shown that the ER has a key function as a regulator of inflammation in a variety of inflammatory diseases.² In particular, ER stress and UPR-related pathways have been reported to play an effective role in human allergic diseases such as asthma.^{13, 32} Because asthma is an inflammatory condition that increases the production of protein complexes such as cytokines, chemokines, and immunoglobulins, it can be inferred that protein complexes can lead to protein misfolding/unfolding conditions.⁴ Elevated levels of ER stress markers such as GRP78/BiP, ATF4, ATF6, and CHOP, have been shown in BALF samples as well as peripheral blood mononuclear cells in asthmatic patients compared to healthy individuals.^{1, 4, 32} For proper protein folding, various molecular chaperones play a role in the ER, such as GRP78/BiP. Under conditions of impaired protein folding in the ER, the synthesis of GRP78 increases.³³ One of the markers that indicate the activation of ER stress is the increased levels of GRP78.⁴ In the present study, elevated GRP78 levels in the OVA-sensitized groups (model and treated groups) indicated activation of ER stress, which may have been involved in airways inflammation. Treatment with different concentrations of crocin (25, 50, and 100 mg/kg) as well as dexamethasone revealed that the activation of ER stress was somewhat blunted.

As a result of activating ER stress, one of the activated signaling pathways is the PERK, which leads to the activation of ATF4 through eIF2 α .⁶ ATF4 is one of the key factors in inducing CHOP apoptosis in cells.³⁴ The results showed that elevated levels of ATF4 occurred in the OVA-sensitized groups, which was significantly inhibited by treatment with crocin and dexamethasone. Elevated levels of protein and gene expression of ATF4, PERK, eIF2 α , and p-eIF2 α in OVA sensitization condition have been shown in previous studies to be consistent with the findings of the present study.^{4, 10}

Also, the second pathway activated in ER stress is the IRE1 α signaling pathway. The IRE1 α pathway is highly associated with inflammatory responses that can lead to the activation of transcription factors such as activator protein 1 (AP1).¹⁰ In addition, IRE1 α affects the survival of cells in the immune system by activating the XBP1 pathway.¹⁰ In this study, it was found that increased expression of XBP1 was induced in OVA-sensitized groups. On the other hand, in the OVA-crocin and dexamethasone-treated groups, the results indicated that only in the OVA-Cr100 group, a decrease in XBP1 expression was observed. The elevated expression level of IRE1 α , XBP1, and AP1 have also been reported in the animal model of asthma.^{4, 10}

The third pathway activated in ER stress is the ATF6 branch, which as a result of its activation is effective in remodeling the airways in asthma.³⁵ The results of our study showed that the expression of ATF6 was significantly increased in the OVA-sensitized groups, which was partially prevented by treatment with crocin 100 and dexamethasone.

CHOP is an ER stress transcription factor and marker that plays a central role in the ER stress-induced apoptosis process.³⁶ Generally, increased GRP78/BiP values are present in ER stress with adaptive conditions, while CHOP is increased in sustained and excessive ER stress during the maladaptive phase.¹ Animal and human studies have reported an increase in CHOP expression as a result of activation of ER stress in asthma, although its mechanism is unknown.^{1, 4, 10} Three classic UPR pathways are capable of activating CHOP, including PERK-eIF2 α -ATF4, IRE1-XBP1, and ATF6 (Figure 1).³⁷ CHOP is also activated via some nonclassical UPR pathways such as IRE1-ASK1-JNK.² CHOP causes mitochondrial-related dysfunction by upregulating the pro-apoptotic gene (Bax/Bak) and downregulating the anti-apoptotic gene (Bcl-2), leading to activation of the caspase cascades, caspase 12 and caspase-9, and ultimately the apoptotic process.² Caspase-12 is located in the ER and is normally available as an inactive procaspase form.³⁸ During ER stress, caspase-12 separates from the ER membrane and cleaves into fragments, activating apoptotic pathways when activated.³⁹ Different upstream pathways have been shown to be able to activate ER stress-related apoptosis such as IRE1, m-calpain, and caspase-7 as well as downstream pathways such as caspase-9 and caspase-3. However, one of the most important pathways for induction of apoptosis is the CHOP-caspase-12 axis.⁴⁰ Therefore, in this study, we examined caspase12, considering that other possible pathways involved in ER stress are also present. Increased expression and protein levels of CHOP in the lung tissue of OVA-sensitized mice were evident in the present study. Treatment with crocin 100 resulted in suppression of

increased CHOP expression and protein levels but was not statistically significant in the dexamethasone group despite inhibition of CHOP expression and protein levels. Elevated levels of caspase 12 protein were also seen in the OVA-sensitized groups, which is suppressed by treatment with crocin 100 and dexamethasone. From the results, it can be inferred that crocin caused adaptive UPR conditions and promotes protein folding by preventing sustained ER stress and maladaptive UPR, which requires further study.

Saffron has strong anti-inflammatory and antioxidant effects due to its various compounds such as crocin, crocetin, and safranal.⁴¹ In vitro, in vivo, and human studies have shown the antioxidant effects of crocin in various pathological conditions such as asthma, COPD, myocardial infarction, and cancers.¹⁶ The results of various studies have shown that crocin exerts antioxidant effects by reducing the production of oxidative factors such as malondialdehyde (MDA), lipid peroxidation (LP), inducible nitric oxide synthase (iNOS), nitric oxide (NO), xanthine oxidase (XO), myeloperoxidase (MPO), protein carbonyls (PC) as well as by increasing antioxidant factors such as glutathione (GSH), total antioxidant capacity, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and glutathione-S-transferase (GST).⁴² Although the direct effects of crocin on modulating ER stress responses are not known, it is thought that crocin plays a core role in ER stress suppression through inhibition of inflammation and free radicals production.^{43, 44} Due to the nature of the activity, the ER has an environment with high production of reactive oxygen species (ROS) and contributes to oxidative stress generation.³¹ Accumulation of misfolded or unfolded proteins in the ER produces large amounts of H₂O₂ which can lead to a depletion in GSH.⁴⁵ Mitochondria also produce more ROS by the take-up of calcium released from the ER under stress conditions.⁴⁶ Activation of various ER stress markers such as CHOP, ATF4, and ATF6 can lead to high oxidative stress in asthma.⁴⁷ On the other hand, the anti-inflammatory effects of crocin on a variety of inflammatory markers such as IL-4, IL-5, IL-13, and IL-17 have been reported in animal studies.⁴⁸ So the use of an antioxidant and anti-inflammatory can be effective in reducing the oxidative stress conditions caused by ER stress. Based on the findings of the current study, at least in part, it can be inferred that the inhibitory effects of crocin on ER stress markers in asthma may have been due to its antioxidant effects, which requires further studies.

One of the limitations of the current study was the lack of further investigation in relation to in vitro studies in addition to the current in vivo study, which could reveal the effectiveness of crocin. The use of an ER-stress pathway inhibitor or activator could also be useful to confirm the overall conclusion of this study regarding the effects of crocin on ER-stress markers. Also, determining protein levels of ER-stress markers, other than CHOP, can be effective in better understanding subsequent changes from posttranscriptional changes.

5 | CONCLUSION

In summary, the results revealed that crocin suppresses lung inflammation in OVA-sensitized mice by inhibiting ER stress markers. In addition, although we did not evaluate the

direct mechanism of crocin on ER stress markers in the current study, it is partially understandable that crocin reduced ER stress by modulating the ATF4-CHOP, ATF6-CHOP, and XBP1-CHOP signaling pathways. Indeed, crocin can modulate maladaptive ER stress conditions by creating adaptive ER stress status and driving protein folding correctly. In addition, the results may support that crocin may be effective in the treatment of asthma as a new therapeutic strategy.

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

AUTHOR CONTRIBUTION

Mohammad Reza Aslani: Literature search, Proposal writing, Data collection, Analysis of data, Interpretation of data, Manuscript preparation, Review of manuscript; Mohammad Amani, Neghin Masrori, Mohammad Hossein Boskabady, Hossein Ali Ebrahimi, Leila Chodari: Proposal writing, Analysis of data, Draft preparation, Review of manuscript.

Open Research



DATA AVAILABILITY STATEMENT

Data openly available in a public repository that issues datasets with DOIs

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