The Effect of Continued Training with Crocin on Apoptosis Markers in Liver Tissue of High Fat Diet Induced Diabetic Rats

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Abstract: Diabetes mellitus (DM) disease can affect process of apoptosis by increasing oxidative stress, nevertheless exercise and crocin can improve apoptosis; therefore present study aimed to investigate the effect of continued training with crocin on apoptosis markers in liver tissue of diabetic rats. In this experimental study 32 diabetic rats based on fasting glucose divided into four groups of eight rats including: 1) sham, 2) training, 3) crocin, and 4) training with crocin also for investigate the effect of DM induction on apoptosis markers, eight healthy rats assigned in healthy control group. During eight weeks groups 2 and 4 ran 60 minutes on treadmill with intensity of 50–55% maximum speed for three sessions per week and groups 3 and 4 received 25 mg/kg/day crocin peritoneally. Shapiro–Wilk, one-way ANOVA with Tukey’s post-hoc tests were used for statistical analysis of data ($P \leq 0.05$). DM induction significantly increased Bcl-2 as well as decreased Bax and P52 ($P \leq 0.05$) nevertheless training and training with crocin significantly decreased Bcl-2 and increased Bax and P53 ($P \leq 0.05$); crocin significantly decreased Bcl-2 and increased P53 ($P \leq 0.05$) and training with crocin had higher effect on increase of Bax and P53 compare to training ($P \leq 0.05$) also increase of Bax compare to crocin. Although training and crocin alone can improve apoptotic markers in diabetic rats, nevertheless training simultaneously with crocin have better effects than training alone.

Keywords: Exercise; crocin; apoptosis; diabetes; tissue

1 Introduction

The prevalence of diabetes mellitus (DM) has been on the rise in recent years. According to various studies, the global prevalence of DM will increase from 2.8% in 2000 to 4.4% by 2030. This growing trend will be 20% in industrialized countries and 69% in developing countries [1]; Also in the seventh

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edition of the Atlas of DM, published in 2015, it was reported that by 2015, 415 million adults worldwide had DM, which is estimated to reach 693 million by 2045 [2]. Obesity is known to be an important factor in insulin resistance and DM [3]. DM is an important factor in increasing oxidative stress and elevating reactive oxygen species (ROS). An increase in ROS is associated with mitochondrial dysfunction. Thus, this condition leads to mutation of the mitochondrial genome, followed by cell death due to apoptosis or necrosis [2,4]. Apoptosis is a type of cellular cell death that is necessary to maintain tissue homeostasis and embryonic development [5]. On the other hand, inhibition of apoptosis is a major cause of tumor formation. Therefore, control and regulation of apoptosis is an important goal in the treatment of related diseases [6]. One of the pathways involved in the induction of apoptosis is controlled by B-cell lymphoma-2 (Bcl-2) family members and, by activating Bcl2-associated X protein (Bax), leads to permeability of the mitochondrial membrane. The P53 is an activator of apoptotic transcription and plays an important role in controlling the cell cycle and apoptosis. During oxidative stress, this molecule increases rapidly, causing cell growth to stop and apoptosis to begin [7]. Under these conditions, the P53 activates caspase-9, followed by caspase-9 from the internal pathway, increasing the expression of the Bax and decreases the expression of Bcl-2 as an anti-apoptotic marker [8,9]. Bcl-2 is a membrane protein found in the outer mitochondrial membrane. In vertebrates, the Bcl-2 protein controls and regulates the internal or mitochondrial pathway of apoptosis [10]. Family of Bcl-2 divided into two functional categories of proteins included anti-apoptotic and pro-apoptotic [11]. Some studies have shown that exercise can reduce apoptosis by reducing oxidative stress [12]. There is clear evidence that regular exercises can inhibit the production of free radicals, thereby reducing ROS, inflammation, ischemia, and oxidative stress. Physical activity seemingly improves apoptosis through the mitochondrial pathway, and physical activity seems to be beneficial in reducing cardiac apoptosis by reducing ROS and preventing the resulting loss of cytochrome C within mitochondria [13]. Also, according to the results of some studies, aerobic endurance exercise and long-term exercise increase the antioxidant capacity [14,15]. On the other hand, some studies have confirmed the protective effect of crocin against tissue damage caused by apoptosis in diabetes [16–18]. Crocin is a coloring carotenoid with 14 active OH groups, one of the most important extracts from the saffron plant (Crocus sativus) [19]. Various studies have examined the effect of crocin on weight loss, oxidative stress, decreased insulin resistance, regulating blood glucose levels, reducing depression, preventing Alzheimer’s disease and improving retinal function [19,20]. The cytoprotic and anti-apoptotic activity of crocin in mammalian cells is mediated by the induction of ROS and the induction of endoplasmic reticulum stress [21]. Due to the antioxidant and anti-inflammatory properties of crocin and the importance of oxidative stress and inflammation in the liver of patients with DM, it seems that supplementation of crocin with continued training may enhance apoptosis in live tissue of patients with DM. Therefore, present study aimed to investigate the effect of continued training with crocin on apoptosis markers in liver tissue of diabetic rats.

2 Methods

In this experimental study, 32 Spragudawly rats with mean age of eight weeks were purchased and after transfer to animal lab, they were kept in standard situation (humidity of 45 to 55%, dark-light cycle of 12–12 hours and temperature of 23 ± 2°C) and free access to water and food (including crude protein 23%, crude fat 3.5%–4.5%, crude fiber 4%–4.5%, ash maximum 10%, calcium 0.95%–1%, phosphorus 0.65%–0.75%, salt 5%–5.5%, humidity maximum 10%, lysine 1.15%, methionine 0.33%, methionine + cysteine 0.63%, threonine 0.72%, and tryptophan 0.25%) for one week to adapt with new environment.

2.1 DM Induction

The present study used a combination of a high-fat diet (HFD) and an injection of streptozotocin (STZ) to induction of type 2 diabetes. For this purpose, all rats were fed a HFD for eight weeks (including 45% of total fat energy (derived from animal oil) containing 24 grams of fat, 24 grams of protein, and 41 grams of
carbohydrates per 100 grams). After eight weeks, induction of diabetes was performed by injecting a single
dose of 30 mg/kg STZ dissolved in sodium citrate buffer with pH = 4.5 intra-peritoneally [22]. To confirm
diabetes, 96 hours after injection of STZ, the rats with fasting glucose levels above 300 mg/dL were selected
as sample [22].

2.2 Grouping

Based on fasting glucose, rats were assigned into four groups of eight rats, including 1) sham, 2)
training, 3) crocin, and 4) training with crocin. Also, to investigate the effects of diabetes induction on
research variables, eight healthy rats were assigned in the healthy control group. Groups 2 and 4 ran on
treadmill for eight weeks, three sessions per week, and 60 minutes per session, with 50–50% of
maximum running speed. Groups 3 and 4 received 25 mg/kg/day crocin (dissolved in normal saline)
intra-peritoneally [23].

2.3 Continued Training Protocol

In order to estimate the maximum running speed, the graded exercise test was performed with a
slope of zero degrees. To perform this test, at first, the running speed was started at 10 m/min, and then
the speed was increased by 1 m/min for every 1 minute. This process continued until the rats were unable
to run (exhaustion). Continued training was started with 25 minutes in first week and reached 50 minutes
in last week.

2.4 Tissue Sampling

Forty-eight hours after the last training session and crocin administration, all rats anesthetized with
ketamine 10% (50 mg/kg) and 2% xylozin (10 mg/kg). Their liver tissue was then extracted by specialists
and placed in liquid nitrogen after being placed in a cryotube and stored at −70°C. RNA extraction was
performed according to the instructions of the RNA extraction kit manufactured by Yekta Tajhiz
Company, using the extraction kit solutions and the proposed protocol of the manufacturer. For molecular
analysis at the gene expression level, first, extraction of RNA from the liver tissue was carried out
according to the manufacturer’s protocol; then, drawing on the light absorbance property at wavelength of
260 nm, the concentration and degree of purity of the RNA sample was quantitatively obtained using the
following equation:

\[ C (\mu g/\mu l) = A260 \times \varepsilon \times d/1000 \]

After extracting RNA with high purity and high concentration from all of the samples, cDNA synthesis
steps were taken according to the manufacturer’s protocol, and then the synthesized cDNA was used for
reverse transcription reaction. Initially, the designed primers for genes were examined, and then genes
expressions were examined by quantitative q-RT PCR method. The sequence of primers used is also
shown in Tab. 1.

| Table 1: Sequence of forward-reverse primers of genes in real-time polymerase chain reaction |
|-------------------------------|-------------------------------|-----------------|
| **Gene** | **Forward (5'-3')** | **Reverse (5'-3')** | **Product Size (bp)** |
|-----------|---------------------|---------------------|---------------------|
| B2M       | CGTGCTTGCCATTCAGAAA | ATATACTCGGTCTCGGTGG | 244                 |
| Bax       | CTGCAGAGGATGATTGCTGA | GATCAGCTCGGGGACTTTAG | 147                 |
| Bcl-2     | ATCGCTCTGTGGAGTCGACTGATAC | AGAGACAGCCAGGAGAATCAAAC | 134                 |
| P53       | GGCTCCGACTATACCCACTATCC | GAGTCTTCCAGCGTGATGATG | 104                 |
2.5 Statistical Analysis of Data

The Shapiro–Wilk test was used to investigate the normal distribution of the data and one-way ANOVA with Tukey’s post-hoc tests were used for investigate the effect of training and crocin on apoptosis markers in SPSS software (version 21) \((P \leq 0.05)\).

3 Results

Gene expression levels of Bcl-2, Bax and P53 in four groups of study are reported in Figs. 1–3 respectively.

![Figure 1](image)

**Figure 1:** Bcl-2 gene expression levels in four groups of study. +++\(P < 0.001\) Significant increase compare to healthy control group. ***\(P < 0.001\) Significant decrease compare to sham group. ###\(P < 0.001\) Significant decrease compare to crocin and training with crocin groups. $$P < 0.01$$ Significant decrease compare to training with crocin group

The results showed that Bcl-2 gene expression levels in sham group were significantly higher than healthy control group \((P = 0.001)\) nevertheless in training, crocin and training with crocin groups were significantly lower than sham group \((P = 0.001)\); in training group were significantly lower than crocin and training with crocin groups \((P = 0.001)\) also in crocin group were significantly lower than training with crocin group \((P = 0.004)\) (Fig. 1).

Bax gene expression levels in sham group were significantly lower than healthy control group \((P = 0.001)\) nevertheless in training and with crocin groups were significantly higher than sham group \((P = 0.001)\) also in training with crocin group were significantly higher than training and crocin groups \((P = 0.001)\) (Fig. 2).

P53 gene expression levels in sham group were significantly lower than healthy control group \((P = 0.03)\) nevertheless in training \((P = 0.01)\), crocin \((P = 0.001)\) and training with crocin \((P = 0.001)\) groups were
significantly higher than sham group; in crocin and training with crocin groups were significantly higher than training group \( P = 0.001 \) also in crocin group were significantly higher than training with crocin group \( P = 0.004 \) (Fig. 3).

4 Discussion

The results showed that diabetes induction significantly increased Bcl-2 and decreased Bax and P53 in the liver tissue of rats. However, eight weeks of continued training significantly decreased Bcl-2 and increased Bax and P53 in the liver tissue of rats with DM. It has been reported that Bax is a strong stimulant of cell death and Bcl-2 function is in order to increase cell survival [24]. Bcl-2 is also an important anti-apoptotic protein in cells, as well as Bax and caspase-3 are important pro-apoptotic markers [25]. P53 plays an important role in regulating mitochondrial hemostasis and apoptosis regulation [26]. P53 can regulate many of the prognostic genes for apoptosis, including Bax and Bid, to induce DNA fragmentation [26]. Studies have shown that Bcl2 expression is significantly decrease by increasing Bax and caspase-3 [25]. Contrary with findings of the present study, Cheng et al. [27] showed that moderate aerobic exercise increases Bcl-2 and decreases Bax and caspase 3 in the heart tissue of STZ-induced diabetic rats. On the other hand, in a study by Ghahremani et al. [24], six weeks of low-intensity exercise reduced the Bax gene expression and increased the Bcl-2 gene expression in rats with myocardial infarction. However, high-intensity exercise increased the Bax gene expression in heart tissue, but did not significantly affect the Bcl-2 gene expression in the heart tissue of rats with myocardial infarction [24]. Also Kanter et al. [12] reported that low-intensity exercise had protective effect in diabetic by decreasing oxidative stress and apoptosis. In this study, it was shown that low-intensity exercise has antioxidant and anti-apoptotic effects. Further researches are needed to investigate the
molecular protective mechanism of low-intensity trainings on apoptosis markers. In the current study, 8 weeks of continued training significantly decreased Bcl-2 gene expression in diabetic rats, which appears to be inconsistent with previous studies. Differences in these findings in the effect of exercise on Bcl-2 can be due to differences in samples, age of rats [28], the type of tissues, the duration and intensity exercise. So that in noted studies, the Bcl-2 and Bax gene expression in heart tissue have been studied, and the mechanism of their changes may be different from liver tissue, so further studies seem to be needed. The results of the present study showed that eight weeks of 30 mg/kg crocin administration significantly decreased Bcl-2 and increased P53 in the liver tissue of diabetic rats. In this regard Thushara et al. [29] reported that crocin protects platelets via reducing the activation of H$_2$O$_2$ due to caspase 3 (apoptotic protein) against oxidative stress-induced apoptosis. Chen et al. [30] showed that crocin significantly suppressed the proliferation of human lung adenocarcinoma cells and significantly increased the mRNA levels of X-P53 protein while decreased Bcl-2. As P53 mutates in approximately 50% of human tumors and the P53 gene is involved in regulating the cell cycle, it controls DNA and apoptosis [31]. According to the findings of present study, eight weeks of crocin administration can significantly increase the P53 gene expression, but cannot enhance the Bax gene expression. Possible mechanisms which crocin modulates apoptosis can be decreasing H$_2$O$_2$ and caspase 3. Crocin also has antioxidant and blood glucose lowering properties, which can be effective in controlling apoptosis [32]. Studies have also shown that saffron carotenoids, including crocin, protect the cell against oxidative stress by reducing the production of free radicals with internal or mitochondrial origin and its blocking role on free radicals.

**Figure 3:** P53 gene expression levels in four groups of study. +$P < 0.05$ Significant decrease compare to healthy control group. ***$P < 0.001$, *$P < 0.05$ Significant increase compare to sham group. ###$P < 0.001$ Significant increase compare to training group. $$P < 0.01$ Significant increase compare to training with crocin group.
These substances react with superoxide anions, other radicals and ROS; stabilizing them and thus protecting the cell from oxidative stress [33].

Regarding the interactive effects, eight weeks of continued training with crocin administration significant decreased Bcl-2 and increased Bax and P53 in the liver tissue of diabetic rats; Also continued training with crocin compared to training alone had a higher effect on increasing Bax and P53, as well as had a higher effect on increasing Bax compare to crocin alone. Regarding the simultaneous effect of exercise and crocin consumption, Ghorbanzadeh et al. [34] showed that exercise with crocin significantly decreased pancreatic P53 gene expression in diabetic rats. It has been reported that crocin may inhibit the increase in ROS via reducing lipid peroxidation, thereby inhibits caspases and P53 as well as prevents the induction of apoptosis. Exercise also blocks apoptosis pathways by increasing the expression and activity of protein kinase B, phosphorylation of Bcl-2 family anti-apoptotic proteins, and inactivation of progressive apoptotic proteins such as Bax, or by direct inhibition of caspase activity. Most importantly, exercise and the simultaneous use of crocin in both interventions increase Bcl-2 and the anti-apoptotic process by inhibiting caspase activity and reducing ROS. Lack of measurement the protein levels of apoptotic markers by Western blotting and ELISA methods were the research limitations of present study; so it is suggested that in future studies in addition to noted methods the hematoxylin and eosin stain (H&E) and TUNEL staining methods should also be used to confirm the findings of present study.

5 Conclusion

Although training and crocin alone can improve apoptotic markers in diabetic rats, nevertheless training simultaneously with crocin have better effects than training alone on improving apoptotic markers in liver tissue of diabetic rats; so it appears that can use protective effect of training along with crocin in diabetes situations.

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