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ORIGINAL ARTICLE

The impacts of yoghurt butter oil on rat testicular morphology and sexual hormones in a 150-day study

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Abstract
The oil derived from traditional produced yoghurt has received much attention over the last two decades. While a high-fat diet (HFD) is assumed to be related to reproductive impairments, fermented dairy products are believed to have positive effects on the regulation of HFD-induced male reproductive damage. The reproductive effects of sheep's yoghurt butter oil (SYBO) have never been studied yet. The current study was to seek for the scientific evidence to determine SYBO effects on male rat reproductive system. In the present study, male Wistar rats were treated by standard diet or standard diet supplemented with 10% (*w*/*w*) or 20% of SYBO for 150 days. Treatment of animals with SYBO (at the both concentrations) did not cause significant alterations in the body weight, testicular morphology and its weight, circulatory concentrations of testosterone, and follicle-stimulating hormone (FSH). However, plasma levels of estradiol and luteinizing hormone (LH) significantly declined ($p < 0.05$) in 10–20% SYBO-treated rats. The evidence from this study supports that high-fat diet made by sheep's yoghurt butter oil had not devastating effects on rat reproductive system even though in long-term period.

Keywords Yoghurt · Butter · Reproduction · Testis · Sex hormones

Introduction

Infertility defines as failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse, affecting 15–25% of couples in Western countries (Thoma et al. 2013). A great body of evidence now exists showing relationship between diet and human fertility (Gaskins and Chavarro 2017). Several studies have reported that dietary fats modified sex hormones and influenced fertility at the both sexes (Hurtado de Catalfo et al. 2009; Mumford et al. 2016; Segarra et al. 2002). Accordingly, hypercholesterolemia and high plasma levels of triglycerides have been associated with poor semen quality and direct adverse effects on the testicular function (Martínez-Martos et al. 2011). Previous studies also indicate that enriched diets with high saturated fats modify the testicular morphology by decreasing seminiferous epithelium height, diameter, and cell proliferation. Moreover, they were negatively related to sperm concentration (Attaman et al. 2012; Campos-Silva et al. 2015).

Yoghurt butter oil is one of the most widely consumed fats in India, Iran, Greece, Turkey, and several other countries (Hosseini et al. 2014; Şenel et al. 2011; Serafeimidou et al. 2012). Yoghurt is a dairy product made by bacterial fermentation mostly from cow's and sheep's milk. Lactic acid bacteria (LAB) through fermentation release components with antihypertensive, antimicrobial, antioxidative, and immunemodulatory activities (Parvez et al. 2006). Moreover, fermentation by the LAB often leads to the enrichment of milk vitamins including vitamin B12, folic acid, and biotin (Hugenholtz and Smid 2002). In the most recent study, we tested long-term effects of traditional yoghurt butter oil on rat plasma lipids, hematology, and liver pathology (Hassanzadeh-Taheri et al. 2018). Surprisingly, in contrast to generally accepted belief, the oil did not cause any metabolic alterations in rat. Hence, the aim of the current work was to improve our knowledge on the effects of long-term

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consumption of traditional sheep's yoghurt butter oil (SYBO) on testicular function of Wistar rats.

Materials and methods

Oil preparation

The SYBO was prepared according to the traditional method. Briefly, fresh sheep's milk was purchased from local dairy market; it was heated to about 80 °C and then cooled down to around 37–40 °C. It was then incubated with the previous day yoghurt sample (Birjand, Iran) and kept at the room temperature. After 1 day incubation, the prepared yoghurt was diluted with cold water (1:1) and churned for about 1 h in a round classic churning machine (ALP, Iran) at 10 °C. Then, the butter fat was collected and gently heated to melt, and the pure butter oil was separated from the sediments. The prepared SYBO was packed and stored at − 80 °C for future use.

Fatty acid profiles of SYBO and standard animal diet chow were determined using a gas chromatograph (YL 6000, Korea) equipped with a CP-Sill 88 capillary column (60 m, 25 μm i.d., 0.2 μm film) blindly at the Standard Research Institute, Karaj, Iran. Fatty acid methyl esters were identified on the basis of ISO 5508 (ISIR 4090) and analyzed according to the ISO 5509 (ISIRI 4091).

Animals and diets

Healthy male Wistar rats (60 days old) were purchased from laboratory animal facility in Birjand University of Medical Sciences, Birjand, Iran. The rats were housed in a temperature-controlled room (22 ± 2 °C) with a 12-h light/dark cycle. All animal procedures were conducted and approved in accordance with the guide for the laboratory animals' care and usage of Birjand University of Medical Sciences, Birjand, Iran (Ethic code: Ir.bums.REC.1396.95). All efforts were made to minimize animal suffering and to reduce the number of animals used. The animals were divided randomly into three equal groups ($n = 10$) with the mean body weight of 257.62 ± 19.78 g. The groups were fed with the following diet: the control group received a standard diet (containing 3% fat), and treatment groups were fed with the standard diet supplemented with 10 or 20% of SYBO, respectively for 20 weeks. The animals had ad libitum access to their respective food and tap water throughout the study.

Determination of blood parameters

At the end of the study (after 20 weeks), the rats were anesthetized with ketamine-xylazine (65:10 mg/kg IP). Blood samples were collected from the heart. The plasma concentration of testosterone (T), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol (E2) were measured using ELISA biochemical kits, for serum testosterone (IBL, Flughafenstrasse, 52a,Hamburg D-22335, Germany); 17β-estradiol (Diametra 20090 Segrate Milano Italy), LH, and FSH (Pishtaz Teb, Iran) were used.

Morphological evaluation

Following the anesthesia and blood exsanguinations, testes were removed, weighted, and fixed in 10% formaldehyde in phosphate buffered saline (0.01 M). Tissue sections of the testes were processed for paraffin-embedding, and serial sections were prepared for staining with hematoxilin and eosin. For each testicle, three random slides were evaluated under a light microscope (UPLAN FI, Japan). For each section, four to six unbiased counting frames were sampled. Seminiferous tubule area (um2), germinal layer area (um2) of each seminiferous tubule, and the ratio of germinal layer area to seminiferous tubule area were measured using Image J Software (1.44p; National institute of Health, USA).

Spermatogenesis examination

Johnsen's score was used to categorize the spermatogenesis. The degree of testicular damages was tested using 1–10 points scale (Table 1). All the morphological and spermatogenesis evaluations were done in blind manner.

Statistical analysis

The statistical analyses were performed using the SPSS 22 statistical software (IBM, USA). The data were statistically evaluated by the one-way analysis of variance (ANOVA) or Kruskal–Wallis test (for nonparametric data). All value expressed as the mean ± standard deviation. The significance between groups was determined by the Dunett t3 post-hoc test or Mann–Whitney U test. Values were considered significant at $p < 0.05$.

Results

Fatty acid composition of SYBO

Fatty acid composition of SYBO and standard animal food (consist of 3% fat) has been summarized in Table 2. The SYBO consisted of 65.8% saturated fatty acids (SFAs); 4.8% poly unsaturated fatty acids (PUFAs); and also 2.9% trans fatty acids (TFAs). The level of monounsaturated fatty acids (MUFAs) was 24.2% of total fatty acids correspondingly in SYBO.

Table 1 Johnsen score description

Johnsen score	Description
10	Complete spermatogenesis and perfect tubules
9	Slightly impaired spermatogenesis, many late spermatids, disorganized epithelium
8	Less than five spermatozoa per tubule, few late spermatozoa
7	No spermatozoa, no late spermatids, many early spermatids
6	No spermatozoa, no late spermatids, few early spermatids
5	No spermatozoa, no late spermatids, many spermatocytes
4	No spermatozoa and spermatids, few spermatocytes
3	Spermatogonia only
2	No germinal cell, Sertoli cells only
1	No seminiferous epithelium

Body weight, testicular weight, and total food intake

The final body weight, 5 months total food consumption as well as energy intake are shown in Table 3. Regarding the SYBO20%, animals showed significant lower relative food consumption during the study ($p < 0.05$). However, compared to the control group, there was no significant difference between these groups in total energy intake.

There was no significant difference ($p = 0.26$) in final body weight between the studied groups. After 150 days of treatment, the mean weights of testes were 1.68 ± 0.11 g, 1.66 ± 0.06 g, and 1.67 ± 0.05 g in control, SYBO10%, and SYBO20% groups, respectively. There was no statistically difference between the groups in testicular weight ($p = 0.95$).

Table 2 Fatty acid composition of sheep's yoghurt butter oil (SYBO) and standard diet

Fatty acid Saturated	SYBO (%)	Basal food (%)	Fatty acid Unsaturated	SYBO (%)	Basal food (%)
C4	0.8	–	C8:1	0.1	–
C6	2.2	19.3	C10:1*trans*	–	–
C8	2.1	0.9	C10:1*Cis*	0.4	–
C10	9.3	6.3	C12:1*trans*	–	–
C12	4.4	4.6	C12:1*Cis*	1.2	–
C13	0.2	–	C14:1*trans*	0.2	–
C14	10.3	3.8	C14:1*Cis*	0.3	–
C15	1.3	–	C16:1*trans*	–	–
C16	24.7	18.8	C16:1*Cis*	1.8	0.5
C17	0.8	0	C17:1*trans*	0.1	–
C18	9.5	4.3	C17:1*cis*	0.3	–
C20	0.1	0	C18:1n-9*trans*	2.6	–
C22	0.1	–	C18:1n-9*Cis*	19.9	19.6
			C18:2n-6	2.8	19.7
			C18:3n-3	2	1.8
			C20:1	0.2	–
			CLA	0.6	0

Values are expressed as percent by weights. Total fats of basal diet were 3%

CLA, conjugated linoleic acid

Sexual hormones

The results of plasma sexual hormones are presented in Table 4. Despite the fact that the SYBO-enriched diet at the both concentration slightly elevated plasma testosterone levels, no statistically difference was found between experimental and control groups ($p = 0.053$).

Significant decreases in LH levels were observed in animals treated with SYBO10%-enriched ($p < 0.0001$) or SYBO20%-enriched ($p = 0.043$) diets, compared to the control group. However, FSH showed no significant difference between studied groups.

Treatment with diet-enriched SYBO at the both concentrations (10, 20% *w*/*w*) caused markedly significant decrease in plasma estradiol levels of rats, compared to the control group ($p = 0.020$, each).

The intra-assay coefficient of variations (CVs) for T, LH, and FSH were 4.16, 4.9, and 3.9% respectively, and the CVs for E2 was 8.1%.

Histopathological assessments

As seen in Fig. 1, the control group showed normal histological structure of the seminiferous tubules with tight and well-arranged spermatogenic cells. Various spermatogenic cells could be clearly identified with spermatogonia, spermatocytes, and radially arranged spermatids. The data of quantitate histological examination of seminiferous tubules are presented in Table 5. The seminiferous area slightly but significantly decreased in SYBO10%-treated animals compared to the control group ($p = 0.005$). However, the area of germinal layer as well as the ratio of germinal layer to seminiferous area showed no significant differences between the studied groups. The Johnsen's score was not statistically significance between the studied groups (Fig. 2).

Discussion

We previously reported that long-term consumption (150 days) of high-fat diet-enriched with 20% (*w*/*w*) tail-fat oil significantly alters testicular morphology and causes hypogonadism in male Wistar rats (Ezi et al. 2016). We speculated that at the least part of these effects (germinal cells

Table 3 Final body weight, energy contents of the diets and total food, as well as energy intake of the groups

	Body weight (g)	Energy (Kcal/g)	Total food intake (g/rat/5 months)	Total energy intake (Kcal/rat/5 months)
Control	327.37 ± 33.67	3.59	2884.68 ± 85.17	10,356.03 ± 305.78
SYBO10%	349.62 ± 26.04	3.94	2724.67 ± 151.95	10,735.22 ± 598.71
SYBO 20%	353.37 ± 39.23	4.44	2470.40 ± 149.54*	10,968.60 ± 663.98

Values are expressed as mean ± SD, $n = 10$

SYBO, sheep's yoghurt butter oil

*Significantly different from the control, ($p < 0.05$)

apoptosis) were because of high content of saturated fatty acids of tail fat, particularly Palmitic acid (around 25%). However, in the most recent study, we found that whereas sheep's or cow's yoghurt butter oils have high contents of saturated fatty acids like Palmitic acid (24.7 and 31.4% respectively), they did not cause metabolic alterations such as hypercholesterolemia and liver dysfunction on rats in a 150-day investigation (Hassanzadeh-Taheri et al. 2018). The biological effects of yoghurt butter oils have not been dealt with in depth. Here, in the present study, food and total energy intake, testicular weight, circulatory sex hormones, and morphological parameters of testicle were measured to evaluate the effects of long-term consumption of SYBO, and traditional fermented milk-based oil on reproductive system of male Wistar rats.

Contrary to expectations, the results of the present study clearly showed that SYBO, as high saturated oil, did not exert significant alterations in spermatogenesis and testicular morphology of treated animals. Nevertheless, SYBO at the both concentrations markedly reduced LH and estradiol levels, compared to control group.

Yoghurt butter is a fat-rich dairy product made traditionally by churning of fermented milk (yoghurt), whereas cream-based butter which produce by churning milk's cream. The Ghee or yoghurt butter oil is one of the widely consumed dietary oils in Asian countries. The fatty acid composition of SYBO showed relatively high degree of saturation (65.8%), with the predominant saturated and unsaturated fatty acids being Palmitic acid (C16:0) with 24.7% and Oleic acid (C18:1) with 22.5%. The traditional butter oils are naturally enriched with conjugated linoleic acid (CLA). In the present study, the SYBO consisted of 0.6% CLA which confirms previous findings in the literature (Fındık and Andiç 2017). A great body of evidence has shown that CLA exerts many beneficial effects including anti-diabetic, anti-obesity, anti-carcinogenic, and cardiovascular protective activities. Moreover, it was clearly proven that lactobacillus bacteria like *Lactobacillus plantarum* which was one of the most abundant micro-organism in sheep's yoghurt convert free linoleic acid (LA) to CLA through fermentation process (Yang et al. 2017). In the current study, circulatory testosterone levels increased in both of SYBO10%- and SYBO20%-treated groups, but the values were not statistically different from those of control. The finding is not in complete agreement with previous research reporting CLA supplementation increases testosterone secretion in Leydig cells via upregulation of CYP17A1 (Barone et al. 2013; Di Felice et al. 2007).

The spermatogenesis is regulated by the sex hormones including FSH, LH, and testosterone. The FSH affects Sertoli cell number as well as function, directly by activating intracellular signaling pathway, and indirectly by enhancing spermatogenesis. The failure of spermatogenesis may be due to a lack of LH and FSH secretion. The action of FSH and testosterone on the process of spermatogenesis is not thought to be directly on the germ cells, but via their action on Sertoli cells, which are triggered to produce several agents necessary for sperm mutation (Brook and Marshall 2001). Recent studies found that FSH plays an important role in intramuscular fat deposition of female rats and chick by affecting genes related to lipid metabolism (Cui et al. 2016). LH controls the rate of testosterone synthesis in Leydig cells by regulating the enzymatic step involved in the conversion of cholesterol to pregnenolone, cholesterol side-chain cleavage. LH and testosterone form the backbone of the hypothalamic–pituitary–testicular axis, where LH stimulates testosterone secretion and the latter exerts negative feedback inhibition on LH secretion

Table 4 Circulating sex hormones in male Wistar rats fed diet enriched with 10 or 20% (*w*/*w*) sheep's yoghurt butter oil (SYBO) for 5 months

	Testosterone (ng/ml)	LH (mIU/ml)	FSH (ng/ml)	Estradiol (pg/dl)
Control	1.29 ± 0.57	2.02 ± 0.46	0.011 ± 0.003	74.90 ± 13.97
SYBO10%	1.92 ± 2.34	0.46 ± 0.39*	0.012 ± 0.004	53.08 ± 21.83*
SYBO20%	2.96 ± 1.67	1.80 ± 1.11*	0.013 ± 0.004	52.98 ± 13.72*

Values are expressed as mean ± SD, $n = 10$

*Significantly different from the control, ($p < 0.05$)

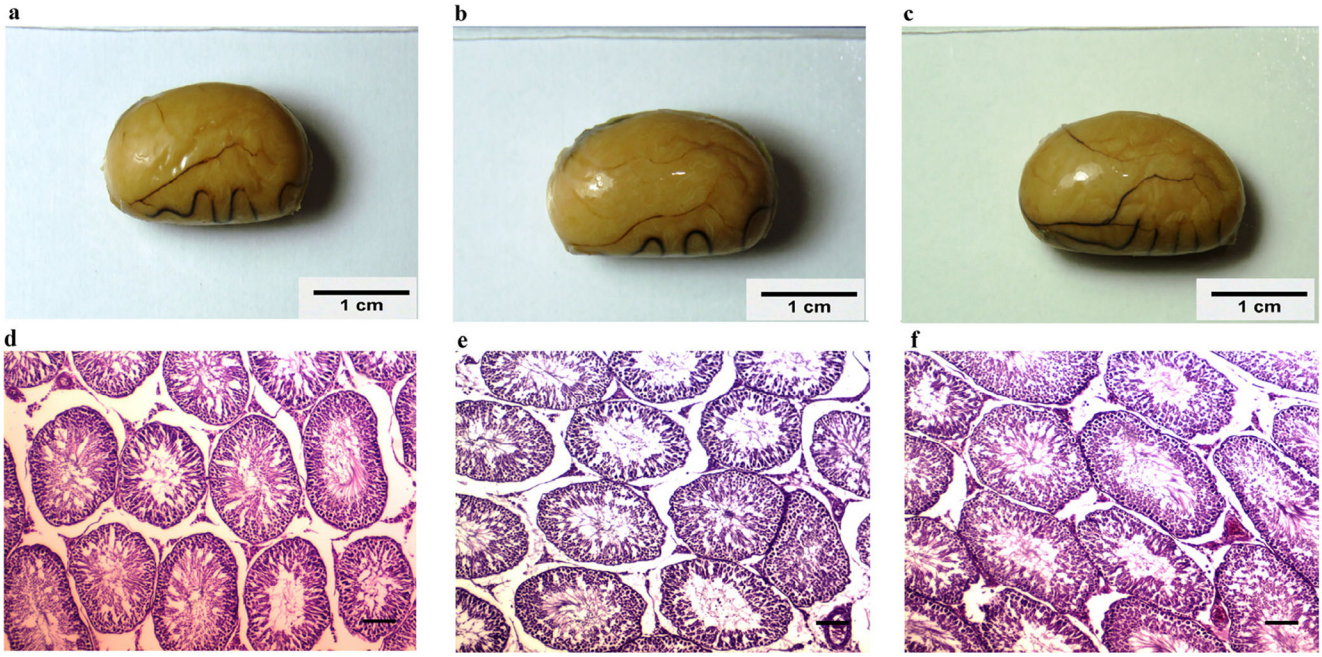

Fig. 1 Effects of 150-day treatment by diet enriched with 10 or 20% sheep's yoghurt butter oil on testicular gross morphology and histological structure. **a**, **d** Control group. **b**, **f** SYBO10% fed group. **c**, **f** SYBO20% fed group. Slides were stained with hematoxylin and eosin dyes, × 100 magnification; scale bar of micrographs indicates 100 μm

(Choi and Smitz 2014). In the present study, LH levels significantly decreased in both of SYBO10%- and SYBO20%-treated groups, in comparison with control group. This finding is in agreement with Olivares et al. study in which they found long-term (180 days) feeding rats with high-fat diet (20% of total calories supplied with saturated fat) exhibited significant decrease of intrapituitary and serum LH concentrations, low serum testosterone levels, and elevated serum 17β-estradiol concentrations (Olivares et al. 2010). Moreover, surprisingly, the results of our study showed that estradiol levels significantly decreased in SYBO-treated animals at the both concentrations. Recent findings demonstrate that estradiol is involved in the regulation of testicular steroidogenesis and spermatogenesis. Estradiol stimulates testicular apoptosis (spermatocytes) without effect on testicular proliferation, CypP450c17 protein levels or enzymatic activity, while it reduces 3β-HSD/I activity during the post-reproductive season (Scaia et al. 2015). Walczak-Jędrzejowska et al. showed that postnatal day (1–15) injection (12.5 μg) of estradiol benzoate decreases the number of spermatocytes per Sertoli cell in male rat pup (Walczak-Jędrzejowska et al. 2013). Despite the fact that previous research found that long-term treatment with saturated high-fat diets caused an elevation in estradiol level in male rats, our findings are significantly different from previous results reported in the literature (Olivares et al. 2010). A study conducted by Carrilo et al. showed that male rats treated with high-fat diet had significantly higher estradiol levels than control animals; based on this study, Wistar rats treated with high-fat diet (34.9% fat) from day 6 of gestation until postnatal day 60 (Carrillo et al. 2017). In addition, they investigated other high-fat-treated male pups by exogenous estradiol and found that estradiol restored the body weight of overnourished males.

Unlike other research carried out in this area, we did not find a significant difference between SYBO-treated animals and control group, in terms of total energy intake and body weight. Several studies have indicated that long-term treatment with standard diet enriched with 10–20% fat enhances

Table 5 Quantitative evaluation of seminiferous tubules

	SA (um2)	EA (um2)	EA coefficient = (EA/SA) × 100
Control	76,584.00 ± 12,442.20	45,270.70 ± 10,144.75	58.06 ± 5.72
SYBO10%	59,285.90 ± 6777.10	38,118.20 ± 6268.18*	63.43 ± 6.46
SYBO20%	70,583.00 ± 15,268.30	38,219.90 ± 211,802.15	54.85 ± 15.62

Values are expressed as mean ± SD, $n = 120$ seminiferous tubules for each group

SA, seminiferous area; *EA*, epithelial area; *SYBO*, sheep's yoghurt butter oil

*Significantly different from the control, ($p < 0.05$)

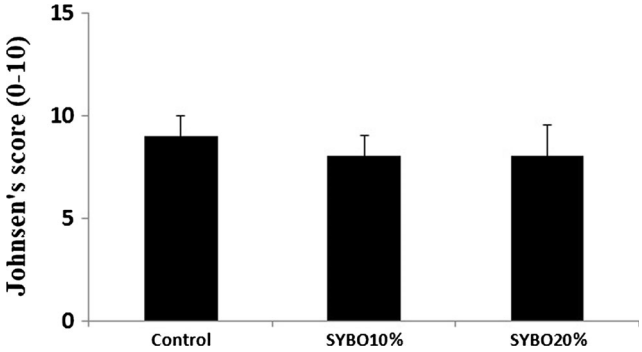

Fig. 2 Johnsen's testicular score: each bar indicates mean ± standard deviation ($n = 30$)

body fat in rat (Wahlig et al. 2012). However, few studies evaluating the effects of yoghurt butter oil on rats exhibited similar findings. In a study conducted by Chaturvedi et al., it was found that 2 months of treatment with 10% (*w*/*w*) yoghurt butter oil enriched diet did not cause significant weight gain, whereas 10% supplementation with sunflower oil significantly increased body weight in rats (Chaturvedi et al. 2016). These results indicate that SYBO by regulation of estradiol levels prevents fat accumulation and weigh gain in rats.

In line with the abovementioned parameters, gross morphology and quantitative histological evaluations of testicular tissues showed that SYBO treatment did not lead to significant histopathological alterations in the animals' testicles. We previously reported that tail-fat oil supplementation with the same protocol and study duration causes obvious hypogonadism with a marked decrease in seminiferous area, germinal epithelium area, as well as its coefficient to seminiferous area (Ezi et al. 2016). Despite the wide use of SYBO in the world, as far as we know, no one has evaluated its effects on reproductive system function either in animals or clinical studies. Colostrum is a full-fat milk product closely similar to SYBO in fatty acid content. We also previously evaluated its effects on sex hormones and testicular morphology in diabetic rats. Our findings revealed that colostrum protects seminiferous epithelium layer in rats via inhibiting lipid peroxidation (Serki et al. 2016). Moreover, there is evidence suggesting that fermented products have beneficial effects on the homeostasis of the lipid metabolism and on alleviating oxidative stress in HFD-induced obese mice (Lei et al. 2015).

Conclusion

There is a growing body of evidence that suggests that yoghurt butter oil has beneficial effects on heart disease, body weight, diabetes, hypertension, and most cancers (Prentice 2014). The evidence from this study supports that yoghurt butter oil has beneficial effects on reproductive system in male Wistar rats, due to its specific formation method as well as enrichment with conjugated linoleic acid.

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Compliance with ethical standards

All the experiments involving animals were performed according to the guide line of the Institutional Animal Ethical Committee, Birjand University of Medical Sciences (the permit code is Ir.bums.REC.1396.95).

Conflict of interest The authors declare that they have no conflict of interest.

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