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Protective Effect of Grape Seed Extract on Dexamethasone-Induced Testicular Toxicity in Mice

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Abstract

Objectives: Protracted and repeated exposure to glucocorticoids such as dexamethasone (Dex) may lead to reproductive dysfunction. In addition, it is a critical cause of male infertility. Grape seed extract (GSE) is an active fraction of a rich source of flavonoids and phenolic procyanidins that is used in traditional Chinese medicine. The purpose of this research was to determine the effect of GSE on testis and testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) in mice with establishing dexamethasone.

Materials and Methods: To this end, 21 adult male Balb/c mice were divided into control, Dex (7 mg/kg/day i.p), and Dex+GSE (100 mg/kg/d dissolved in 0.5 mL of olive oil) groups. All the animals in the above-mentioned groups were sacrificed after 35 days, followed by evaluating testosterone, FSH, and LH levels, spontaneous acrosomal reaction, and testis stereological structure.

Results: Significant changes were observed in the normal range of testosterone, FSH, LH serum levels, spontaneous acrosomal reaction, as well as the number of Sertoli and Leydig cells, spermatogonia, spermatocytes, and spermatids in Dex group compared to the control rats. These parameters changed to a less extent in Dex+GSE animals compared to Dex rats as well.

Conclusions: Our findings propose that GSE might have a curative potential on the reproductive system function and its impairment. It is regulated by Dex and reproductive-related hormones.

Keywords: Testosterone, Follicle-stimulating hormone, Luteinizing hormone, Dexamethasone, Grape seed extract

Introduction

Glucocorticoids (GCs) are usually used in conditions like adrenal gland failure, inflammatory, infectious diseases, and some cancers. In recent years, the administration of dexamethasone (Dex) has increased dramatically (1). Various biological systems may change the exposure to GCs like the reproductive system and the high level of GCs can alter the quality of semen (2). In addition, GCs are globally utilized as a suggested treatment for immunosuppressive and anti-inflammatory conditions (3). Some researchers recommend that Dex, as GCs, may change the reproductive system. Testosterone, as the most important sex hormone, has a key role in male physiological function and a normal level of testosterone regulates the functions and the control survival of all types of spermatogenesis process. The inadequate changes in the concentration of testosterone can induce male infertility as well (3,4). According to these researchers, the weight and volume of adrenal gland increased while pituitary, testis, and seminal vesicle demonstrated a reduction because of the Dex in rats that associated with a decreased number of spermatozoa, leading to male infertility (3). Dex significantly increases the concentration of the gonadotropin-releasing hormone, follicle-stimulating hormone (FSH), luteinizing hormone (LH) in males whereas it reduces testosterone concentration in the plasma that causes poor spermatozoa quality. Therefore, the first goal of the present study was to evaluate the effect of Dex on testosterone, LH, and FSH concentrations, along with spontaneous acrosomal reaction on spermatozoa cells and stereology study. Several different approaches are applied to treat this impairment although this chemical treatment demonstrates various side effects. Accordingly, it seems essential to find a suitable supplement herbal

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therapy for neutralizing or decreasing their negative side effects. Grape seed extraction (GSE), which is a sold in dietary supplement form and is claimed to have numerous health benefits, has a broad collection of pharmacological and biological anti-inflammatory, anti-carcinogenesis, and antioxidant activities (5). Further, GSE, with a high level of vitamins C and E, and bioflavonoid, is known as the most powerful antioxidant that can regulate oxidative damage. Likewise, this supplement protects the cells from oxidative injury, inflammatory mediator, and carcinogenic effects (6). Recently, studies have shown that GSE has anti-apoptotic and antioxidant effects. (7-10). The other studies suggested that GSE has potential in reducing drug toxicity as a free radical scavenger in different diseases. Some researchers also showed the beneficial effects of GSE on testis damages. The current study further aimed to introduce a protective agent, which could be available to the public without any problem and can prevent the damage effects of Dex on the testis. To this end, GSE was used as a supplement herbal treatment (10,11). Considering the above-mentioned explanations, the present study sought to find answers to the following questions.

- Does Dex change the spontaneous acrosomal reaction and testis stereological structure?
- Does Dex change testosterone, FSH, and LH serum level analysis?
- Does GSE protect the mice testis and reproductive-related hormones that are exposed to Dex?

Materials and Methods

Animals

The experimental groups included 21 adult male Balb/c mice (6–8 weeks old and weighing 25-30 g) which were obtained from the Experimental Research Center of Shiraz University of Medical Sciences. The animals were kept in a temperature and light-controlled room and had *ad libitum* access to food and water as well.

Experimental Design

According to the protocols of Razmaraii et al (12) and Belviranli et al (13), 21 adult male Balb/c mice were assigned to control, Dex (7 mg/kg/d i.p), and Dex + GSE (100 mg/kg/d dissolved in 0.5 mL of olive oil) groups and were then sacrificed after 35 days. They were sacrificed under halothane anesthesia as well. Similarly, testis tissues were harvested and semen samples were taken from the tail of epididymis after taking the blood samples through cardiac puncture. Finally, testosterone, FSH, and LH levels, as well as spontaneous acrosomal reaction and testis stereological structure were evaluated accordingly.

Hormonal Assay

In general, 1.5 mL of mice blood sample was centrifuged for 15 minutes at 3000 rpm and supernatants (serum) were collected for hormonal assay, and then frozen at -20°C up to 2 weeks. The testosterone concentration level was measured by radioimmunoassay kit (R and D Systems, Minneapolis, MN, USA), Serum FSH (ELISA kit, #MBS283243), and LH (ELISA Kit, #MBS2607965). All the samples were analyzed simultaneously (14).

Semen Analysis

The right cauda epididymis of each mouse was cut and placed in a Petri dish including 5 mL phosphate-buffered saline (PBS) with a pH of 7.2 and the spermatozoa were allowed to diffuse into the solution. Then, the suspension was gently shaken to homogenize and spread the spermatozoa at 37°C for 5-10 minutes (15). The semen samples were extended on a Neubauer hemocytometer and analyzed using an optical microscope. Additionally, the spermatozoa heads were manually counted and the data were expressed as the total number of spermatozoa/mL. To minimize the error, the count was repeated at least five times for each mouse by 2 or 3 coworkers (16). Then, the spermatozoa suspension samples were located on a slide warmed at 37°C. Next, 10 microscopic fields were evaluated and each field was selected randomly. Eventually, the motile spermatozoa were defined as the mean number of motile spermatozoa ×100/the total number of the spermatozoa (Figure 1).

Acrosomal Reaction Assessment

All the samples were incubated and washed in PBS and centrifuged at 1600 rpm for 5 minutes and then were fixed with paraformaldehyde 2% at 4°C for 30 minutes After fixation, the samples were centrifuged and washed in PBS, and finally, incubated in fluorescein isothiocyanate-conjugated to the peanut agglutinin (PNA) lectin (Vector Laboratories, Burlingame, USA) 10µL/mL for 2 hours at 37°C. The final sample was washed in PBS twice and the spermatozoa reaction to PNA was measured via FL1 channel flow cytometry which was equipped with FlowJo software (17).

Stereological Study

After dissecting the testis, the weight and volume of each animal were measured and then the samples were fixed in 10% formalin.

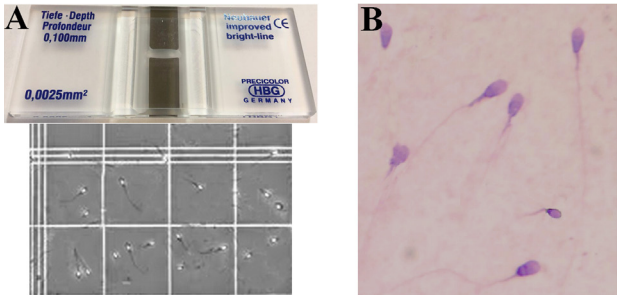

Figure 1. Semen Analysis. Note. (A) Neubauer hemocytometer and counting spermatozoa and (B) Morphometric parameters.

Estimation of Testis, Seminiferous Tubule, and Germinal Epithelium Volume and Weight

The Cavalieri method was used to measure the volume of the testes (18) by embedding them in paraffin and serially sectioning by using a microtome (4 µm and 20 µm thickness), followed by utilizing 4-µm sections of each testis to measure the total testis volume applying the following formula:

V(testis): $\Sigma A \times T$
Vv(tubule/testis) = P(tubules)/P(testis)
V(tubule) = Vv(tubule/testis) × V final testis
V(epithelium) = Vv(epithelium/tubules) × V (tubule).

Estimation of the Number of Sertoli and Leydig Cells, Along With Spermatogonia, and Spermatids

The total number of Sertoli and Leydig cells, as well as spermatogonia, spermatocytes, and spermatids (round and long) was estimated in 25-µm thick sections employing an optical dissector method (Figure 2). Then, systematic random sampling technique was used to estimate the numerical density "Nv" by following formulas (17):

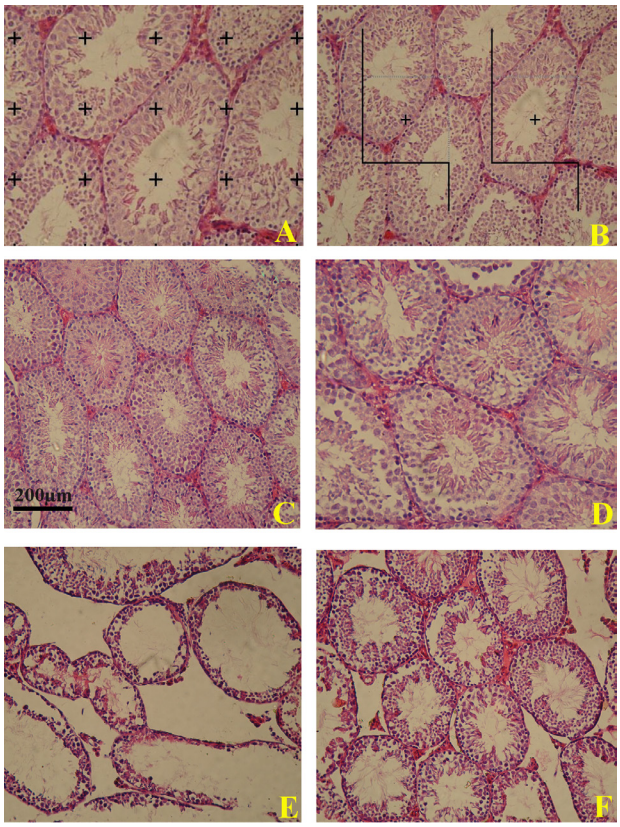

Figure 2. The Point-counting Method for Obtaining the Volume Density of the Components.
Note. B. H & I. Two optical sections of the testis for estimating the numerical density of the cells by an unbiased counting frame. The comparison of the changes of seminiferous tubules and histology scattering cells exposed to the dexamethasone (DEX) and grape seed extract (GSE). C. Control; D. GSE; E. DEX; F. DEX+ GSE. Remarkable differences can be detected between groups regarding the population cells.

Nv (cells/unit volume) = $\Sigma Q/(\Sigma A \times h) \times (t/BA)$.

Statistical Analysis

All data were presented in mean (±) standard deviations (SD). In addition, the parametric test (ANOVA) was applied to analyze the hormone assay and acrosomal reaction assessment, and nonparametric test (i.e., the Mann-Whitney U) was applied for stereology study. These differences were considered significant at $P < 0.05$.

Results

Serum Testosterone, Luteinizing Hormone, and Follicle-Stimulating Hormone Concentration

The serum testosterone concentration reduced while LH and FSH levels showed a significant increase in Dex animals compared to the corresponding control rats ($P < 0.01$). This parameter diminished to a lesser extent in the Dex + GSE treated animals compared to the Dex rats ($P < 0.01$), the related data are presented in Table 1.

Semen Analysis

Spermatozoa Counting

As shown in Table 1, there was an average 50% reduction in the spermatozoa counting in the Dex group when compared to the corresponding controls ($P<0.01$). This parameter decreased but to a lesser extent in Dex + GSE animals in comparison to the rats which only received Dex ($P<0.01$).

Spermatozoa Motility

Based on the data in Table 2, the percentage of motile spermatozoa in the Dex group reduced to one-third of the motile spermatozoa of the control rats ($P<0.01$). The reduction of motility occurred to a lesser extent in Dex + GSE animals as compared to the rats which only took Dex ($P<0.01$).

Spermatozoa Morphology

Likewise, the percentage of normal spermatozoa represented a decrease in the Dex group in comparison with the control spermatozoa ($P<0.01$). Further, the number of abnormal sperm decreased but to a lesser extent in Dex + GSE rats in comparison to the spermatozoa of the rats which only received Dex ($P<0.01$), the details of which are summarized in Table 2.

Table 1. Mean ± Standard Deviation of the Serum Testosterone, LH and FSH (ng /mL) in Control, Dex and GSE+Dex groups

Groups	Testosterone	LH	FSH
Control	7.1±6.1	3.4±0.4	13.2±0.4
Dexamethasone	4.4±2.0*	9.9±0.3*	18.3±0.1*
GSE+Dex	6.4±9.4*	5.4±0.6*	16.9±0.2*

*$P<0.01$ (Control vs. Dex) or (GSE+Dex vs. Dex) or compared with Control group.

Table 2. Mean ± standard Deviation of the Spermatozoa Count (×10⁶), Morphology, (%) Motility (%) in Control, Dex and GSE+Dex Groups

Groups	Count	Normal Morphology	Motility			
			Rapid	Slow	Non-progressive	Immotile
Control	7.1±6.1	90.4±0.4	42.1±5.4	18.3±3.1	15.6±2.4	23.8±6.2
Dexamethasone	4.4±2.0*	74.9±0.3*	10.7±2.6*	10.4±2.3*	26.3±5.2*	52.4±6.4*
GSE+Dex	6.4±9.4*	88.4±0.6*	24.0±4.0*	13.0±2.6*	21.4±6.5*	38.3±1.9*

*P<0.01 (Control vs. Dex) or (GSE+Dex vs. Dex) or compared with the control group.

Spermatozoa Reaction to Peanut Agglutinin

PNA can be applied as the marker of the intactness of the acrosome. As shown in Figure 3 and Table 1, a significant decline was found in the Dex group regarding the percentage of the PNA-reactive spermatozoa as compared with the control group ($P<0.01$). The level of reaction improved in Dex + GSE spermatozoa in comparison to the spermatozoa of rats which received Dex ($P<0.01$).

Stereology

Weight and Volume of the Structures of Testis, Seminiferous Tubule, and Germinal Epithelium

Based on the data in Figure 2, the weight and volume of the testes, seminiferous tubule, and germinal epithelium reduced in Dex-treated animals compared to control mice ($P<0.05$). These volumes were also reduced in the Dex+GSE group, but to a lesser extent compared to Dex-treated mice ($P<0.05$).

Numbers of Cells

The results (Figure 4) demonstrated that the total number of Sertoli and Leydig cells, along with spermatogonia A and B, spermatocytes, and elongated and round spermatids decreased in the Dex group in comparison with the control mice ($P<0.05$). Furthermore, the total number of the above-mentioned cells changed to a lesser extent in the Dex+GSE group as compared to those mice in the Dex group ($P<0.05$).

Discussion

Previous studies confirmed that Dex could change normal homeostasis on various bodies and disrupt an endocrine system (19,20). Some other studies reported that Dex changes the pituitary-testicular axis (21,22) by sex hormone reduction, delayed ejaculation, low sexual desire, and low spermatozoa quality, leading to male infertility (23-25). The findings of the current study revealed the elevated concentration of FSH and LH in mice that were exposed to Dex. Moreover, Sertoli and Leydig cells were controlled by the concentration of LH and FSH (19). In the Dex group, a decline in testosterone led to a decrease in the responsiveness of Leydig cells to LH through inhibiting testosterone production. On the other hand, the enhancement in serum FSH concentration indicated the destruction of spermatogenesis in the experimental group and reflected the germ cell loss or Sertoli cell damage. Likewise, the reduced levels of serum testosterone with the increased levels of FSH and LH in experimental rats represented a damaged pituitary-testicular axis. According to Wang et al, a reduction in testosterone level could be explained by an increase in cortisol concentration that might suppress the Leydig cells through binding to the GC receptors on the cell surface (26). Our results demonstrated that GSE can improve the reproductive hormone (i.e., testosterone, FSH, and LH) levels in the Dex mice. This might be due to the protective effect of GSE on Leydig cells (8). In line with our findings, previous research approved the protective effects of GSE by using properties such as anti-apoptotic, anti-oxidative, and antigenotoxic (27). In addition, several researchers like Abarikwu et al, Hawkley et al, and Enyeart et al reported that GSE inhibited cortisol secretion by suppressing adrenocorticotropic hormone and increasing mRNAs coding for steroid controlling proteins (28-30).

According to previous evidence, serum testosterone concentration significantly increased after GSE treatment (8). In this study, a significant reduction in testosterone, as well as an increase in serum cortisol, LH, and FSH concentrations strongly led to a decrease in the following parameters:

- Spermatozoa counting, motility, and normal morphology;
- The percentage of the peanut agglutinin-reactive spermatozoa;
- The total testis;
- The somniferous tubule;
- The germinal epithelium weight and volume;
- The total number of Sertoli and Leydig cells, spermatogonia A and B, spermatocytes, elongated, and round spermatids in the Dex group.

Other studies recognized Dex as a strong mediator of apoptosis and showed that Dex significantly increases the levels of gonadotropin-releasing hormone, FSH, LH (3,31), which corroborates with the results of the present study. Khorsandi et al also indicated that Dex reduced spermatozoa counting, motility, and the normal morphology of spermatozoa. Similarly, it was reported that Dex leads to an apoptotic loss of germ cells and the Leydig cells (3). Correia et al believed that a change in C^{+2} may cause an acrosomal reaction and GC changes this channel (32). Additionally, Shannon et al. and Lee et al. found that Dex-induced reactive oxygen species (ROS) production in spermatogenesis cells and increased DNA damage were considered as the mechanisms throughout

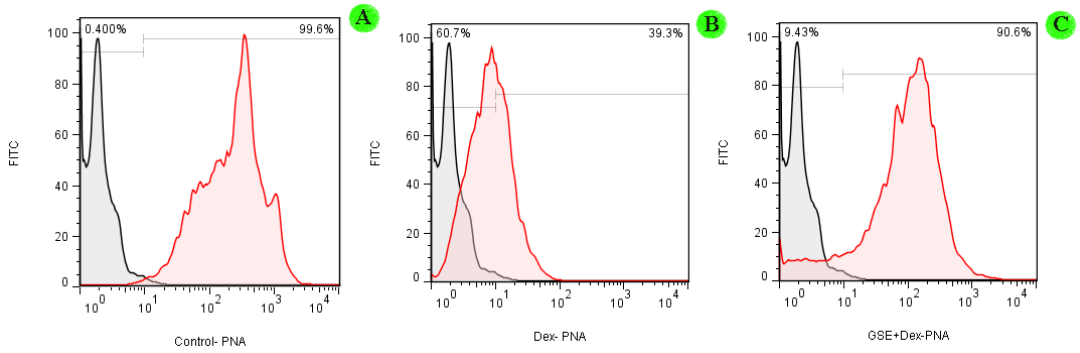

Figure 3. Lectin Histochemistry of the Spermatozoa of Mice at Exposure to the DEX and GSE.
Note. A. Control PNA; B. DEX PNA; C. DEX+GSE PNA after incubation; DEX: Dexamethasone; GSE: Grape seed extract; PNA: Peanut agglutinin.

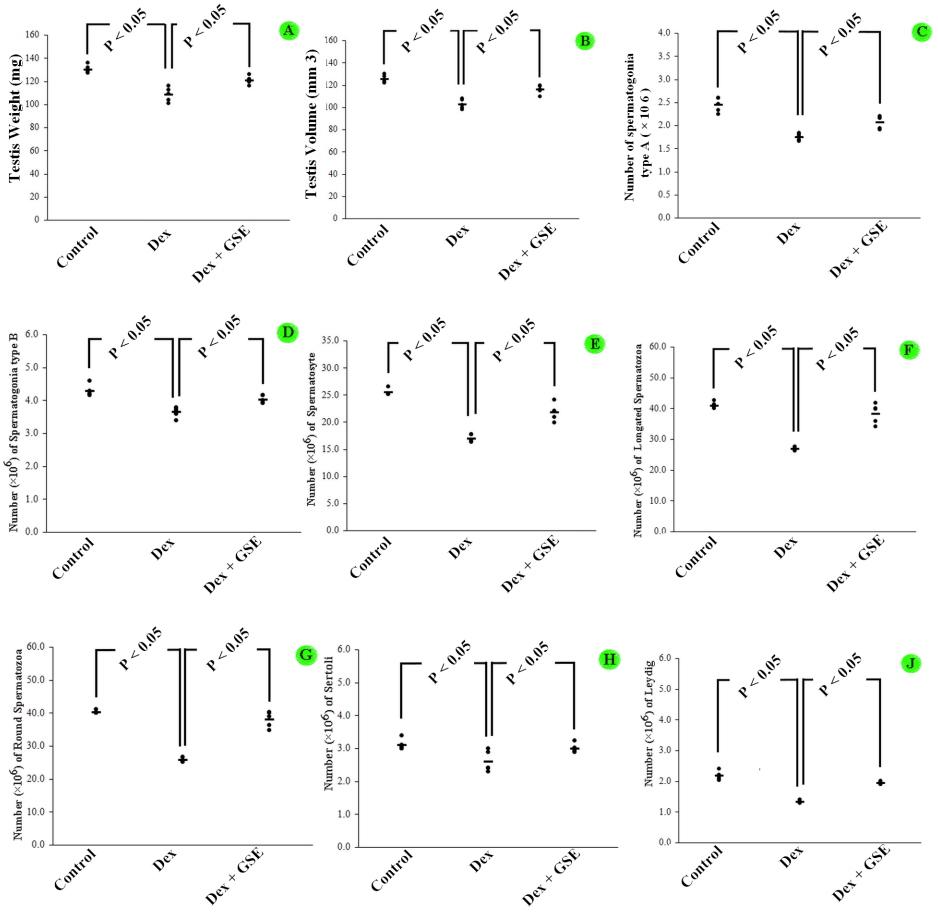

Figure 4. The Scatter Plots of the Study Parameters in Control, DW, DEX, GSE, and DEX+ GSE Groups.
Note. A. The weight of testis; B. The volume of testis; C. The total number of spermatogonia A; D. Spermatogonia B; E. Spermatocyte; F. Elongated spermatid; G. Round spermatid; H. Sertoli cells; J. Leydig cell of the animals; DEX: Dexamethasone; GSE: Grape seed extract. Each dot represents an animal and the horizontal bar is the average of the values of animals in each group.

the Dex (33). In addition, GCs were shown to increase the susceptibility of unusual testis regions, particularly the Leydig cells. Further, GCs can increase the production of ROS in Leydig cells (33,34). Gao et al and Juárez-Rojas et al demonstrated that GCs control mitosis and apoptosis induction in testicular cells (31,35). Based on the findings of our study, GSE increased testosterone while reducing LH and FSH concentrations, which might be due to the protective effect of GSE on the Leydig cells. Furthermore, GSE improved the reduction of counting, motility, and the normal morphology of spermatozoa. This is also in conformity with the findings of Abdel-Kawi et al which

showed that GSE as a phenolic compound can inhibit cortisol secretion, improve spermatogenesis, control the stimulation of gonadal function, cause the enhancement of testosterone concentration and spermatozoa production, and reduce FSH and LH (36). GSE inhibited the apoptosis of Leydig cells and led to high motility and counting of spermatozoa by increasing testosterone concentration (8). Moreover, GSE, as herbal medicine which has an antioxidant effect, can improve spermatogenesis, which is in agreement with the results of Abdel-Kawi et al and Alkhedaide et al, which revealed that herbal phenolic compounds including GSE might improve spermatogenesis due to the stimulation of gonadal function, as well as the enhancement of testosterone concentration and spermatozoa production (8, 36). Our results showed that Dex considerably damaged testis structure and acrosome reaction, which could be protected by GSE. Thus, future studies can evaluate the impact of the signaling pathway of Dex and GSE on the male reproductive system.

Conclusions

Overall, exposure to Dex altered the hormonal levels, semen parameters, spermatozoa membrane glycoconjugates, and testis structure in the mice. In addition, our findings showed the possible protective role of GSE, as an easily available natural component, in recovering the changes of testis after exposure to Dex.

Acknowledgements

This study was conducted in the Histomorphometry and Stereology Research Center, Shiraz University of Medical Sciences (Shiraz, Iran) and the Neuroscience Research Center of Iran University of Medical Sciences (Tehran, Iran).

Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Issues

The experimental protocols were performed according to the Ethics Guideline of Iran University of Medical Sciences. Moreover, the care and treatment of the animals were in accordance with the Ethical Guidelines of the Committee (approved No. NRC-96-1).

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