Pharmaceutical Sciences
September 2017, 23, 182-188
doi: 10.15171/PS.2017.27
http://journals.tbzmed.ac.ir/PHARM

Research Article

Effect of Crocin and Voluntary Exercise on P53 Protein in Pancreas of Type2 Diabetic Rats

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Article Info

Article History:
Received: 16 September 2016
Accepted: 2 March 2017
ePublished: 30 September 2017

Keywords:
-Crocin
-Voluntary exercise
-Apoptosis
-P53 protein
-Type 2 diabetes

ABSTRACT

Background: Excessive apoptosis of the pancreatic beta-cell has been associated with type 2 diabetes. Hyperglycemia significantly stimulates pancreatic islet cell apoptosis. We evaluated the role of crocin and voluntary exercise on apoptosis of pancreas tissue in type2 diabetic rats.

Methods: Animals divided into 5 groups as: control (Con), diabetes (Dia), diabetic-crocin (Dia-Cro), diabetic-voluntary exercise (Dia-Exe), diabetic-crocin-voluntary exercise (Dia-Cro-Exe). Type 2 diabetes was induced by high-fat diet (4 weeks) and injection of streptozotocin (STZ) (i.p, 35 mg/kg). Animals received crocin orally (50 mg/kg), voluntary exercise performed alone or together for 8 weeks. At the final of study, blood glucose levels and HbA1c were detected. Also p53 protein levels of pancreas tissue were measured by ELISA.

Results: P53 levels in pancreas tissue of diabetic group were significantly higher than control group. Crocin and exercise significantly decreased the blood glucose, HbA1c levels and p53 expression in treated diabetic groups compared to diabetic group. The glucose, HbA1c and p53 levels were also significantly lower in crocin-voluntary exercise group in comparison to the other experimental groups.

Conclusion: Our results reveal that both crocin and voluntary exercise reduce apoptosis of pancreas through reduction of p53 levels. Moreover, treatments with crocin and voluntary exercise have synergistic anti-apoptotic effects on pancreas tissue of type 2 diabetic rats. Protective effects of these interventions probably perform through the decreasing of glucose and HbA1c levels in blood of rats suffering from diabetes.

Introduction

Type 2 diabetes exhibits itself in individuals who lose the ability to produce sufficient amounts of insulin to maintain normoglycemia in the face of insulin resistance.[1] In patients with Type 2 diabetes there is a loss of beta cells and/or impaired augmentation of beta-cell mass consequent to increased demand.[2] The endocrine pancreas has a remarkable capacity to adapt to conditions of increased insulin demand by increasing its functional β-cell mass.[3] Long-term adaptation of the β -cell mass to conditions of increased demand occurs by increasing the β–cell apoptosis. In type 1 diabetes, β -cell mass is reduced by 70 – 80% at the time of diagnosis. However, the mechanisms lead to beta cell loss in type 1 diabetes are not well defined, previous studies has been proposed a role for high glucose and cytokine in p53-dependent apoptosis.[4,5] Crocin is the major active constituents of saffron which used in the traditional medicine for the treatment of numerous diseases including diabetes, depression, cognitive disorders and cancer.[6,7] Some investigations indicate that saffron and its constituents, crocin, have many pharmacological effects on diabetic animal models such as stimulation of glucose uptake by peripheral tissues,[8] inhibition of intestinal glucose absorption,[9] correction of insulin resistance,[10] stimulation and regeneration of beta cells in islets of langerhans to release more insulin.[11,12] As an interest, Hoshyar*et al*. (2013) reported that Crocin triggers the apoptosis through increasing the Bax/Bcl-2 ratio and caspase activation in human gastric adenocarcinoma.[13]

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Samarghandian *et al*. (2013) showed that saffron induces apoptosis in alveolar human lung cancer cells through caspase-dependent pathways activation.[14] Also Rezaee *et al*. (2014) displayed that crocin administration caused a mild increase apoptosis on human myeloma cells.[15] Regarding many reports concerning pro-apoptotic effects of crocin on cancer cells, there was not enough study of crocin effects on non-cancer cells.

Intervention studies of physical exercise training strongly support its efficacy for diabetes prevention and management.[16] In addition, training exercise upgrades insulin sensitivity in humans [17] and decreases the risk of developing type 2 diabetes in animal models.[18] Kiraly*et al*. exhibited that regular swimming exercise in diabetic rats elevates β-cell mass by increasing the number of β-cells.[19] Comparable results were found by Delghingaro-Augusto et al. that examined diabetic rats subsequent 6 weeks of voluntary wheel running.[20] Nevertheless, exhaustive exercise may be problematic, as they are stressful through production of reactive oxygen species and can cause damage to muscle tissue and other organs,[21] voluntary exercise may be a better model with more beneficial effects.[22] In the animal model of voluntary exercise, the animal has free access to a running wheel and uses the wheel according to its physiological threshold for physical activity.

Therefore, the influence of crocin combination with voluntary exercise on pancreases apoptosis following type 2 diabetic rats remains unknown. In this study, we used the type 2 diabetes animal model to investigate the effects of crocin combined with voluntary exercise on p53 pro-apoptotic protein in high fat diet induced-type 2 diabetic rats.

Materials and Methods

Animals

Male wistar rats (200-250g) were obtained from Tabriz medical faculty (Iran-Tabriz). They were housed four per cage excluding rats in exercise groups that was singly. Animals housed in a room with a constant temperature of 24˚C, a relative humidity of 50%, and a 12h dark / light cycle with access to food and water ad libitum. The present study was approved by the Animal Ethics Committee (document number 92197) in accordance with the instruction for the care and use of laboratory animals prepared by Tabriz University of Medical Sciences.

Experimental design

Animals were randomly divided into five groups (n=7). Group 1 received NaCl 0.9 %Solution as a control group. Group 2 (positive control group) received excessive-fat diet (4 weeks) and single dose of STZ (35mg/kg; i.p.) as a diabetic group (Dia). Group 3 received a single dose of crocin (50 mg/kg) for eight weeks after confirmation of Diabetes (Dia-Cro). Group 4 performed voluntary exercise for eight weeks after confirmation of Diabetes (Dia-Exe). Group 5 received crocin and simultaneously performed voluntary exercise for eight weeks after confirmation of Diabetes (Dia-Cro-Exe). Crocin (Sigma) was gavaged (50 mg/kg) 6 days per week during 8 weeks (Figure 1). For the assessment of voluntary exercise, rats were housed individually in a cage containing a wheel (1.00 m circumference, Tajhiz Gostar). This stainless-steel running wheel equipped with a digital magnetic counter that is activated by wheel rotation. Each exercising rat had a separate running wheel in its cage that allowing it to run voluntarily during 8 weeks of the study. Rats that their running distance was lower than ~2000 m per day were eliminated before statistical analysis.[23]

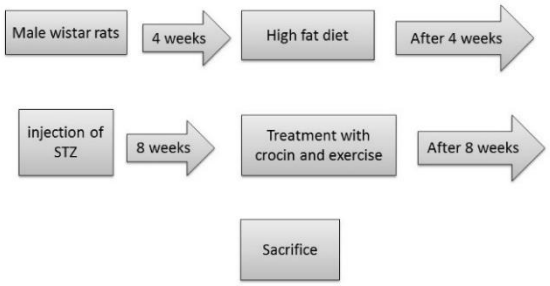

Figure 1. Experimental design: high-fat diet-induced diabetic rats treated with crocin and voluntary exercise.

Development of type 2 diabetes

After1 week ad libitum exposure to drinking water and standard rat pellet diet, rats were nourished with high fat diet comprising 22% fat, carbohydrate 48% and protein 20% in blend with standard laboratory chow consisting of 5% fat, 53% carbohydrate and 23% protein for 4 week. The composition and preparation of high fat diet were as described Previously.[24,25] Following the period of dietary manipulation, diabetes was induced in by an intraperitoneal injection (35 mg/kg BW) of streptozotocin (STZ) (Sigma).[24] Afterwards 72 hours of streptozotocin administration, fasting blood sugar levels were measured. Animals with fasting blood glucose value > 300 mg/dl were considered as diabetic. Animals with fasting blood glucose concentration < 300 mg/dl were excluded from the study.

Blood collection and preparation of pancreatic tissue

On the final day of experiment, rats were sacrificed under anesthesia with ketamine/xylazine (88/10 mg/kg, i.p.). Blood samples were collected from the inferior vena cava and stored in tubes at –70 °C until determination of blood glucose concentration and Hemoglobin A1c (HbA1c) measurement. HbA1c was measured using the immunoturbidimetric assay.[26]

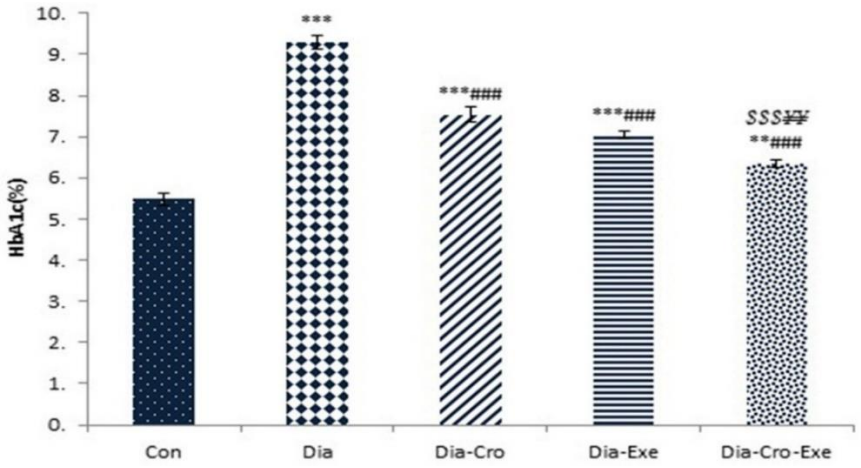

Figure 2. Effect of crocin and voluntary exercise on HbA1c levelsAfter 8 weeks of treatment in diabetic rats. Data are shown as mean ± S.E.M for n=7 animals, ***P<0.001 and **P<0.01 indicated significantly change compared with control group, $^{\#\#\#}$P<0.001 indicated significantly change compared with Dia group. $^{\$\$\$}$P<0.001 indicated significantly change compared with Dia-Cro group and $^{¥¥¥}$ P<0.001 indicated significantly change compared with Dia-Exe group.

Pancreas tissue shortly removed and washed with saline 0.9%. Then pancreas were excised, frozen in liquid nitrogen and stored at deep freeze (-70 °C) for p53 protein measurements.

P53 protein assay

For the detection of p53 protein, we used a p53 pan-ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany). This assay for the quantification of wild-type and mutant p53 of human, mouse, and rat origin, is based on a quantitative sandwich ELISA principle. The biotin-labeled capture antibody is pre-bound to the streptavidin-coated micro titer plate. During a single incubation step, the p53-containing sample reacts with the capture antibody and the peroxidase-labeled detection antibody to form a stable immunocomplex. Subsequent to the washing step, the peroxidase bound in the complex is developed by tetramethylbenzidine as a substrate. The resultant absorbance is proportional to the concentration of p53. Tissue homogenates were prepared from tissues, as described in the p53 pan ELISA kit and collected supernatants were analyzed according to the manufacturer's instructions.[27]

Statistical analysis

All values were expressed as means±SEM. The between-group parameters were analyzed using two-way ANOVA followed by Tukey post-hoc test. Differences were considered statistically significant when P<0.05.

Results

Effects of crocin and voluntary exercise on the blood HbA1c levels

Two-way ANOVA showed significance difference between HbA1c levels in diabetic groups versus control group (all P<0.001 except Dia-Cro-Exe P<0.01). After 8 weeks of medication of diabetic rats with crocin and voluntary exercise, HbA1c levels of Dia-Cro and Dia-Exe were significantly (P<0.05) lower than Dia group (Figure 2). In crocin together with exercise group, the HbA1c levels significantly decreased compared to diabetic animals (P<0.001). A substantial difference was found between Dia-Cro-Exe group and Dia-Cro (P<0.001) or Dia-Exe groups (P<0.01). Figure 2 indicates that crocin combined with voluntary exercise has an additive effect in HbA1c levels.

Effects of crocin and voluntary exercise on the blood glucose levels

According to the Figure 3, two-way ANOVA indicates that blood glucose levels significantly increased in the diabetic groups in comparison with control group. Treatment accompanied by crocin and exercise significantly reduced the blood glucose levels in Dia-Cro (P<0.05), Dia-Exe (P<0.05), and Dia-Cro-Exe (P<0.001) in comparison with diabetic group. Also blood glucose levels in Dia-Cro-Exe group were significantly lower than those of other treatment groups (P<0.05). These findings exhibit that increasing of blood glucose by diabetes is reversed by crocin and voluntary exercise.

Effects of crocin and voluntary exercise on the pancreas levels of p53

As depicted in Figure 4, there were significant difference in protein levels of p53 in rats with diabetes compared to control group (P<0.001). After 8 weeks of treatment of diabetic rats with crocin and voluntary exercise, there was a significant decrease in p53 content in treatment groups relative to diabetic group (P<0.001). P53 was also lower in Dia-Cro-Exe group than in Dia-Cro and Dia-Exe groups (P<0.001). No difference was found between Dia-cro group and Dia-Exe groups for pancreas level of p53 (P>0.05).

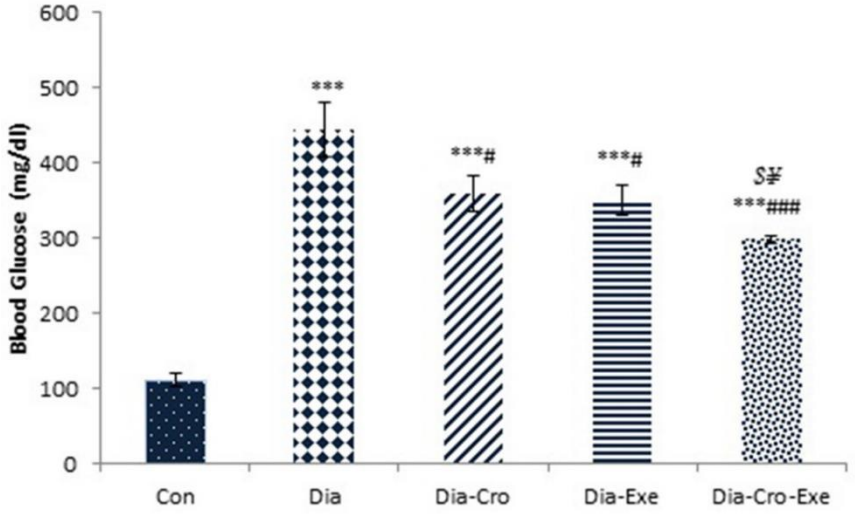

Figure 3. Effect of crocin and voluntary exercise on blood glucose levels.crocin and exercise significantly reduced the blood glucose levels in diabetic rats. Data are shown as mean ± S.E.M. for n=7 animals, ***P<0.001 indicated significantly change compared with control group, $^{\#\#\#}$P<0.001 and $^{\#}$P<0.05 indicated significantly change compared with Dia group. $^{\$}$P<0.05 indicated significantly change compared with Dia-Cro group and $^{¥}$P<0.05 indicated significantly change compared with Dia-Exe group.

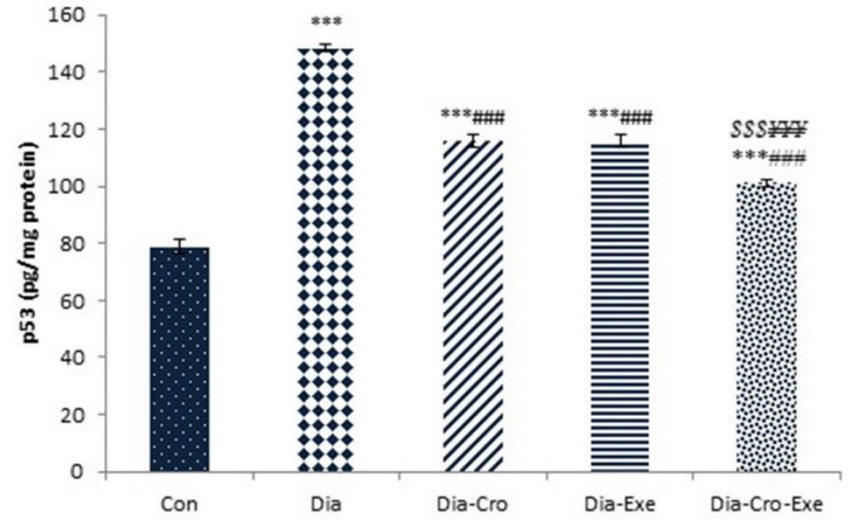

Figure 4. p53 levels after 8 weeks treatments of diabetic rats with crocin and voluntary exercise was measured by ELISA. Data are shown as mean ± S.E.M. for n=7 animals, ***P<0.001 indicated significantly change compared with control group, $^{\#\#\#}$P<0.001 indicated significantly change compared with Dia group. $^{\$\$\$}$P<0.001 indicated significantly change compared with Dia-Cro group and $^{¥¥¥}$P<0.001 indicated significantly change compared with Dia-Exe group.

Discussion

The results of the present study showed significant increase in p53 expression in high fat diet/STZ induced type 2 diabetic rats. Administration of crocin and exercise reduced the p53 levels of pancreas tissue in diabetic rats after eight weeks of medication. Furthermore, crocin and voluntary exercise significantly decreased the HbA1c and glucose levels in the blood of type 2 diabetic rats. Also combination of crocin and voluntary exercise lead to a drastic reduction of p53 expression, HbA1c and Blood glucose levels in type 2 diabetic rats.

Type 2 diabetes is characterized by hyperglycemia and progressive β-cell failure. Loss of the β-cell functions is defined by a loss in tolerance to glucose and sensitivity to insulin. It has been demonstrated that in type 2 diabetes the β-cell mass decreases up to 60%, due to increase in apoptosis rate.[28] Several studies indicated that hyperglycemia triggers the cells apoptosis through p53 activation.[4,29] P53 regulates transcription of pro-apoptotic genes and causes expression of inhibitors of survival-related proteins. Fiordaliso et al.(2001) has been proposed a role for p53 in high glucose-induced ventricular myocyte apoptosis.[4] Keim et al. (2001) reported that $p53^{-/-}$mice exhibited differential induction of apoptosis in response to high glucose concentrations, suggesting that p53 is required for high glucose-induced apoptosis. Hyperglycemia promotes p53 phosphorylation which correlates with activation of pro-apoptotic proteins such as Bax.[29] Furthermore, Hinault et al. (2011) demonstrated that in p53 knockout mice, the β-cell population regenerate and rescue the diabetic

phenotype in this animal model.[30] According to above mention statements, it can be stated that up regulation of the apoptosis pathways are implicated in the development of type 2 diabetes. Therefore, reduction of apoptosis in β-cells has considerable importance for the improvement of pancreas functions.

Practice training is clearly established as being protective against T2DM development. Many studies of exercise training strongly support its efficacy for diabetes prevention and management .The study of Krotkiewski et al. (1989)was the earliest to investigate aerobic training in individuals diagnosed with T2DM and they concluded that exercise training made better insulin secretory capacity to oral glucose due to elevated β-cell mass.[31] Studies indicated which exercise training promoted β-cell growth dramatically and growth restricted animals had partial β-cell mass restoration (by 60–68%) if they were regularly exercising.[32] Kiraly et al. (2007) demonstrated that swimming exercise in type 2 diabetic rats elevates β-cell mass by increasing the number of β-cells per.[19] In the present study, β-cell apoptosis through measuring of p53 levels in pancreas tissue of type 2 diabetic rats were decreased by voluntary running. The effects of voluntary exercise on p53 expression level possibility due to the decreasing effect of voluntary exercise on glucose and HbA1clevel.Previous study proved that subjects undergoing exercise training improved many of the abnormalities associated with the type 2 diabetes specially improvement of glycemia control.[33] Although other results suggest that reduction of glucose is not a contributing factor to the exercise-induce reduction in apoptosis of pancreas. Wisniewski et al. (2015) reported that exercise reduced significantly the inflammatory markers, oxidative damage and brain apoptosis in high fat fed animals.[34]

Our results also revealed that administration of crocin leads to significantly decreasing in the level of P53 pro-apoptotic protein. Since crocin has been shown to have pro-apoptotic effects on tumor cells and many reports claim crocin to be a promising cancer therapeutic agent, our finding showed its anti-apoptotic effects in diabetic rats. Also we revealed that blood glucose and HbA1c levels in crocin-treated diabetic rats was significantly lower compared to the normal control rats. High blood glucose level causes deterioration of pancreatic β-cells due to oxidative stress that the generation of reactive oxygen species rapidly induces apoptotic cell death via both mitochondria-dependent and-independent pathways.[35] With regard to the reduction of pancreatic apoptosis through decreasing p53 expression levels, effect of crocin may be related to the antioxidant properties and its effects in reducing blood glucose and HbA1c levels. In addition, Xu et al (2006)demonstrated that crocin has preventive effects on the cell apoptosis induced by hydrogen peroxide through antagonising apoptosis.[36] Thus, hara et al (2013) suggested that crocin protects platelets from oxidative stress-induced apoptosis through reducing H2O2-evoked activation of caspase-3 (pro-apoptotic protein).[37] Therefore we suggest that the anti-apoptotic effect of crocin may due to antioxidant effects, lowering blood glucose and reduction of p53 protein in pancreas tissue. Finally, in the present study, we found that crocin combination with voluntary exercise have an additive effects on blood glucose, HbA1c and p53 expression levels in pancreas tissue of type 2 diabetic rats. Thereby, our data suggest that crocin together voluntary exercise is a potential therapeutic candidate in the treatment of pancreatic β-cells loss in type 2diabetes.

Conclusion

In conclusion it can be articulated that crocin combined with voluntary exercise can be implemented in the treatment strategy for type 2 diabetes. These interventions exert excellent anti-apoptotic effects on pancreatic apoptosis through reduction of p53 expression levels. They show the ability to retard the various events of apoptosis pathway probably mediated by its blood glucose lowering effects and antioxidant activity. Although the detailed mechanism by which crocin and exercise act remains unknown, we suggest that additional experiments should conduct on the effects of crocin and voluntary exercise on apoptotic gene/protein modulation, e.g., the expression of Bcl-2 family proteins, release of cytochrome c and activation of caspase-3 that might provide useful information in pancreatic β-cells apoptosis in type 2 diabetes.

Conflict of interests

The authors claim that there is no conflict of interest.

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