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Effect of Total Suspended Particulate Matter in the Air on Inflammation Factors and Apoptotic Markers in Diabetic Rats: The Protective Effect of Insulin and Crocin

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Abstract

Background: The effect of total suspended particulate matter (TSP) was investigated on the expression of inflammatory and apoptotic factors in diabetic rats, and the effect of crocin and insulin was examined on these factors.

Methods: Fifty-four adult male wistar rats were divided into nine experimental groups: control group, crocin group (received crocin, 50 mg/kg), diabetic group (received a single dose of alloxan at 120 mg/kg, IP), TSP group (5 mg/kg TSP instilled intratracheally), diabetic-crocin group (received crocin at 50 mg/kg after the induction of diabetes by alloxan (120 mg/kg)), diabetic-insulin group (received regular insulin **(**5 U/kg), crocin-TSP group (received crocin at 50 mg/kg, IP, and then 5 mg/kg TSP was instilled intratracheally), diabetic-TSP-insulin group (after receiving alloxan (120 mg/kg) and instilling TSP (5 mg/kg, intratracheally), a single dose (5 U/kg) of regular insulin), and diabetic-TSP-crocin group (after receiving alloxan (120 mg/kg) and instilling TSP (5 mg/kg, intratracheally), a single dose of crocin (50 mg/kg, IP)). Quantitative real-time PCR was performed to measure the expression of the mRNAs of apoptotic (Bax and Bcl2) and inflammatory mediators (TNFα, COX2, iNOS/eNOS) in Wistar rats.

Results: In diabetic and TSP groups the inflammatory factors and BAX/Bcl2 ratio significantly increased compared to the control group. In diabetic-TSP-insulin and diabetic-TSP-crocin, a significant decrease was observed in the rate of inflammatory factors and BAX/Bcl2 ratio.

Conclusions: The results suggested that diabetes and exposure to TSP increase the rate of apoptosis and inflammation, and also demonstrated the anti-apoptotic and anti-inflammation role of insulin and crocin.

Keywords: Apoptosis, Crocin, Diabetes, Inflammation, Insulin, TSP.

Introduction

The number of people with diabetes is expected to increase from 425 million adults in 2017 to 629 million in 2045 (1). Inflammation is a common feature of diabetes (2). There is a direct relationship between inflammation and progression from the prediabetes phase. A high plasma glucose level may modulate the balance of pro-apoptotic and anti-apoptotic Bcl proteins toward apoptosis (3). The total suspended particulate matter (TSP) is

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introduced into the atmosphere from the variability of anthropogenic and natural sources (4). Environmental pollution may be a neglected risk factor for diabetes (5). Exposure to particulate matter (PM) has been proved to increase the level of inflammatory cytokines, cyclooxygenase-2, iNOS, caspase-3, and Bax and decrease the expression of anti-apoptotic protein Bcl-2 (6-15). Antioxidants such as crocin, a natural carotenoid found in Crocus sativus L. (saffron), and Gardenia jasminoides J. Ellis flowers reduce the inflammation and oxidative stress prompted by diabetes and obesity (16, 17). The comprehensive antioxidant and anti-inflammatory effects of insulin as a metabolic hormone have been shown with potent effects on glucose and lipid metabolism (18, 19). Because of its anti-inflammatory action, insulin can be used as an anti-inflammatory drug (20). Therefore, this study investigated the effect of TSP on the expression of inflammatory factors such as COX2, TNFα, and iNOS/eNOS, as well as the expression of Bax and Bcl2 in diabetic rats, and examined the effect of crocin and insulin on these factors.

Materials and Methods

Studied area

Abadan is a city in Khuzestan Province in southwestern Iran, located at 30°20′21″N 48°18′15″E. It is one of the few hottest populated places on Earth and experiences many sand and dust storms. The backward trajectory of dust storm and wind direction (~300 °) during dust storm in Figure 1 shows that the sources of it are Iraq and Saudi Arabia Pennsylvania. Air pressure was in the lowest level at 9 hours prior to the highest PM_{10} concentration during the investigated 72-hour. This probably increased wind speed and PM_{10} concentration into the atmosphere. As a result, dew point, air temperature and relative humidity decreased after the highest PM_{10} peak (Fig. 1).

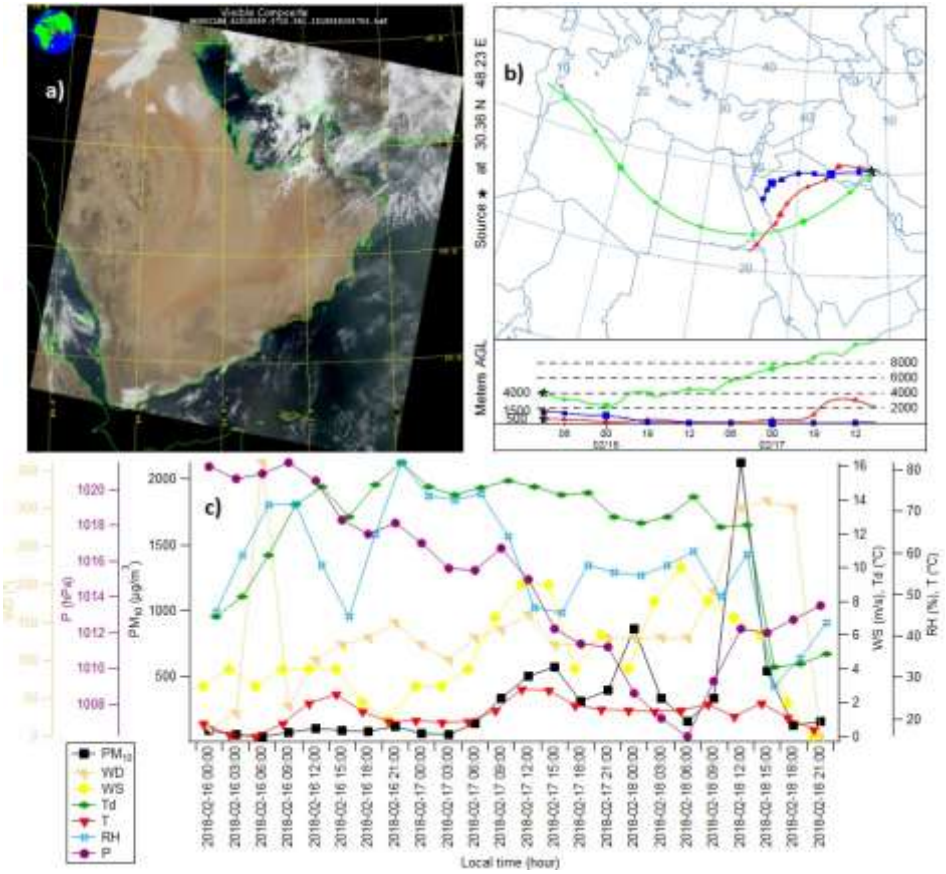


Fig. 1. Properties of the dust storm that occurred on February 18 2018 (**a**) 48 hours backward trajectory (**b**) MODIS imagery from air flow to the region (**c**) meteorological condition during the dusty day and two preceding days of it (P: Pressure; WD: Wind Direction; T: Temperature; Td: Dew Point; RH: Relative Humidity; WS: Wind Speed).

Chemicals

Alloxan monohydrate (Sigma Chemical Company Inc, USA), a High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Germany), and an RT-PCR kit (TAkARA BIO INC, Japan) were employed. Ketamine HCl (10%) and xylazine (2%) were obtained from Alfasan Co. (the Netherlands). Crocin sodium was purchased from Sigma-Aldrich Co. (USA), and insulin (LANSULIN) was purchased from Exir Pharmaceutical Company (Iran).

Ethical standard statement

Animals were treated according to the guidelines of the Animal Care and Use, Ethics Committee of Laboratory Animals, Abadan Faculty of Medical Sciences. Animals were maintained at standard conditions (50% humidity, 12-hour dark-light cycle, and 22±2 °C temperature), with free access to tap water and standard rat chow diet. Ethical Approval was with IR.ABADANUMS.REC.1395.163 as a reference number.

Instruments, reagents and sample preparation heavy metals concentration

High-purity concentrated nitric acid (HNO_3, 65%), perchloric acid ($HClO_4$, 70%) and standard solutions of heavy metals including nickel (Ni), mercury (Hg), chromium (Cr), arsenic (As), lead (Pb), and cadmium (Cd) were supplied from Merck (purity higher than 99%). Ultrapure water was prepared by a Milli-Q system from Millipore (USA). The concentrations of heavy metals such as Ni, Hg, Cr, As, Pb, and Cd in the soil sample collected on February 16th, 2018 from Abadan, Iran. Heavy metals have been determined by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7500, and American). Instrumental conditions of the ICP- MS are: Nebulizer Gas flow rates: 0.8 $Lmin^{-1}$; Auxiliary Gas Flow: 0.8 $Lmin^{-1}$; Plasma, auxiliary, and nebulizer gas: Argon; Plasma Gas Flow: 12.2 $Lmin^{-1}$; Lens Voltage: 7.25 V; ICP RF Power: 1200 W; Background correction and Sample: Fixed point and aspiration rate: 2 mL min^{-1}; RF generator Power:1200W; Sample uptake time; 260 total (S); Type of detector Solid state: CCD; Type of spray chamber cyclonic: Modified Licht. The samples were analyzed with an ICP-MS by using wavelengths: As 188.98 nm, Cd 214.43 nm, Cr 267.71nm, Hg 184.88 nm, Ni 231.64 nm, and Pb 220.35 nm.

Acid digestion was used by mixture HNO_3 and $HClO_4$. The samples were dried at 105 ^{0}C for 3h. In the Erlenmeyer, one gram of dried soil sample was poured, and then was added 10 ml of HNO_3 65%. The new mixture was stored 24 hours in the room temperature. After that, 5 ml of 70% $HClO_4$ was added to the soil samples. The samples were digested in water bath at 80 °C for 5 hours. After digestion, the samples placed at ambient temperature to cool and filtered through a Whatman filter 0.45 µm. The final sample was collected in a volumetric flask and its volume increased to 25 mL using ultra-pure water (21). After preparation samples, heavy metal concentrations were measurement by using an ICP MS.

Animals and Treatments

Fifty-four adult male wistar rats with body weight of 200-250 gr were divided into nine experimental groups (n= 6 in each): control group, crocin group: received crocin, 50 mg/kg (19); crocin was suspended in 0.1 ml of normal saline and administered to the rats via intraperitoneal injection); diabetic group: received a single dose of alloxan at 120 mg/kg, IP (22) to induce diabetes; TSP group: 5 mg/kg TSP instilled intratracheally; diabetic-crocin group: received crocin at 50 mg/kg as a single intraperitoneal injection after the induction of diabetes by alloxan (120 mg/kg); diabetic-insulin: received regular insulin **(**5 U/kg) as a single subcutaneous injection after the induction of diabetes by alloxan (120 mg/kg) (23); crocin-TSP group: received crocin at 50 mg/kg, IP, and then 5 mg/kg TSP was instilled intratracheally; diabetic-TSP-insulin group: after receiving alloxan (a single dose of 120 mg/kg, IP) and instilling TSP (5 mg/kg, intratracheally), the animals were given a

single dose (5 U/kg) of regular insulin by subcutaneous injection; diabetic-TSP-crocin group: after receiving alloxan (a single dose of 120 mg/kg, IP) and instilling TSP (5 mg/kg, intratracheally), the animals were given a single dose of crocin (50 mg/kg, IP).

Induction of diabetes

The basal glucose level of the animals was measured before the induction of diabetes. Alloxan monohydrate (Sigma Chemical Company Inc.) was dissolved in sterile normal saline and injected intraperitoneally at a single dose of 120 mg/kg to five groups of rats. Diabetes was confirmed three days after alloxan injection. Rats with fasting blood sugar >150 mg /dl were considered as moderate diabetic and were included in the study (22, 24).

TSP intratracheal instillation

Each particle sample (TSP) was suspended in 0.1 ml of saline and mixed. The animals were anesthetized with an IP injection of ketamine-xylazine [xylazine (10 mg/kg) and ketamine (50 mg/kg).

The saline-suspended TPS was instilled into the trachea through the intubation tube. Twenty-four hours later, the animals were anesthetized with an IP injection of ketamine-xylazine. Blood samples were collected directly from the heart of the rats 30 min after crocin administration, transferred into EDTA-containing tubes, centrifuged at 4000 g for 10 min to obtain plasma, and stored at -80 °C for RNA extraction analysis.

RNA Extraction and cDNA Synthesis

Total RNA of each sample was extracted from the plasma using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. The quality and quantity of the RNA were evaluated by ratios of 260/280 using nanodrop spectrophotometry (Nano Drop 2000 Thermo Scientific), and cDNAs were synthesized using a PrimeScript 1st strand cDNA Synthesis Kit (Takara). The cDNA was used as the template in qPCR reactions. Real-time PCR was performed using a LightCycler 96 Detection System (Roche) in a final volume of 20 μL reactions. The reagents used in reactions were from the RT-PCR kit (Takara) and included RNase free water and RT-PCR Master Mix. Inflammatory factors and BAX/Bcl2 were calculated using the $2^{-\Delta\Delta Ct}$ method (25). A list of sequences of the PCR primers used for the experiments is given in Table 1.

Table 1. Sequence of RT-PCR primers for interest and reference genes.

Oligo name	Sequence (5′→3′)
Bcl-2-Forward	TGGTACCTGCAGCTTCTTTC
Bcl-2-Reverse	ATCTCCAGTATCCCACTCGTA
TNF-α-Forward	TGATCCGAGATGTGGAACTG
TNF-α -Reverse	AACTTCTCCTCCTTGTTGGG
eNOS-Forward	GACCCTCACCGATACAACAT
eNOS- Reverse	AACTTCTCCTCCTTGTTGGG
iNOS-Forward	CGATAAAGGGACAGCGTCA
iNOS- Reverse	ACTGGGGGAAACCATTTTGA
Cox2 Forward	TCAAACTCAAGTTCGACCCA
Cox2 Reverse	GCGATTGGAACATTCCTTCC
BAX-Forward	TTTTCCTGGGATGAATGGGG
BAX-Reverse	TGAGGTTTATTGGCACCTCC
GAPDH- Forward	TCCACCACCCTGTTGCTGTAG
GAPDH- Reverse	ACACCCACTCCTCCACCTTTG

Statistical analysis

The data were analyzed using GraphPad Prism 8 and expressed as mean±SEM**.** One-way analysis of variance (ANOVA) followed by LSD was used for comparison tests, and $p< 0.05$ was considered statistically significant.

Results

Heavy metals concentrations

The heavy metals in soil sample were analyzed using ICP-MS after acid digestion. In the present work, according above-mentioned conditions the concentrations of some contamination were evaluated in a soil sample. The results of heavy metals concentrations using the proposed methods are summarized in Table 2. In this study the mean concentration of Cr and Ni same as and ranged 1.2 ppm. According to the results, the concentrations of Cr and Ni were lower than the toxicity limits for heavy metals in natural soil.

Table 2. The heavy metals concentration in sample

Heavy metals	Concentration ppm
As	0.05
Cd	0.10
Cr	1.20
Hg	0.05
Ni	1.20
Pb	4.50

According to the results obtained, the lowest heavy metals content was as and Hg. The mean concentration of as and Hg obtained 0.05. The highest concentrations of selected heavy metals were observed in Pb.

The results showed that the concentration of Cd in the sample amount 0.1 ppm, which was below the standard limit. According to the results, the concentrations of six selected heavy metals were lower than the toxicity limits for heavy metals in natural soil. Also, there was no significant deviation from EPA, WHO standards and Europe action level of soil.

Expression of COX2 RNA

The expression of COX2 mRNA in the diabetic group showed a significant increase compared to the control group ($p< 0.0001$). In the crocin group, a significant reduction in COX2 levels was observed compared to the control group ($p< 0.0001$). In diabetic groups receiving crocin or insulin, a significant decrease in the COX2 mRNA level was observed compared with the control group. This decrease was higher in the diabetic-crocin group. In the TSP group, a significant increase in COX2 mRNA was observed compared to the control group ($p< 0.0001$). In the diabetic-TSP-insulin group, there was a significant decrease in COX2 mRNA compared to the TSP group and diabetic group ($p< 0.0001$). In diabetic-TSP-crocin group, a significant decrease in COX2 mRNA was observed compared with the diabetic group and TSP group (Fig. 2A) ($p< 0.0001$).

Expression of TNFα RNA

The TNFα RNA level in the diabetic group demonstrated a significant increase compared to the control group ($p< 0.0001$). In the crocin group, a significant decrease in TNFα expression was seen compared to the control group ($p< 0.0001$). In diabetic-crocin group and diabetic-insulin group, a significant reduction in TNFα expression was found compared with the diabetic group ($p< 0.0001$). This decrease was greater the in diabetic-insulin group than the diabetic-crocin group.

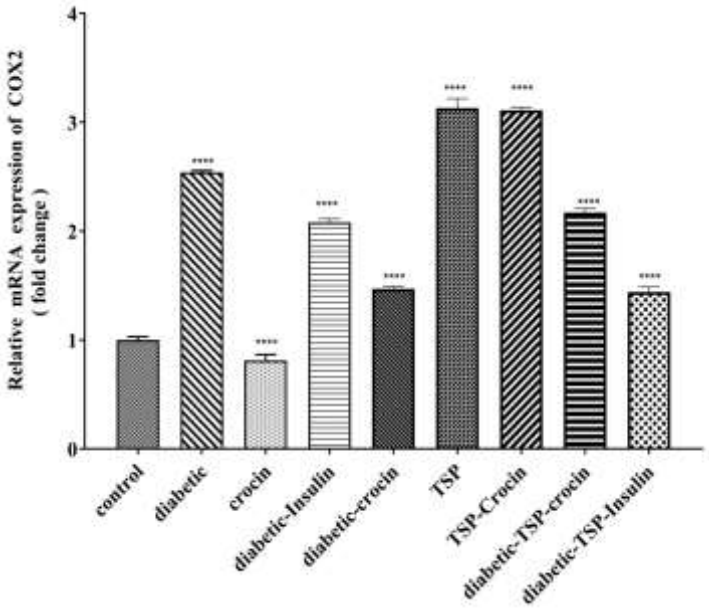
In the TSP group, a significant increase in the expression of RNA of TNFα was observed compared to the control group ($p< 0.0001$)**.** In the TSP- crocin, there was a significant decrease in TNFα expression compared to the TSP group ($p< 0.0001$).

In the diabetic-TSP-crocin group, there was a significant decrease in the expression of RNA of TNFα compared to the TSP group and diabetic group ($p< 0.0001$). In the diabetic-TSP-insulin group, there was a significant decrease in the expression of RNA of TNFα compared to the TSP group and diabetic group ($p< 0.0001$). This decrease in insulin was more severe than that in crocin, indicating the important role of insulin and crocin in reducing the level of inflammation in diabetics exposed to TSP (Fig. 2B).

Expression of eNOS RNA

The eNOS level in the diabetic group showed a significant decrease compared to the control group ($p< 0.0001$). In the crocin group, there was a significant decrease in eNOS compared to the control group, but a significant increase compared to the diabetic group ($p< 0.0001$). In the diabetic-insulin group and diabetic-crocin group, a significant increase was seen compared to the diabetic group ($p< 0.0001$), which was higher in the diabetic-insulin group. In the TSP group, there was a significant reduction in eNOS expression compared to the control group ($p< 0.0001$), which was significantly increased due to crocin reception compared to the TSP group ($p< 0.0001$). Moreover, there was a significant increase in eNOS expression in diabetic-TSP-insulin group and diabetic-TSP-crocin group compared with the diabetic group ($p< 0.0001$). This increase was higher in the insulin-receiving group than the crocin-receiving group. There was a significant increase in eNOS expression in diabetic-TSP-insulin group compared with the TSP group ($p< 0.0001$) but no significant relationship was observed in diabetic-TSP-crocin group compared to TSP group (Fig. 3A).

A:



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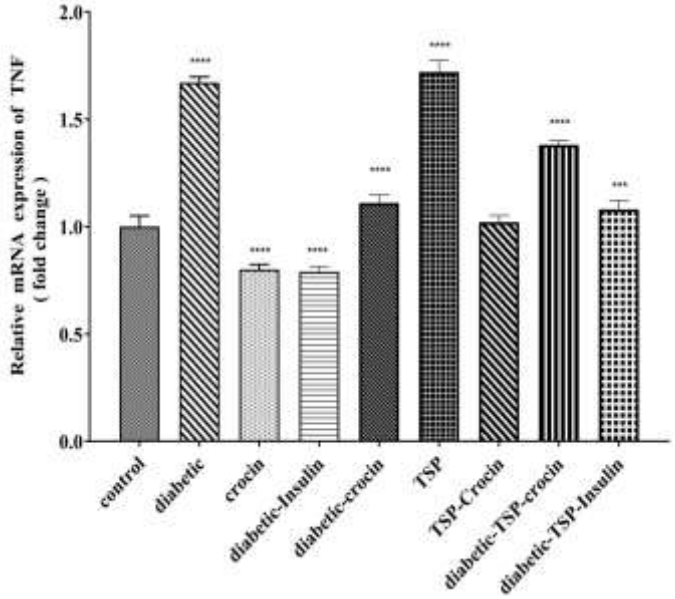
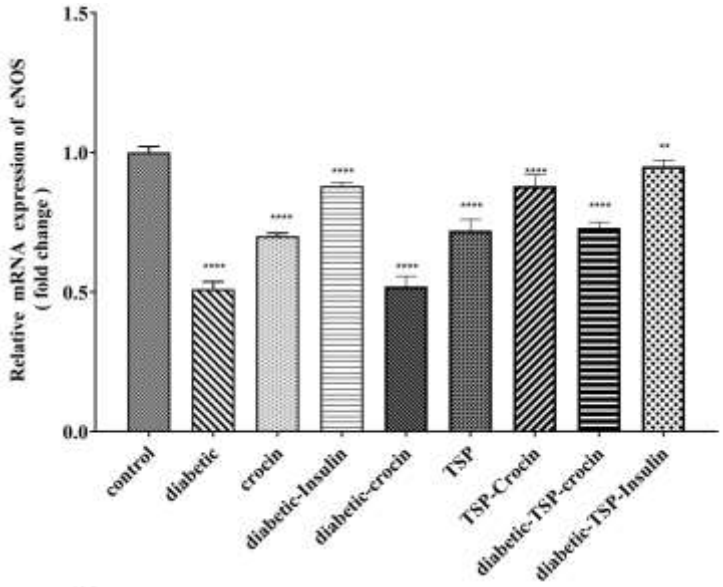


Fig. 2. Effect of crocin, insulin, TSP on A: relative mRNA expression of COX2 (fold change) and B: relative mRNA expression of TNFα (fold change) in diabetic rats. For total test one way ANOVA followed by Fisher s LSD test was used; ***P<0.001, ****P<0.0001 Vs. control group.

A:



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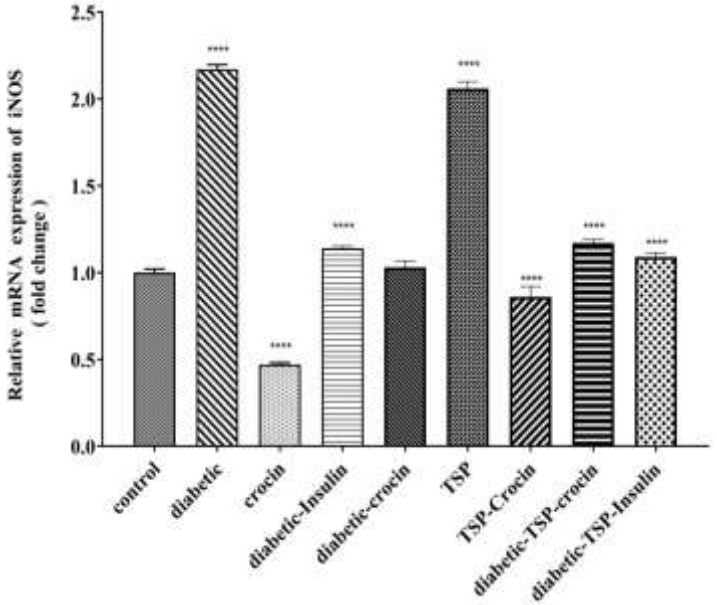

C:

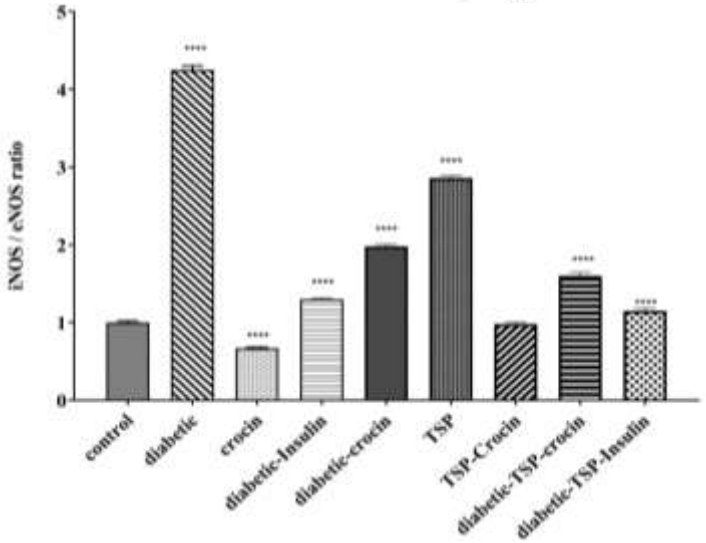

Fig. 3. Effect of crocin, insulin, TSP on A: relative mRNA expression of eNOS (fold change) B: relative mRNA expression of iNOS (fold change) and C: iNOS/eNOS ratio in diabetic rats. For total test one-way ANOVA followed by Fisher s LSD test was used; ***p< 0.001, ****p< 0.0001 Vs. control group.

Expression of iNOS RNA and iNOS/eNOS Ratio

There was a significant increase in the expression of iNOS RNA and the ratio of iNOS/eNOS in the diabetic group compared to the control group ($p< 0.0001$). In the crocin group, there was a significant reduction in the expression of iNOS RNA and the ratio of iNOS/eNOS compared to the control group ($p< 0.0001$). In the diabetic-crocin group, there was a significant decrease in the ratio of iNOS/eNOS compared to the diabetic group ($p< 0.0001$). In the TSP group, a significant increase in iNOS and the ratio of iNOS/eNOS was observed compared to the control group ($p< 0.0001$). In the TSP-crocin, there was a significant decrease in iNOS and the ratio of iNOS/eNOS compared to the TSP-group ($p< 0.0001$). In diabetic-TSP-insulin and diabetic-TSP-crocin, a significant decrease in iNOS and the ratio of iNOS/eNOS was observed compared with the TSP group and diabetic group ($p< 0.0001$) (Figs. 3B and 3C).

Expression of Bcl2 RNA

There was a significant reduction in Bcl2 expression in the diabetic group compared to the control group ($p< 0.0001$). In the crocin group, a significant increase in Bcl2 expression was observed compared to the control group ($p< 0.001$). In diabetic-crocin group and diabetic- insulin group, there was a significant increase in Bcl2 expression compared to the diabetic group ($p< 0.0001$), which was higher in the diabetic-crocin group than diabetic- insulin group. In the group exposed to TSP, there was a significant decrease in Bcl2 expression compared to the control group ($p< 0.0001$). Furthermore, in diabetic-TSP-insulin and diabetic-TSP-crocin, there was a significant increase in Bcl2 expression compared with the diabetic group and the TSP group ($p< 0.0001$) (Fig. 4A).

Expression of Bax RNA and Bax /Bcl2 Ratio

The expression of Bax RNA and the ratio of Bax /Bcl2 in the diabetic group showed a significant increase compared to the control group ($p< 0.0001$). In the crocin group, a significant decrease in the expression of RNA of Bax and the Bax /Bcl2 ratio was seen compared to the control group ($p< 0.0001$). In the diabetic-crocin group and diabetic-insulin group, a significant decrease in the expression of Bax RNA and the ratio of Bax /Bcl2 was found compared to the diabetic group ($p< 0.0001$). In the TSP group, there was a significant increase in the expression of Bax RNA and the ratio of Bax /Bcl2 compared to the control group ($p< 0.0001$), which was significantly reduced in the TSP-crocin group compared to the TSP group. In the diabetic-TSP-crocin group, there was a significant decrease in the expression of Bax RNA and the ratio of Bax /Bcl2 compared to the TSP-group and diabetic group ($p< 0.0001$). In the diabetic-TSP-insulin, a significant decrease in the expression of Bax RNA and the ratio of Bax /Bcl2 was observed compared to the diabetic group, but no significant change was observed in comparison with the group exposed to TSP (Figs. 4B and 4C).

Discussion

Our results showed that in diabetic groups, the mRNA expression of the studied inflammatory factors COX2, TNFα, iNOS and the ratio of iNOS/eNOS significantly increased compared to the control group, while the gene expression of eNOS significantly decreased. These changes indicated that the rate of inflammation increases in diabetes. These results are in agreement with the findings of Szerafin et al. who reported that COX-2 mRNA expression increased in coronary arterioles in diabetes (26). iNOS is related to tubular damage in renal failure, and the NO increase in diabetes may describe the endothelial dysfunction associated with diabetic complications (27).

In the present study, in the diabetic groups, the expression rate of Bax as an apoptotic factor showed a significant increase compared to the control group, whereas the expression rate of the Bcl2 factor showed a significant decrease. Accordingly, the ratio of eNOS/iNOS was decreased. Several studies have reported that the expression of Bax is increased in the blood vessels of diabetic patients, and that long hyperglycemia prompts apoptosis in the

endothelial cells of diabetic ulcers (28, 29).

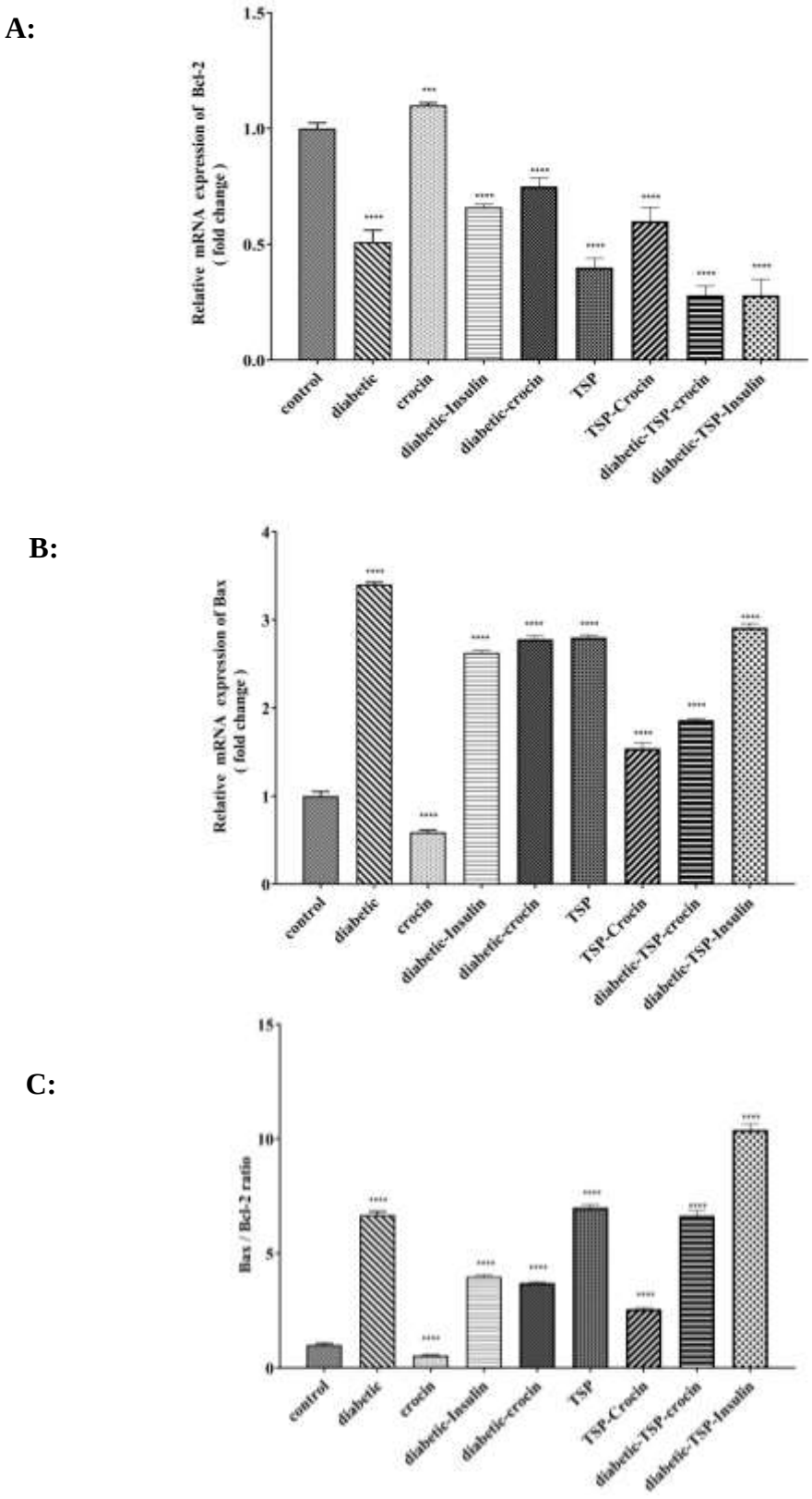

Fig. 4. Effect of crocin, insulin, TSP on A: relative mRNA expression of Bcl2 (fold change), B: relative mRNA expression of Bax (fold change) and C: Bax/Bcl-2 ratio in diabetic rats. For total test one way ANOVA followed by Fisher s LSD test was used; ***p< 0.001, ****p< 0.0001 Vs. control group.

In our study, in the group exposed to TSP, a significant increase was observed in inflammatory factors (COX2, TNFα, iNOS, and the ratio of iNOS/eNOS), and a significant decrease was seen in eNOS expression. In the study by Peng et al., high doses of PM increased pro-inflammatory factors significantly (30) and promoted the apoptosis of EPCs in association with increased ROS production and serum TNF-α and IL-1β levels (31).The PM10 sample significantly increased the tendency of COX-2, iNOS, and ICAM-1 expression (32).

The results of our study revealed that exposure to TSP reduced Bcl2 expression and increased Bax expression. In another study, PM up-regulated pro-apoptotic p53, Bad, p21, and Bax and enhanced the mitochondrial localization of Bax and decreases Bcl-2/Bax ratios (33, 34).

Based on our findings, in the diabetic groups receiving crocin, a significant decrease in the rate of inflammatory factors was observed, suggesting the effective role of crocin in reducing the rate of inflammation in diabetes. The results of this study demonstrated that crocin improves apoptotic factors (decreases Bax but increases Bcl2) in diabetes, and plays a more effective role than insulin in reducing the expression of Bax mRNA and the ratio of Bax/Bcl2. Based on our results, receiving crocin in rats exposed to TSP improves inflammatory factors (decreases the expression of COX, TNF, iNOS, and the ratio of iNOS/eNOS, but increases eNOS). Receiving crocin in rats exposed to TSP improves apoptotic factors as well (decreases Bax but increases Bcl2).

Crocin has been reported to lowers the IL-1β and TNF-α levels, reduces the inflammation in diabetic rats, and inhibits/activates internal and external apoptotic stimulations, which could be effective in diabetes (35).

The results of the present study also showed that insulin can reduce the expression of inflammatory factors as well as the expression of TNFα, COX2, and iNOS in diabetes, but increase the expression of eNOS in diabetic groups. This decrease in the insulin-receiving diabetic group was greater than that of the crocin-receiving diabetic group, indicating a stronger anti-inflammatory role of insulin than crocin. Our results also showed that insulin improves apoptotic factors (decreases Bax expression and increases Bcl2) in the diabetic group.

It has been reported that insulin increases Bcl-2 levels and decreases the expression of Bax, indicating its contribution to the prevention of cell death by increasing the expression of anti-apoptotic genes and decreasing the expression of pro-apoptotic genes (36). Insulin suppresses endotoxin-induced pro-inflammatory transcription factors and the genes regulated by them, and induces the expression of eNOS, endothelial NO synthase (37, 38).

Based on our findings, the increased ratio of Bax/Bcl2 resulting from diabetes was improved both by insulin and crocin; in this case, the role of crocin was more effective. The results also showed that in the group exposed to TSP, the Bax/Bcl2 ratio showed a significant increase, indicating a significant decrease with crocin. The current findings also revealed that inflammatory factors increased due to diabetes. With insulin or crocin, a significant reduction in inflammatory factors was observed in diabetics, suggesting the strong anti-inflammatory role of crocin and insulin, and the results of this experiment confirmed the stronger anti-inflammatory role of insulin compared to crocin. Moreover, the rate of inflammation increased due to exposure to TSP, and insulin and crocin reduced the rate of inflammation in these groups. The results of this study suggest that diabetes and exposure to TSP may increase the rate of apoptosis and inflammation, and confirm the anti-apoptotic and anti-inflammation role of insulin and crocin.

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