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Effect of Crocin and Treadmill Exercise on Spermatogenesis and Testis Structure in Streptozotocin-Induced Diabetic Rats: An Experimental Study

Hassan Hamidi[1], Asghar Tofighi[1*], Javad Toluei Azar[1], Amir Afshin Khaki[2], Mazdak Razi[3]

Abstract

Objectives: This study aimed to evaluate the effect of crocin and treadmill exercise on oxidative stress, sperm parameters, and testis structure in streptozotocin (STZ)-induced diabetic rats.

Materials and Methods: In this experimental study, 64 diabetic rats induced by a single intraperitoneal (IP) injection of STZ (50 mg/kg) were assigned to the following groups (n=8/each): healthy control group, diabetic control group, diabetic group treated daily with crocin at a dose of 50 mg/kg, and one healthy group receiving daily crocin at a dose of 50 mg/kg for 56 days. Some groups such as the controls and diabetics exercised with treadmill, as well as the healthy and diabetic groups performed daily exercise with treadmill and crocin. After crocin treatment, all rats were anesthetized, their blood samples were taken, and the serum level of insulin, glucose, and oxidative stress markers were measured. Finally, the testicles and epididymis were removed and sperm parameters were assessed.

Results: Treatment of diabetic rats with crocin along with treadmill exercise significantly replaced the testicular tissue damage, sperm parameters, glucose, and insulin plasma levels ($P=0.001$). In diabetic rats, moreover, the level of malondialdehyde (MDA) was increased and the levels of superoxide dismutase (SOD) and catalase (CAT) enzymes activity were reduced in the testicular tissue ($P=0.001$). Crocin administration (50 mg/kg BW) and exercise significantly improved these parameters ($P<0.05$).

Conclusions: Our results confirm the antioxidant role of crocin and the positive role of treadmill exercise in improving the sperm parameters and testicular oxidative damage caused by diabetes.

Keywords: Oxidative stress, Diabetes mellitus, Treadmill exercise, Crocin, Testis, Sperm parameters

Introduction

Diabetes is one of the most common metabolic disorders worldwide, which is associated with the increased serum levels of glucose. Diabetes is caused by either defects in insulin secretion (Insulin-dependent diabetes) or the resistance of hormone in peripheral tissues (non-insulin-dependent diabetes) with a decrease in the secretion of hormone from pancreatic islets β-cells. Sexual dysfunction is one of the most significant complications of diabetes in men, and its other complications include a decline in testicular weight, sperm quality parameters, levels of testosterone in plasma, abnormal sperm counts, and infertility (1,2). High blood glucose in a diabetic patient can cause an advanced glycation end-products, changes in the protein kinase C activity, an imbalance in prostanoids, and enhanced production of mitochondrial superoxide. These effects, in turn, result in increased "oxidative stress" due to free radical excess. Several studies have shown that enhancing the antioxidant system can reduce the complications of diabetes (3-5).

Therefore, the oxidative stress management can be a key therapeutic approach to treat diabetes and its complications. A habitual physical exercise is a strong tool in preventive medicine, especially for the diabetic patients. Despite possible risk of acute exercise related to oxidative stress in diabetic patients, studies have shown that regular moderate exercise and fitness may protect diabetic men against oxidative stress (6).

Although several chemical medicines have been developed to treat diabetes, using the herbal compounds has drawn much attention recently due to their lower risk for diabetic patients.

Crocin and crocetin in saffron are the most important "carotenoids", and are responsible for the color of saffron. In the metabolized body, crocin is converted to crocetin. Crocetin with various therapeutic properties is known as a strong antioxidant and anti-inflammatory agent in laboratory animals. Crocin acting as an activator for DNA repair enzyme cleavage prevents DNA damage. Although this hypothesis has not yet been proved or refuted, other

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[1]Department of Exercise Physiology and Corrective Movement, Faculty of Sport Sciences, Urmia University, Urmia, Iran. [2]Department of Anatomical Sciences, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran. [3]Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
*Corresponding Author: Asghar Tofighi, Tel: +9843-32752741-044, Email: a.Tofighi@urmia.ac.ir

Key Messages

- Diabetes leads to testis damage and reduction of sperm quality.
- Treatment with treadmill exercise, crocin can improve testicular damage and sperm parameters.

mechanisms have been suggested for its protective effect (7).

Given the above discussion, this study aimed to evaluate the effect of crocin and treadmill exercise on oxidative stress, sperm parameters, and testis structure in streptozotocin (STZ)-induced diabetic rats.

Materials and Methods

Animals and Experimental Design

A total of 64 adult male Wistar rats (weight rage: 200–250 g) were included in this experimental study. All rats were kept under the same environmental and nutritional situation in a room with normal temperature of 23 ± 2°C and humidity of 40-50% in a 12 hours/12 hours light/dark cycle. Standard laboratory diets and drinking water were provided for the animals. However, the rats were randomly divided into 8 groups (n = 8/each) as follows:

1. Healthy control group (control or G1);
2. Diabetic control group (G2);
3. Diabetic group treated with crocin (50 mg/kg/d) (8) for 8 weeks (G3);
4. Healthy control group treated with crocin (50 mg/kg) for 8 weeks (G4);
5. Healthy group exercised for 8 weeks;
6. Healthy group exercised on a treadmill for 8 weeks and received crocin daily;
7. Diabetic group exercised on a treadmill for 8 weeks and received crocin daily;
8. Wistar rats were trained in a diabetic group for 8 weeks.

Based on the previous study, diabetes in the experimental groups was induced by intraperitoneal (IP) injection of a single dose of 50 mg/kg STZ (Sigma-Aldrich, Germany) dissolved in 0.01 mM citrate buffer (pH = 4.5) (9). Then 72 hours after the STZ injection, the blood glucose levels were checked using samples collected from the tail vein tip. The rats with blood glucose levels more than 250 mg/dL were confirmed to be diabetic (9).

Treadmill Exercise Protocol

After conducting the treadmill exercise protocol (TEM) for 72 hours, the adapted TEM with a duration of 8 weeks, 1 h/d, 5 d/wk at 0° of the slope was initiated. The first week was aimed at adapting the rats to moderate-intensity exercise, with a progressive increase in time and speed. From the second week, every session comprised three periods: (*a*) "warm-up" lasting 5 minutes at 30% of Smax1; (*b*) "Moderate intensity exercise" lasting 50 minutes at 60% of Smax1; and (*c*) "Recovery" lasting from 5 minutes to 30% of Smax1. A low-intensity electric stimulus (1.5-2.0 mA) placed at the back of every lane was utilized to stimulate the animals (10).

Histopathological Examination of Testis

To estimate the testis histopathological changes in "seminiferous tubules", testicular tissues were dehydrated after fixation and then embedded in paraffin. Finally, the slides were prepared to evaluate the histopathological damage and the number of germ cells was counted (at 400× magnification) (11).

Biochemical Assays

In order to assess the changes in the plasma level of insulin and glucose, the blood samples were centrifuged immediately after sampling, and the serum sample was removed and stored at -80°C until analysis. The glucose condensation was measured using commercial kits (Parsazmun, Iran). Plasma insulin levels were measured adopting the ELISA method and using the commercial kit of Rat Insulin (Mercodia).

Measurement of Lipid Peroxidation

The lipid peroxidation level was demonstrated by measuring malondialdehyde (MDA) in the testis tissue. To this end, 375 mg of thiobarbituric acid (TBA) was initially dissolved in 2 mL of hydrochloric acid (HCL) to prepare the TBA-trichloroacetic acid (TCA)-HCL solution. Then, this solution was added to 100 mL of 15% TCA. A water bath at 50°C was used to finalize the sediment dissolution. For achieving a 10% homogenized mixture, a slice of testis tissue was weighed and immediately homogenized with 5.1% potassium chloride solution. Then, 1cc of homogenized tissue mixture was added to 2 mL of TBA-TCA-HCL solution and heated for 45 minutes in a boiling water bath (pink-orange solution), cooled rapidly, and centrifuged at 1000 rpm for 10 minutes. A spectrophotometer was used to read the absorption (A) at 535 nm.

Determination of Superoxide Dismutase Activity

The concentration of superoxide dismutase (SOD) in the testis tissue was assessed using an ELISA reader (Antus) according to the manufacturer's protocols (Ransod, UK).

Catalase Activity Assays

The catalase (CAT) activity was determined by evaluating the reduction in absorbance of a reaction mixture including 30 mM H2O2, in sodium phosphate buffer (pH = 7), and prerequisite volume homogenized tissue at 240 nm. The specific activity was calculated and expressed as units/mg of total protein.

Evaluation of Sperm Parameters

The epididymis from both testes of rats was removed, cleared from blood, placed in 5 mL HAMS F10 medium,

and cut into smaller segments. Afterwards they were put in the incubator of CO2 37°C for 30 minutes and removed the 100 µL of the solution dissolved in 900 µL from HAMS F10. This method was repeated for new solutions. One drop of the solution was mixed carefully and added to Neuberger's chamber. The sperm count was conducted based on the standard Protocol. Finally, the total sperm count was multiplied by the correction factor, $\times 10^6$ m in order to evaluate the sperm morphology. After preparing the smears of sperm, the slides were fixed with 96% alcohol and dried in exposed to the air. This enabled our research team to access to sperm morphometry. All slides were stained with H & E. To this end, 100 sperm were counted in each slide of each sample. In the end, the number of normal and abnormal sperms was determined and expressed as percentages (12).

Data Analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS, version 19.0 for Windows; SPSS Inc., Chicago, IL). The results were calculated as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's range test was used to analyze the data. *P* values less than 0.05 were considered statistically significant.

Results

Serum Glucose Level

During the third and sixth weeks of the study, a significant increase was observed in the serum glucose levels in the diabetic group compared to those in the control group ($P=0.001$). Additionally, a significant decrease was observed in the serum glucose levels in the third and sixth weeks in the diabetic group treated with crocin and treadmill in contrast to those in the diabetic control (Table 1).

Serum Level of Insulin

Comparing the serum insulin levels of the groups showed that diabetes caused a significant decrease in the levels of serum insulin in contrast to control group ($P=0.001$). Treatment of diabetic rats with crocin along with treadmill was found to improve the decreasing levels of serum insulin compared with the diabetic group ($P=0.03$) (Figure 1).

The Histological Finding

The result of germ cell count showed that the number

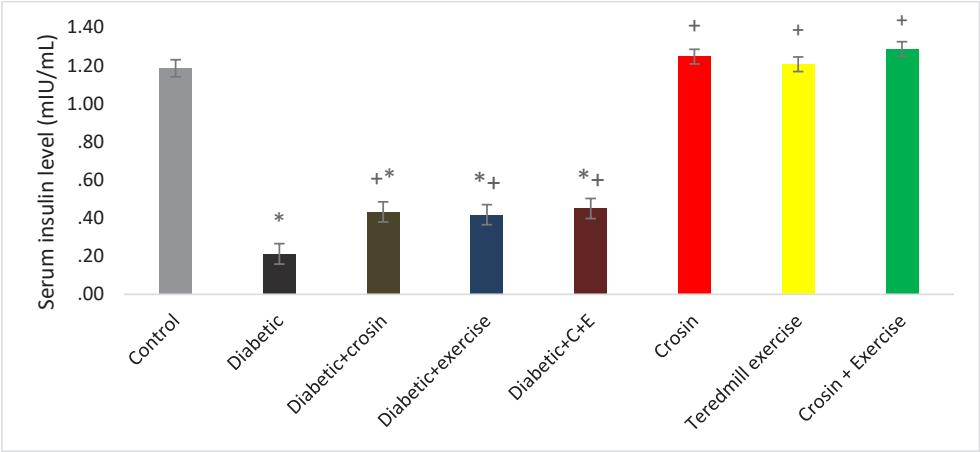

Figure 1. The Serum Level of Insulin in Study Groups. Asterisk sign (*) shows significant difference with control and Plus sign (+) shows significant difference with diabetic.

Table 1. The Serum Level of Glucose in Study Groups

Groups	One Week Before Diabetes	One-Week After Diabetes	At the End of Study
Control	94.5 ± 5.34	102.42± 7.25^{+}	92.4±9.34^{+}
Diabetic	103.2 ± 2.6	350.25± 4.57*	367.6±38.73*
Diabetic + crocin	97.5 ± 5.89	294.5± 4.93^{+*}	186.2±6.7^{+}
Diabetic + exercise	92.5 ± 4.5	290.82± 3.70^{+*}	134.2±15.38^{+}
Diabetic + crocin + exercise	98.65 ± 2.6	297.5± 5.2^{+}	117.75±6.1^{+}
Crocin	93.60 ± 7.6	95.25± 3.7^{+}	89.8±3.21^{+}
Treadmill exercise	104.25 ± 2.6	102.37± 7.2^{+}	87.8±4.21^{+}
Crocin + exercise	99.45 ± 3.4	95.8± 6.7^{+}	92.3±8.05^{+}

Asterisk sign (*) shows significant difference with control and Plus sign (+) shows significant difference with diabetic.

of germ cell was notably reduced in the diabetic group compared to the control group ($P<0.05$). In the crocin-treated group at a dose of 50 mg/kg and treadmill exercise such as diabetic rats, on the other hand, the number of germ cells was significantly higher than that of the diabetic control ($P<0.05$). Histopathological examination showed that the seminiferous tubule was irregular in the diabetic group with a reduction diameter and thickness of eminiferous tubule. Also, hemorrhage was observed between the seminiferous tubules. In the other groups, crocin treatment along with treadmill exercise improved this damage (Figure 2, Table 2).

The Oxidative Stress Markers Levels in the Testis Tissue

A highly significant increase was observed in MDA levels in the testes of diabetic rats compared to those in the control group ($P=0.001$). Treatment with crocin and treadmill exercise in the diabetic rats remarkably declined the increasing level of MDA in testicular tissue caused by diabetes ($P=0.001$). These findings suggested that diabetes caused a significant decrease in the CAT enzyme activity compared to the control group ($P=0.001$). Treating diabetic rats with crocin and treadmill exercise produced significantly different results in comparison with findings obtained in untreated diabetic group ($P=0.001$). Our findings showed that the activity of SOD in the diabetic group was significantly decreased compared to that in the control group ($P=0.001$). Comparison between the treatment groups and the diabetic group showed a significant increase in SOD enzyme activity ($P=0.001$) (Table 3).

The Sperm Count, Morphology and Motility

The number of sperms in the diabetic group was significantly decreased compared with that in control group ($P=0.001$). In both therapeutic groups, the number of sperms was significantly increased compared with that in the diabetic group ($P=0.001$). The number of abnormal sperms was significantly increased in diabetic group compared with the control group ($P=0.001$). Also, crocin treatment along with treadmill exercise significantly decreased the number of abnormal sperms compared with the diabetic group ($P=0.001$). On the other hand, sperm motility in the diabetic group was significantly decreased compared with that in the control group ($P=0.001$). Treatment of diabetic rats with crocin and treadmill exercise significantly improved the sperm motility in both therapeutic groups (Table 4).

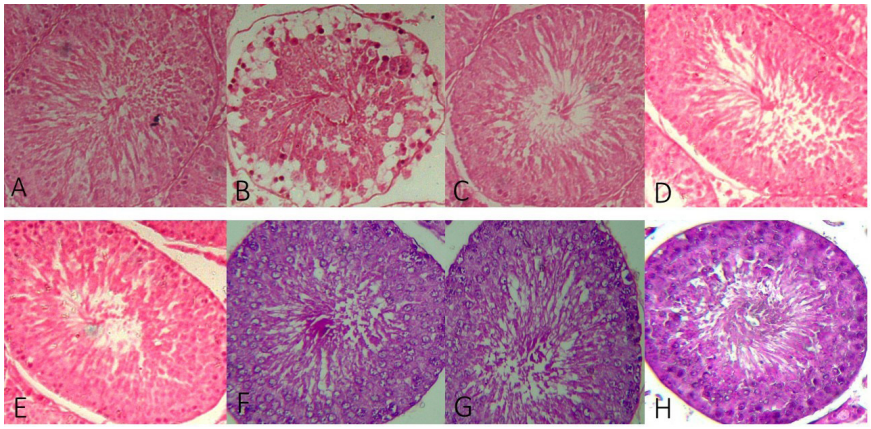

Figure 2. The Histological Assessment of Testicle in Study Groups. (A) Control, (B) diabetic group, (C) diabetic + crocin, (D) diabetic+ exercise, (E) diabetic+ exercise + crocin, (F) crocin, (G) exercise and (H) exercise + crocin.

Table 2. The Count of Germ Cell in the Study Groups

Groups	Round Spermatid	Primary Spermatocytes	Spermatogonia Cells	Sertoli Cells
Control	219.01 ± 3.85	110.41 ± 4.25	35.41 ± 1.05	27.04 ± 2.17
Diabetic	97.41 ± 5.07	64.12 ± 2.60	18.87 ± 2.35	19.22 ± 1.65
Diabetic + crocin	185.65 ± 2.45*+	98.41 ± 3.17*+	26.54 ± 2.03	24.12 ± 2.35
Diabetic + exercise	175.34 ± 1.90*+	95.54 ± 4.36*+	24.54 ± 1.42*	23.41 ± 1.15
Diabetic + crocin + exercise	180.55 ± 4.35+*	98.71 ± 3.45+*	25.39 ± 1.09	23.06 ± 1.07
Crocin	224.55 ± 6.35+	145.71 ± 5.45+	37.41 ± 1.05	27.06 ± 1.17
Treadmill exercise	214.55 ± 5.15+	135.71 ± 7.45+	32.87 ± 2.35	26.56 ± 1.7
Crocin + Exercise	215.33 ± 3.25+	103.55 ± 4.15+	36.54 ± 2.03	26.14 ± 1.2

* shows significant difference between control group and diabetic group.
+ shows significant difference between diabetic group and diabetic treatment groups ($P<0.05$).

Discussion

The present study examined the meliorative effect of crocin and treadmill exercise on damages induced by diabetes in the male reproductive system. Diabetes causes male reproductive dysfunction, and treatment with crocin and exercise improves these functional deficits by producing antioxidant and anti-diabetic effects. Moreover, regular treadmill exercise can regulate the oxidative stress markers and improve the antioxidant enzyme activity (13,14). Our findings showed that crocin and exercise decreased the blood glucose levels as well as the levels of oxidative stress markers in the testes of diabetic rats. The hypoglycemic activity of crocin and treadmill contributes to increasing the sensitivity of tissues to insulin (15,16).

In diabetic patients, in addition to an increased amount of blood glucose, the balance between production and elimination of free radicals is suspended. As a result, free radicals increase and cause oxidative stress (5,17). Oxidative stress results in cell injury through mechanisms such as lipid peroxidation and DNA and protein oxidative damage (18-23). Our findings revealed that diabetes remarkably increased the MDA levels (as lipid peroxidation marker) in the testicles of diabetic rats. This finding was consistent with the results from various previous research on oxidative stress in the testis of diabetic rats (5,18). Hence, increasing MDA levels in the testis of the diabetic group is indicative of the increase of lipid peroxidation. In this research, treatment of diabetic rats with crocin and regular exercise caused a significant decrease of MDA concentration in testis tissue. Several studies have reported that flavonoids in crocin scavenge the free radicals generated during lipid peroxidation (19,20). Thereby a decline in testis MDA concentration in the treated groups with the crocin and treadmill exercise may have been due to antioxidant effects of crocin as well as regular treadmill exercise.

According to our study results, the SOD activity was extremely declined in diabetic rats. These findings were in line with the results from previous studies in this regard (17). SOD is known as one of the most important enzymes of the antioxidant system, while its main action is the catalysis of superoxide anion radicals to H_2O_2. This method decreases toxicity of "superoxide" and no "free radicals" from superoxide are produced (21). In our study, on the other hand, SOD activity was significantly increased in testes of diabetic rats being under treatment with crocin and treadmill exercise in contrast to the diabetic group.

CAT as an antioxidant enzyme has detoxification effects against free radicals (22). In the present study, the reduction of CAT enzyme activity in diabetic rats was more significant than that in the control group, whereas CAT was significantly increased in groups treated with

Table 3. The Concentration of MDA, CAT, and SOD in Rat's Testes Tissues of 4 Groups

Groups	MDA	CAT	SOD
Control	0.782 ± 6.5	5.85 ± 0.057	1.63 ± 0.21
Diabetic	1.485 ± 7.35*	2.88 ± 0.048*	0.60 ± 0.11*
Diabetic + crocin	0.977 ± 5.7*+	4.22 ± 0.072+	1.16 ± 0.18*+
Diabetic + exercise	1.00 ± 5.25*	3.95 ± 0.10+	1.37 ± 0.24*+
Diabetic + crocin + exercise	0.95 ± 2.25*	4.32 ± 0.084+	1.27 ± 0.14*+
Crocin	0.705 ± 6.5+	5.97 ± 0.037+	1.63 ± 0.21+
Treadmill exercise	0.752 ± 2.2+	5.50 ± 0.077+	1.60 ± 0.15+
Crocin + Exercise	0.801 ± 3.5+	6.05 ± 0.059+	1.65 ± 0.25+

Data presented as Mean±(SE). *In comparison with control group (P=0.001) and + In comparison with diabetic group (P=0.001).

Table 4. The Comparison of Sperm Parameters after Treatment Period

Groups	Sperm parameters				
	Sperm Count (Mean ± SE) x10^6	Morphology		Sperm Motility	
		Normal	Abnormal	Motile	Immotile
Control	72.60 ± 3.48	75.4 ± 1.07	24.6%±1.07	73.6 ± 1.13	26.4 ± 1.13
Diabetic	18.5 ± 2.59+	26.4 ± 0.50+	73.6%±.50+	12.25 ± 1.03+	87.75 ± 1.23+
Diabetic + crocin	52.45 ± 2.98*	50.12 ± 1.17*	49.88%±1.17*	49.45 ± 0.84*	50.55 ± 0.84*
Diabetic + exercise	47.85 ± 3.18*+	49.62 ± 0.59*+	50.38%±.59*+	45.5 ± 0.75*+	54.5 ± 0.75*+
Diabetic + crocin + exercise	45.60 ± 3.48*+	44.4 ± 1.07*+	55.6%±1.07*+	43.6 ± 1.13*+	56.4 ± 1.13*+
Crocin	72.5 ± 9.16*+	70.87 ± 0.51+	29.13%±0.51+	70.75 ± 1.20*+	29.25 ± 1.22+
Treadmill exercise	68.5 ± 6.07*+	65.87 ± 0.45+	34.13%0.75±+	74.75 ± 1.06*+	29.25 ± 1.08+

*Shows significant difference between control group and diabetic group and + shows significant difference between diabetic group and diabetic treatment groups (P<0.05).

crocin and exercise compared to that in the diabetic group. In this study, the decline in the activity of CAT may have been attributed to the increase in H2O2 production due to autoxidation of glucose and non-enzymatic protein glycation which produces oxygen-free radicals (3). It is known that antioxidant therapy and regular exercise cause an increase in the activity of CAT, as was also confirmed in our study.

Our findings indicated that diabetes reduced the sperm parameters (count, motility, and morphology), and crocin therapy and treadmill exercise increased the sperm count and improved the sperm motility and morphology in diabetic rats. This may have been attributed to the antioxidant activity of crocin and the effect of regular exercise on the activation of antioxidant enzymes that can counteract free radicals. In line with our findings, the results from previous study indicated that medical plants with flavonoids compounds had the potential to improve sperm parameters and testosterone levels (5,18,23).

Possible mechanisms by which testicular "oxidative stress" can be improved in diabetic rats by crocin and exercise can be described as follows: crocin with anti-oxidant properties can decrease blood glucose levels and improve insulin secretion. Furthermore, regular exercise can activate the antioxidant enzyme (19,20,24,25).

Conclusions

It was concluded that diabetes may have adversely affected testis and sperm quality through oxidative stress. Crocin and treadmill exercise were found to significantly contribute to activating the antioxidant system and reducing the oxidative stress induced by diabetes. However, it was recommended that further studies should be carried out in order to clarify and confirm our results.

Authors' Contribution

Hassan Hamidi, Asghar Tofighi, Javad Toluei Azar, Amir Afshin Khaki, and Mazdak Razi planned and designed the experiments and performed the experiments. Hassan Hamidi and Asghar Tofighi analyzed the data. Hassan Hamidi, Asghar Tofighi, Javad Toluei Azar, Amir Afshin Khaki, and Mazdak Razi wrote the manuscript.

Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Issues

The Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran approved the study protocol (Code: IR.TBZMED.VCR.REC.1398.347).

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