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ORIGINAL PAPER

Sex-Independent Cognition Improvement in Response to Kaempferol in the Model of Sporadic Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is associated with neural oxidative stress and inflammation, and it is assumed to affect more women than men with unknown mechanisms. Kaempferol (KMP) as a potent natural antioxidant has been known to exhibit various biological and pharmacological functions, including antioxidant and anti-inflammatory. We aimed here to evaluate the role of gender difference in response to KMP on the rat model of sporadic AD. Forty-six female and male Wistar rats were divided into six groups of sham, streptozotocin (STZ) + saline (SAL), STZ + KMP. Female rats were ovariectomized, and then all animals received an intracerebroventricular bilateral injection of STZ (3 mg/kg) to induce the AD model. KMP (10 mg/kg) was intraperitoneally administered for 21 consecutive days. Afterward, spatial learning and memory were assessed via the Morris water maze task (MWM). Finally, the hippocampus level of superoxide dismutase (SOD), glutathione, and malondialdehyde were measured using calorimetric kits. Data showed a significant cognition deficit in STZ + SAL compared with the sham. To sum up, we reported that chronic KMP treatment increase significantly improved acquisition and retrieval of spatial memory as evident by longer TTS (total time spent) and short-latency to the platform in MWM. In addition, KMP increased the levels of SOD and glutathione in the hippocampus of rats. Also, KMP decreased hippocampal levels of malondialdehyde in both genders. In conclusion, KMP successfully restores spatial memory impairment independent of gender difference. This memory restoration may at least in part be mediated through boosting the hippocampal level of SOD and glutathione.

Keywords Neurodegenerative disorder · Streptozotocin · Flavonoids · Gender difference · Oxidative stress · *Mespilus germanica*

Introduction

Alzheimer's disease (AD) is pathologically associated with the accumulation of amyloid-β deposits [1], and behaviorally with cognition deficit [2]. There are contradictory findings of gender difference and cognition performance which vary from no significant differences [3, 4], outperform of male vs. female's hippocampus-dependent memories [5–8], and better object recognition in females vs. males [9]. Epidemiological studies reported that AD affects women more than men in both prevalence and severity [10, 11], especially after menopause. Multiple mechanisms are hypothesized for gender difference most likely, sex steroid hormones, antioxidant defenses system, and neurotransmitters [12, 13].

Imbalance in oxidant and antioxidant defense systems is assumed to be a contributing factor to the progression of AD [14, 15]. For example, oxidative stress leads to the generation of reactive oxygen species (ROS) and promotes the synthesis and deposition of amyloid-β, the main hallmark protein in AD [16, 17]. Also, free radicals produced during oxidative stress reduces the endogenous antioxidants, causes lipid peroxidation [18, 19], and releases glucocorticoids from the adrenal cortex. Cortisol elevation leads to shrinkage in hippocampal volume and memory impairment [18]. Finally, oxidative stress leads to neuroinflammation and apoptosis [20]. Therefore, targeting oxidative stress and boosting antioxidant activities might slow down the progression of AD [21, 22].

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Currently, there is no appropriate treatment for AD. Evidence suggests that natural anti-oxidants found in plants; mostly flavonoids, may delay the pathogenesis of the disease. Flavonoids exhibit anti-inflammatory and neuroprotective activities [20, 23]. Kaempferol (KMP) is a natural flavonoid with free radical scavenging activity, and it is found in many vegetables and fruits [24] such as *Mespilus germanica.* It is a small tree from the *Rosaceae* family which has been for traditional medicine for many years in Europe, and Asia [25]. Our recent study has demonstrated that alcoholic extract of *Mespilus germanica* leaves consisted of KMP, chrysin, luteolin, myricetin, naringenin, quercetin, and rutin, with a higher percentage of KMP (70%) than other components [26]. Numerous preclinical studies have shown that KMP has a wide range of pharmacological activities, including antioxidant, anti-inflammatory, anticancer, and neuroprotection [27, 28]. The neuroprotective effect of KMP has been shown in many studies. For example, Yang et al. [29] demonstrated that KMP protects neurons by regulating the expression levels of various apoptosis-associated proteins. Also, Zarei et al. [30] demonstrated that KMP improves memory via the cholinergic system in scopolamine-induced memory impairment.

Considering the hypothesis assuming that men and women differ in cognition performance and susceptibility to AD, the present study was designed to compare the effectiveness of KMP on the cognitive deficit in female and male rats with sporadic AD. To approach this, we used streptozotocin (STZ) to induce sporadic AD as it was approved before [31]. Moreover, cognition deficit was assessed by Morris water maze (MWM).

Materials and Methods

Ethics Statement

Experiments were conducted according to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and was approved by the ethics committee of Guilan University of Medical Sciences, Rasht, Iran (IR.GUMS.REC.1396.51).

Animals, Surgical Procedure, and Drug Treatment

A total of forty-six, 3-month-old, female (n = 8/group) and male (n = 7–8/group) Wistar rats (220–250 g) were used in this study. Animals were kept in a standard condition (22 ± 2 °C, 12-h light/dark cycle, light on 7:00 A.M.), ad libitum access to food and water. At the beginning of the experiment, female rats were anesthetized with 75 mg/kg ketamine (Rotexmedica GmbH, Trittau, Germany) and 5 mg/kg xylazine (SciENcelab, Houston, Texas, USA), and ovariectomized to cease the sexual cycle according to our previous study [32]. Rats of each gender were divided into three groups of STZ + saline (SAL), STZ + KMP, and sham. Then all animals were subjected to stereotaxic surgery for cannulation in the lateral ventricles based on the coordination of anterior–posterior = − 0.8 mm, medial–lateral = ± 1.5 mm, and dorsal–ventral = − 3.4 mm, according to Paxinos and Watson atlas [33]. Finally, each rat received 10 µl of ICV injection of STZ (3 mg/kg) (Sigma-Aldrich, MO, USA) on days one and three after surgery.

Plant Extraction

Mespilus germanica leaves were collected from Guilan province of Iran in spring and were confirmed (Herbarium code of 6157) by a specialist from the herbarium center of the Guilan University. The leaves were washed, dried, and powdered. Then 5 g of the powdered leaves were dissolved in 104 mL of 70% ethanol and kept on a 40 °C heater at a speed of 40 m/s. Then 2 M of hydrochloric acid and ethyl acetate were mixed and transferred to a rotary evaporator to achieve pure flavonoids. Two-dimensional paper chromatography was used for detecting all components of extract (at 366 and 254 nm). Then KMP was isolated by thin layer chromatography and high-performance thin layer chromatography according to reported methods. KMP (10 mg/kg), or saline was intraperitoneally infused for 21 consecutive days [27]. The experimental protocol is explained in Fig. 1.

Morris Water Maze

Morris water maze was used to test animal spatial learning and memory abilities. MWM consist of a circular pool (148 cm in diameter, 60 cm in height) was filled with 26 °C water and divided into four presumptive quadrants (north, south, east, west). A black hidden-platform (1.5 cm below the water surface) was submerged in the northwest quadrant. MWM included one block (4 trials, 90 s) per day for 4 consecutive days. The probe trial without the platform was performed to evaluated spatial reference memory on the 5th day; and then the animal's vision was assessed by visible test, with appearing the platform from water. Total time spent in the target quadrant (TTS), latency time to reach the platform location, and swimming speed were recorded using the camera and "Ethovision 11 Noldus" tracking system (Netherlands).

Biochemical Measurements

Animals were decapitated under anesthesia with a mixture of ketamine/xylazine. The hippocampal tissue was removed from the brain on ice and was homogenized in lysis buffer (containing Tris-HCl, pH 8.0,

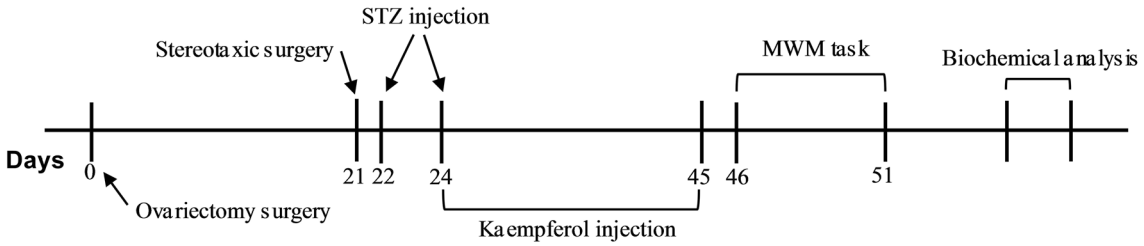

Fig. 1 The experimental protocol of this study. This Fig shows time course of experiment. Female rats were ovariectomized, and then both genders received intracerebroventricular injection of streptozotocin on days 1 and 3 after surgery. Intraperitoneal injections of kaempferol were performed from day 3 beginning from day after STZ for 21 consecutive days. Afterward, spatial learning and memory were assessed via the Morris water maze task. Finally, brain homogenates were used for biochemical analysis

NaCl, sodium deoxycholate, SDS, EDTA, Triton X100, 1 ml diluted protease inhibitor), then supernatants collected after centrifugation (at 4000 rpm/10 min) were used to measure biochemical biomarkers. Endogenous antioxidants of superoxide dismutase (SOD) and glutathione (GSH) were determined using a Super Oxide Dismutase Assay Kit (Zellbio GmbH, Ulm, Germany) and a Glutathione Assay Kit (Zellbio GmbH, Ulm, Germany), according to the manufacturer's instructions. After adding the appropriate reagents, samples, and standards into the wells, and following the details of incubation and washing, the absorbance was measured colorimetrically at 532 nm, and 412 nm by a microplate reader (Awareness Technology Inc, Palm City, FL, USA); respectively. Malondialdehyde (MDA) as a marker of lipid peroxidation were determined using a MDA Assay Kit (Zellbio GmbH, Ulm, Germany), and absorbance was measured colorimetrically at 532 nm using a spectrophotometer (UNICCO, Houston, TX, USA).

The brains of four male rats from the groups of sham and STZ + SAL and one from the STZ + KMP group, and also two in the female's sham group were excluded from the antioxidants assessments due to technical problems in western blotting.

Statistical Analysis

Parametric data (checked with Shapiro–Wilk test) were presented as means ± SD. Data related to the acquisition phase were evaluated by repeated measure and one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Data related to the probe trial and biochemical parameters were analyzed by two-way ANOVA and one-way ANOVA as well, followed by Tukey's post-hoc test, assuming $P < 0.05$. SPSS software was used for statistical analysis (version 22, IBM Corporation, Chicago, USA).

Results

Effect of STZ on Spatial Learning and Memory in Female and Male Rats

One-way ANOVA followed by Tukey's post-hoc result for MWM parameters is given in Fig. 2. Data showed a significant difference between groups among one gender in four blocks (B) during the acquisition phase. So that escape latency was significantly increased during B_2, B_3, and B_4 in the STZ + SAL group compared with the sham group in female ($P = 0.003$, $P = 0.001$, $P = 0.001$) and male ($P = 0.021$, $P = 0.020$, $P = 0.001$) rats (Fig. 2a, b). In the probe test, latency time was significantly longer in the STZ + SAL group in female ($P = 0.002$) and male ($P = 0.001$) rats, compared with the sham counterparts, respectively (Fig. 2c). According to Fig. 2d, e, TTS was significantly decreased in the STZ + SAL group compared with the sham group in female ($P = 0.001$) and male ($P = 0.001$) rats (in the probe phase).

Effect of KMP on Spatial Learning and Memory in Female and Male Rats with Sporadic AD

Female and male rats of AD model which received KMP, demonstrated a significant reduction in escape latency during B_2, B_3, and B_4 compared with the sham group; in female ($P = 0.002$, $P = 0.001$, $P = 0.001$) and male ($P = 0.018$, $P = 0.027$, $P = 0.001$) rats (Fig. 2a, b). Latency time in the probe trial was significantly shorter in the KMP receiving group compared with the STZ + SAL group in female ($P = 0.003$) and male ($P = 0.001$) rats (Fig. 2c). Besides, during the retrieval phase, TTS was significantly increased in the STZ + KMP group compared with the STZ + SAL in female ($P = 0.001$) and male ($P = 0.001$) rats (Fig. 2d, e).

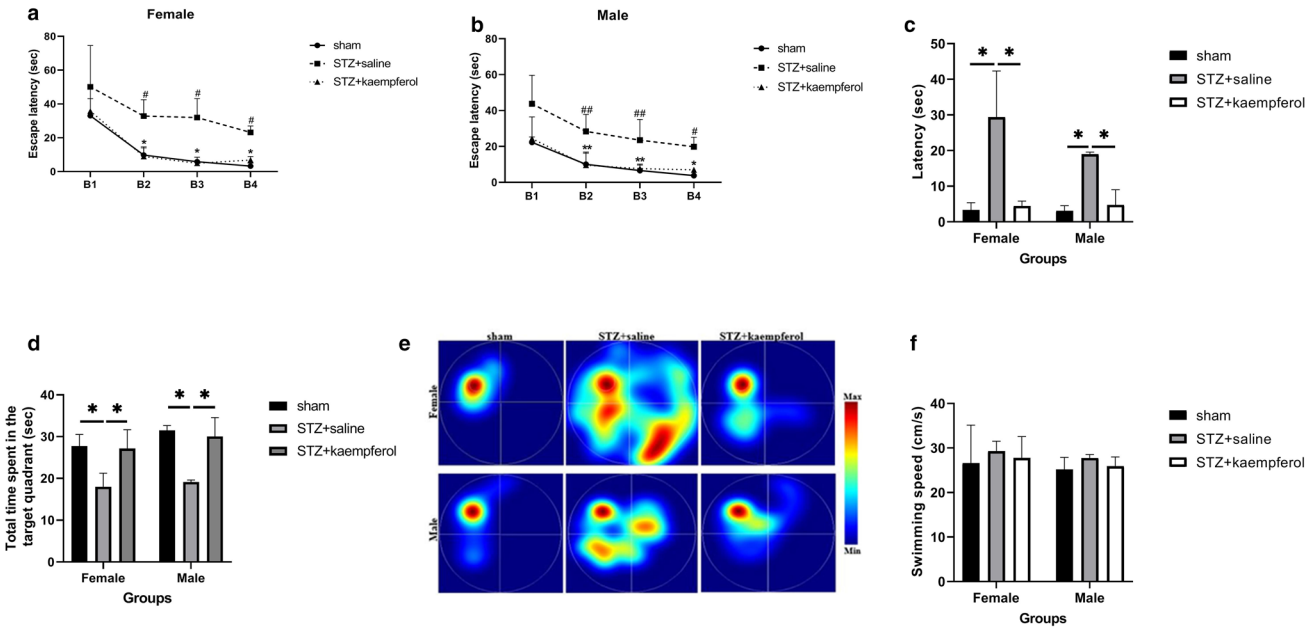

Fig. 2 Effect of KMP on spatial learning and memory in female and male rats with sporadic AD. Escape latency in the acquisition phase in **a** female rats and **b** male rats (in female rats: $^{\#}P<0.01$ vs. sham group; $^{*}P<0.01$ vs. STZ + SAL group; and in male rats $^{\#\#}P<0.05$, $^{\#}P<0.01$ vs. sham group; $^{**}P<0.05$, $^{*}P<0.01$ vs. STZ + SAL group). **c** Latency time during the retrieval phase between groups in female and male rats ($^{*}P<0.01$). **d** Total time spent in the target quadrant during probe trial between groups in female and male rats ($^{*}P<0.01$). **e** Occupancy plots during the Morris water maze probe test in female and male rats. Increasing color intensity (arbitrary scale) represents increased time spent. **f** Average swimming speed of animals in the Morris water maze. Values are expressed as means ± SD and statistically evaluated by repeated measures, two-way ANOVA and one-way ANOVA followed by Tukey's post-hoc test. female rats: n = 8/group, male rats: n = 7–8/group

Comparison of Gender Differences in Spatial Learning and Memory

Comparing two genders, didn't show any significant difference in escape latency during the acquisition phase [$F_{3.6,\ 32.4} = 0.401$, $P = 0.787$], analyzed by repeated measure. Also, in the probe phase, comparing two genders didn't show any significant difference in latency time [$F_{2,\ 43} = 1.43$, $P = 0.251$] and TTS [$F_{2,\ 43} = 0.783$, $P = 0.464$], analyzed by two-way ANOVA. There were no significant differences in swimming speed between groups among one gender [$F_{2,\ 43} = 1.37$, $P = 0.266$], analyzed by two-way ANOVA (Fig. 2f).

Effect of STZ on Spatial Oxidative Stress Biomarkers in Female and Male Rats

One-way ANOVA followed by Tukey's post-hoc result for oxidative stress parameters is given in Fig. 3. The hippocampal level of SOD was significantly lower in the AD model of female rats ($P = 0.004$) and also male rats ($P = 0.001$) compared with the sham gender counterparts (Fig. 3a). As

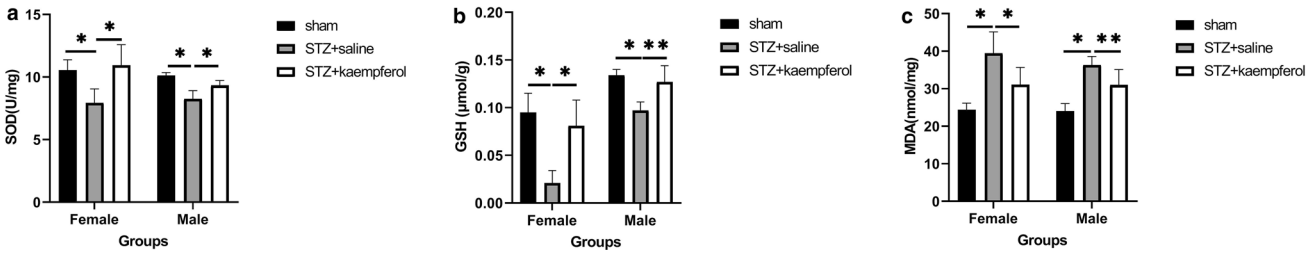

Fig. 3 Effect of KMP on oxidative stress biomarkers in female and male rats with sporadic AD. The hippocampal levels of **a** SOD, **b** GSH, and **c** MDA in female (n = 6–8/group) and male (n = 4–6/group) rats with sporadic AD ($^{*}P<0.01$; $^{**}P<0.05$). Values are expressed as means ± SD and statistically evaluated by two-way ANOVA and one-way ANOVA followed by Tukey's post-hoc test. *SOD* Superoxide dismutase; *GSH* glutathione; *MDA* malondialdehyde

shown in Fig. 3b, the level of GSH was significantly reduced in the STZ treated group compared with the sham group in female ($P=0.001$) and male ($P=0.006$) rats. MDA level was significantly increased in the female and male group of STZ + SAL compared with control female ($P=0.001$) and male ($P=0.001$) of the sham (Fig. 3c).

Effect of KMP on Oxidative Stress Biomarkers in Female and Male Rats with Sporadic AD

SOD activity was significantly elevated in the STZ + KMP compared with the STZ receiving group in female ($P=0.001$) and male ($P=0.009$) rats (Fig. 3a). GSH was significantly enhanced in the female group of STZ + KMP compared with the STZ + SAL group ($P=0.001$) and also male rats ($P=0.012$) (Fig. 3b). MDA level was significantly decreased in the STZ + KMP compared with the STZ + SAL group in female ($P=0.004$) and male ($P=0.049$) rats (Fig. 3c).

Comparison of Gender Differences in Oxidative Stress Biomarkers

KMP treatment showed no significant change between genders in the hippocampal level of antioxidants and lipid peroxidation [SOD: $F_{2, 33}=2.55$, $P=0.094$; GSH: $F_{2, 33}=2.41$, $P=0.106$; MDA: $F_{2, 33}=0.462$, $P=0.634$], analyzed by two-way ANOVA.

Discussion

The present study showed that: (1) ICV injection of STZ in both female and male rats significantly impaird spatial memory and reduces the endogenous antioxidants of SOD and GSH, but increased the lipid peroxidation marker of MDA in the hippocampus of both genders, (2) KMP (10 mg/kg for 21 consecutive days) reversed STZ-induced memory impairment and increased the level of SOD and GSH parallel with a reduction in MDA in both genders.

Our data confirmed the previous findings that STZ disturbs brain metabolism, induces insulin resistance [34], promotes amyloid-β deposition, synaptic dysfunction, tau hyperphosphorylation, [35–37], and cognitive dysfunction [38]. Moreover, change in brain level of acetylcholinesterase, brain-derived neurotrophic factor (BDNF), has been introduced following STZ administration [38, 39].

Moreover, STZ administration induced more memory impairment in female rats compared with male rats. The study of Klambatsen et al. [9] demonstrated that male and ovariectomized female rats had a deficit in a novel object recognition task, while intact females did not show this deficit; which is probably due to the elimination of sex hormones, like our study. However, contradictory with this study carried out by Bao et al. [40] and Biasibetti et al. [10], in which they showed poor performance in the MWM and object recognition tasks in males than females after injection of STZ. Also, the biasibetti's study emphasized the importance of sex dependence in brain response to injury. The contradictory results probably refer to the protocol and animals used, so that our female rats were ovariectomized 1 month before the initial of experiment, and this time was quite sufficient for estrogen withdrawal [41], however, their animals were non- ovariectomized and intact.

Furthermore, the administration of KMP significantly restored the STZ-induced cognitive dysfunction and improved the hippocampal antioxidant parameters independent of genders. Although the baseline level of learning and memory capability was worse in females than males 3 weeks after ovariectomy, both male and female rats learned to find the platform after treatment with KMP significantly.

In addition, the memory restoration was parallel with elevation in hippocampal levels of two antioxidants of SOD and GSH in an independency to gender. The findings of neuroprotective roles of KMP are in agreement with the study carried out by Lopez-Sanchez et al. [42], in which they reported that KMP decreased ischemia-induced brain damage in male rats due to antioxidant properties [42]. This finding confirms the previous studies of KMP antioxidant effects in rat models of Parkinson's disease [43] and diabetic rats [44]. KMP chronic administration successfully decreased hippocampal level of MDA, which is in agreement with [45]. Also, it seems that KMP chronic administration exerts an anti-inflammatory effect as well [27, 46]. Taken together, our findings consistent with the previous studies support anti-inflammatory, antioxidant and neuroprotective properties for KMP in a dose used here.

To answer the question raised here, why KMP didn't show a gender difference response, it can be explained that although female rats exhibited worse navigation in MWM compared with their male counterparts after STZ administration, the neuroprotective and antioxidant properties of KMP seems to override the gender difference. Although males and females differentially rely on different learning strategies during spatial navigation tasks [47, 48]. For example, male rodents prefer hippocampus-dependent place strategies and females prefer striatum-dependent cue strategies when ovarian hormones are low [47, 48]. In another word, male rats use geometric cues whereas female rats use landmark and visual cues to solve spatial tasks in both [49]. Despite, a sex difference favoring males exists in the standard reference memory version of the MWM task, but generally there is no sex difference observed during a cue competition task in which subjects can use both the place and response strategy to solve the task [50].

Finally, our data support that KMP improves spatial memory independently of gender by increasing hippocampal level of SOD, GSH, and, MDA. In conclusion, based on our data, the ability of KMP to improve cognitive performance equally in female and male rats could involve an ability to improve redox signaling or to prevent the accumulation of molecular oxidative damage, via up-regulation of cellular antioxidative defenses.

One limitation of the study was not being able to measure the inflammatory and apoptotic markers. Also losing some brains during western blotting for hippocampal CREB level. In the clinical importance of view, this compound could be used to slow down the progression of AD in both genders. We suggest for future study including non-ovariectomized female rats and other learning and memory tasks such as object recognition and Y maze. Comparing changes in the ROS molecular pathway of nuclear erythroid 2-related factor 2 and also inflammatory cytokines such as tumor necrosis factor and interleukin following kaempferol administration in the AD model are suggested.

Author Contributions PB designed this study, performed experiments, wrote and revised the paper for important intellectual content. KE performed experiments and read the manuscript. SK performed experiments, statistical analysis, and participated in paper writing. All authors approved the final version of the paper.

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Data Availability The datasets and supporting materials generated and/or analyzed during the current study will be available from the corresponding author upon reasonable request.

Declarations

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

Ethical Approval Experiments were conducted according to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and was approved by the ethics committee of Guilan University of Medical Sciences, Rasht, Iran (IR.GUMS.REC.1396.51).

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