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Research Paper: The Effect of Different Doses of Melatonin on Learning and Memory Deficit in Alzheimer Model of Rats

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ABSTRACT

Background: Alzheimer Disease (AD) is an age-related neurodegenerative disorder with a progressive impairment of cognitive function. The pineal gland hormone melatonin (MEL) has been known as a protection agent against AD. However, the effect of melatonin in various doses is inconsistent.

Objectives: In this study, we aimed to investigate two doses of MEL on learning and memory in the amyloid-βeta (Aβ)-induced AD in the rats.

Materials & Methods: Forty-eight male Wistar rats were used in the experiment and randomly divided control, sham, vehicle, AD, AD+MEL10 mg/kg, and AD+MEL 20 mg/kg groups. Intracerebroventricular injection of Aβ1–42 was used to develop the animal model of AD. Also, MEL-treated groups received an intraperitoneal injection of MEL for 4 next weeks. The Morris Water Maze (MWM) and Passive Avoidance Learning (PAL) tests were used to examine animals' learning and memory. The brain of animals was removed for immunohistochemistry for anti-Amyloid Precursor Protein (APP).

Results: Intra-peritoneal injection of MEL significantly improve learning and memory in MWM (P=0.000) and PAL test (P=0.000), but there were no significant changes in the two groups that received the melatonin (P>0.05). Histopathological analysis revealed that the clearance of APP deposition in the AD+MEL20 group was considerable compared with the AD+MEL10 group (P=0.000).

Conclusion: Our findings indicate that 10 and 20 mg/kg doses of melatonin have similar results on learning and memory in the AD model. But 20 mg/kg of melatonin has significantly more effect on the clearance of APP deposition.

Keywords: Melatonin; Alzheimer disease; Memory; Learning

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Highlights

- Melatonin has effect on memory function, especially spatial memory in Alzheimer's model of rats.
- No difference is between the effect of two doses of 10 and 20 mg/kg on memory in Alzheimer's model of rats.

Introduction

Alzheimer Disease (AD) is an age-related neurodegenerative disorder characterized by a progressive impairment of cognitive function [1, 2]. AD targets vital brain cells through which it affects thinking, memory, and behavior: these effects compromise work and social life [3].

Pathogenesis of AD has not been fully understood; however, it is evident that Amyloid-βeta (Aβ) accumulation is an integral part of this disease [4], as it plays an essential role in the AD-related neurodegenerative process [3]. Aβ is a peptide containing 37–43 amino acids, and it is produced from Amyloid Precursor Protein (APP) [5] through a proteolytic cleavage mediated by β- and γ-secretases [4]. Intraventricular injection of Aβ is known to induce learning and memory deficits [3]. Toxic effects of Aβ in the brain may exert through an increase in neuroinflammation, mitochondrial dysfunction, and Reactive Oxygen Species (ROS), through which it induces oxidative stress in the brain [4, 6-8]. Oxidative stress plays a key role in learning and memory impairment [9].

Melatonin (MEL) (N-acetyl-5-methoxytryptamine) is a production of the pineal gland [10] and a multifunctional molecule that participate in a variety of physiological functions, including antioxidant [11-14] and radical scavenger against harmful effects of free radicals on biological membrane lipids, Deoxyribonucleic Acid (DNA), and proteins [12, 15]. Melatonin appears effective and safe in improving sleep quality in patients with AD [16]. There is growing evidence that sheds light on the protective role of MEL in memory deficit [9], aging, and Alzheimer disease [17]. MEL can be served as a neuroprotective hormone against mitochondrial damage [18], oxidative stress resulting in amelioration of learning deficits and neurodegenerative diseases [19, 20]. MEL has anti-amyloid properties through inhibition of Aβ generation [21]. Also, MEL stimulates the nonamyloidogenic pathway over the amyloidogenic pathway in the cultured neuronal and non-neuronal cells [22-24].

It has been suggested that melatonin has sufficient high concentrations in the cerebral tissue [25], and melatonin analogs are employed in higher densities [26]. However, different doses of melatonin have been shown to have different effects on memory in animal models of memory impairment; for example, one study indicated that melatonin at a dose of 10 mg/kg improves memory [27], while another study found no effect on memory [28]. In another study, a dose of 20 mg/kg melatonin improved memory [29], suggesting that doses greater than 10 mg/kg may improve memory, whereas, in their study, a dose of 30 mg did not affect memory [30]. Thus, this study aimed to evaluate the treatment role of two different MEL dosages on the learning and memory, Amyloid Precursor Protein (APP) deposit and migration of microglia in AD rats.

Materials and Methods

Animals

A total of 48 male Wistar rats weighing 250-300 g were obtained from the Medical Faculty of Hamedan University of Medical Sciences. The rats were placed in standard conditions of illumination (12 h light-dark cycle), humidity (55%-65%), and temperature (22°C). They were allowed to have easy access to food and water ad libitum for one week to be acclimatized to the new environment. All procedures were performed by reliance on the guides from the Ethics Committee of Hamedan University of Medical Sciences compliance to the institutional and national guidelines for animal care and use.

Experimental design

Animals were divided randomly into 6 groups, including control, sham, vehicle, AD, AD+MEL10, and AD+MEL 20. AD received Aβ1-42 on the first day of experiments. After two weeks, MEL (Sigma) at two doses of 10 mg/kg and 20 mg/kg was administered intraperitoneally once per week for 4 weeks [28, 29]. Then, the learning and spatial memory of the rats were evaluated. Morris Water Maze (MWM) and Passive Avoidance Learning (PAL) tests were used to assess memory function. Also, APP microglia was assessed by histological study, respectively.

Surgery and injection of Aβ (1–42)

The procedure was done according to previous studies [31, 32]. The animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and submitted to a stereotaxic apparatus (Stoelting, USA). According to the bregma, the coordinates for the bilateral intracerebroventricular were AP: 1.2 mm; ML: 2 mm; and DV: 4 mm [33]. Aβ (Tocris Bioscience, UK) solution (1 µg/µL, 5 µL) was bilaterally microinjected into the region. Sham rats only sustained surgery procedure without any injection. Vehicle groups received the same volume of vehicle. Following injections, the skin was sutured, and the animals were allowed to recover in their home cages.

Passive Avoidance Learning (PAL)

Apparatus

PAL was evaluated using a step-through apparatus [34] consisted of two compartments: a light compartment (20 cm × 20 cm × 30 cm) made from transparent plastic and a dark compartment made from dark opaque plastic (20 cm × 20 cm × 30 cm). A stainless steel rod with a diameter of 3 mm was applied to the floor of the compartments. A shock generator (Behbood Pardaz Co. Tehran, Iran) was used to electrify the dark compartment floor. A rectangular opening (6 cm × 8 cm) capable of closing by an opaque guillotine door was located between the two compartments [34].

Training

First, each rat received two trials to become familiarized with the apparatus. For this purpose, the rats were located in the light compartment facing away from the door for 5 s. Then, the guillotine door was raised. Typically, the rats have an instinctive tendency to the dark environment. When the rats entered the dark compartment, the guillotine door was closed, and the animals were kept for 30 s in this compartment. This trial was repeated after 30 min interval. The entrance latency to the dark compartment (STLa: Step-through latency in the acquisition) was tested when the rats were placed in the dark compartment [35].

After the rats entered the dark compartment spontaneously, the guillotine door was closed for 30 s, and a 0.5 mA electrical shock was applied for 3 s. The procedure was repeated after 2 min. The training was finished when animals stayed in the light compartment for 120 consecutive s. The number of trials, which are entries to the dark compartment, was recorded [35].

Retention test

In the retention test, the rats were placed in the light compartment for 5 s, and then the guillotine door was raised. The delay in the entrance to the dark compartment (i.e., staying in the light compartment) or Step-Through Latency in the retention trial (STLr) and the Time spending in the Dark Compartment (TDC) was recorded up to 300 s. The retention test was finished when the animal did not enter the dark chamber within 300 s [36].

Morris Water Maze (MWM)

Evaluation of spatial working and reference memory was performed using MWM. Briefly, a large circular black pool (180 cm diameter and 60 cm height) filled to a depth of 35 cm with opaque water (22°C±1°C) was used. Non-motile external cues were located around the pool as a guide for rats to find their roots. The pool was divided into North (N), East (E), West (W), and South (S) quadrants. A hidden platform was placed in the center of the northern quadrant at 1 cm below the water surface. Rats were assessed on 5 consecutive days between 10:00 AM and 12:00 PM. The rats were allowed to do training that consisted of two blocks each includes 4 trials. Evaluation of visual test was carried out at day one by placing the clear covering platform on the water surface. The platform (with no cover) was placed at 0.5 cm below the water surface during trial days 2, 3, and 4 for checking short-term or working memory. On day 5 (probe stage), the platform was removed, and rats were allowed to swim for 60 s to evaluate long-term or reference memory. Videotaping of animal performance was performed using a video camera (Nikon, Melville, NY, USA) that was installed above the pool, and it was connected to a tracking system to record the animal's performance. In the first 4 days of training, following placing in each of the quadrants, the rats were permitted to find the platform in 90 s. About 30 s rest was allocated between the two trials, and 5 min rest was allowed between the two blocks. Animals with no capability of finding the platform during each trial by themselves were manually placed on the platform. During the probe stage, all animals were placed in the pool from the western side. In these animals, recognition of the exact location of the place of platform was calculated by recording the percentage of time spent in the target quadrant [37].

Tissue preparation and Immunohistochemistry

After 60 days, the animals were deeply anesthetized with ketamine and xylazine, perfused transcardially with 150–200 mL PBS and fixed with 4% paraformaldehyde.

After whole-brain primary fixation, the hippocampus was harvested and re-fixed with 4% paraformaldehyde. Specimens were embedded in paraffin (Merck, Germany), and 5-µm paraffin sections were prepared for immunohistochemistry evaluation. After deparaffinization and antigen retrieval, the sections were rinsed with PBS, treated with blocking solution, and incubated with anti-amyloid precursor protein antibody (ab49385. 1:2000, Abcam) primary antibodies overnight at 4°C. The next day, after washing with PBS, the sections were incubated with the secondary antibody: Goat anti-rabbit (ab6721, Abcam) for 2 h at room temperature. After extensive washing in PBS, the sites of antibody binding were visualized using the avidin-biotin-peroxidase method (ABC Standard kit, VECTASTAIN, VectorLabs). DiaminoBenzidine (DAB) was used as a chromogen. The sections were counterstained with hematoxylin and cover-slipped with entellan [38].

Statistical analysis

Homogeneity of variances (Levene's test) was checked, and data revealed normal distribution (The Shapiro-Wilk's test). All values were presented as Mean±SD. One-way Analysis of Variance (ANOVA) using SPSS V. 22 (SPSS Inc., Chicago, IL) was applied for data analysis. MVM, STLr, and TDC showed unequal variances, thereby Tamhane used as a Post Hoc Test. ANOVA results for STLa and the number of trials was not significant. P≤0.05 was considered statistically significant.

Results

PAL test findings

The STLa recording was performed in animals placing in the dark compartment without receiving an electric shock. Entrance latency to the darkness compartment showed no considerable difference (Figure 1A).

The number of trials showed significant differences in the AD+MEL 10 and AD+MEL 20 groups compared with the AD group (P=0.000). However, there were no significant differences in the number of trials to acquisition between the AD+MEL10 and AD+EL20 groups (Figure 1B).

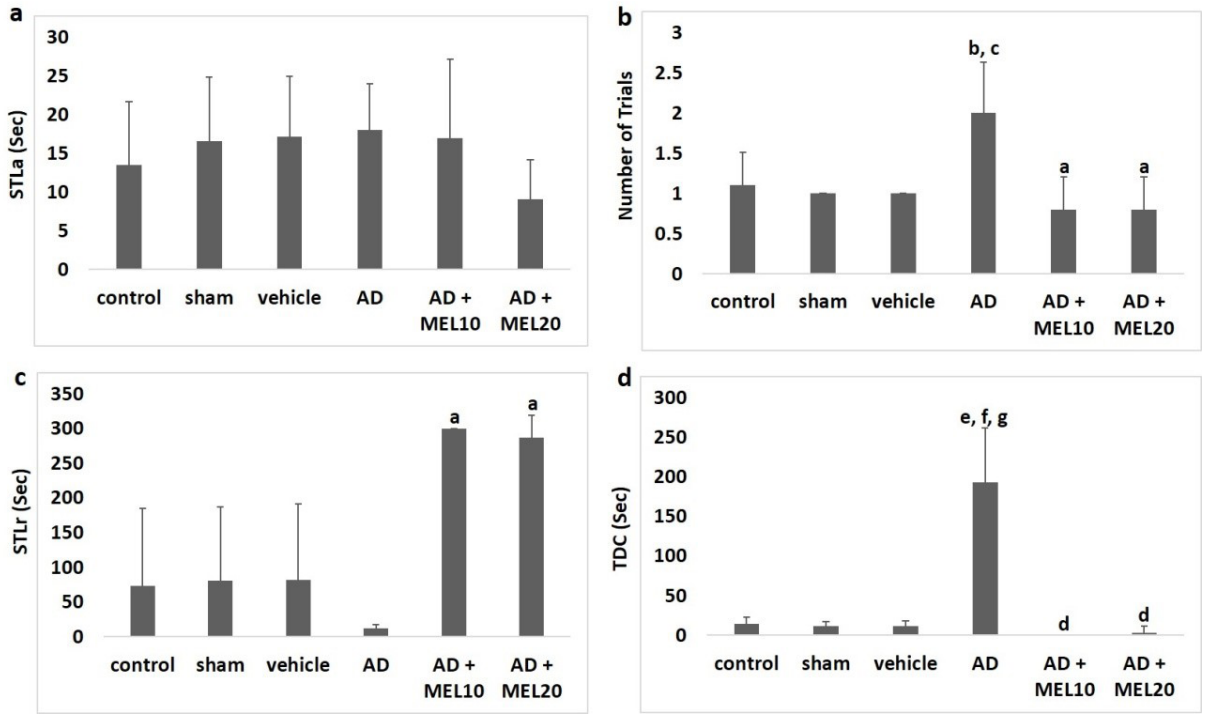

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Figure 1. Effects of Melatonin (MEL) administration in dosages of 10 mg/kg and 20 mg/kg on the passive avoidance learning (PAL) test

A. Step-Through Latency (STLa) in the acquisition trial; B. Number of trials to acquisition; C. Step-through latency in the retention trial (STLr); and D. The Time spending in the Dark Compartment (TDC) (n=8).

A. P<0.001 vs. AD; B. P<0.001 vs. vehicle and sham; C. P=0. 009 vs. control; D. P<0. 01 vs. AD; E. P<0. 01 vs. vehicle; F. P<0.01 vs. sham; and G. P<0. 01 vs. control.

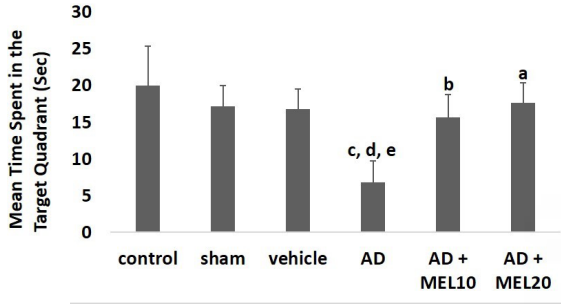

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Figure 2. Evaluation of the meantime spending in the target quadrant by Morris Water Maze (MWM) to test the role of Melatonin (MEL) administration in dosages of 10 mg/kg and 20 mg/kg on spatial memory in the model of Alzheimer Disease (AD) induced by Aβ

A. P<0.001 vs. AD; B. P=0.007 vs. AD; C. P=0.002 vs. vehicle; D. P<0.001 vs. sham; and E. P<0.01 vs. control.

The STLr (Figure 1C) and TDC (Figure 1D) were also recorded in animals. In the retention test, MEL-treated groups showed a rise in the STLr and a fall in the TDC. The STLr and TDC for the AD+EL10 group were 300±0 s (P=0.000 vs. AD group) and 0±0 s (P=0.000 vs. AD group). The STLr and TDC for the AD+MEL20 group were 286.6±32.65 s (P=0.000 vs. AD group) and 2.8±8.1 s (P=0.000 vs. AD group), respectively. Both STLr and TDC showed no significant changes in the AD+MEL 20 group compared with the AD+MEL10 group.

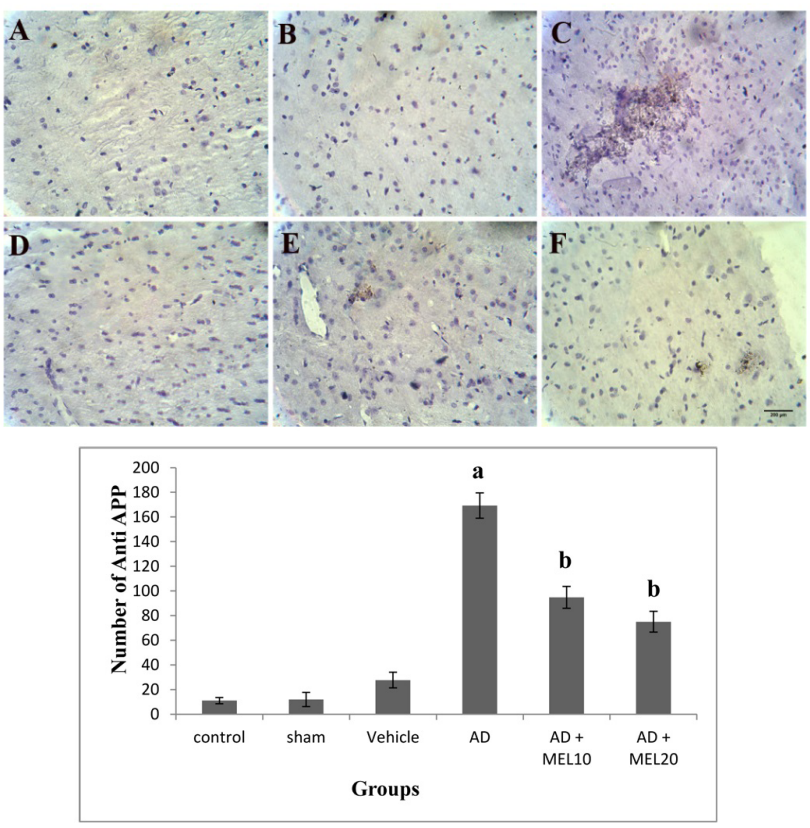

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Figure 3. Immunohistochemistry images of the anti APP

A. Control group; B. Sham; C. AD; D. Vehicle; E. AD+MEL10; F. AD+MEL 20;

B. Number of anti-APP by image G;

A. Compared to the control group; B. Compared to AD groups; P<0.05.

MVM test results

The mean time was spent in the target quadrant was recorded in the probe trial stage. We found that the two MEL-treated groups spent less time in the target quadrant. One-way ANOVA showed a significant difference between experimental groups. The Mean±SD for AD+MEL10 and AD+MEL20 groups were 15.67±3.09 (P=0.000 vs. AD group) and 17.64±2.7 (P=0.000 vs. AD group), respectively. There were no significant changes in the AD+EL10 group compared with the AD+MEL 20 group (P>0.05) (Figure 2).

Histological findings

Our finding indicated that the number of APP deposit was significantly decreased in the brain of both groups received melatonin (P=0.000). However, their numbers in the MEL 20 groups were less than in the MEL10 group (Figure 3).

Discussion

In the present study, effects of MEL application at doses of 10 mg/kg and 20 mg/kg were evaluated in the Aβ-induced AD. Evaluations of learning and spatial memory, level of APP deposit was performed to target these effects.

Here, we found no changes in the learning of passive avoidance tests in all groups. The AD+MEL 10 and AD+MEL 20 groups showed a considerable fall in the number of acquisition trials compared to the AD group, possibly showing improvement in the acquisition.

TDC is considered as an indicator of inhibitory avoidance behavior [3]. In the retention test, we found that the AD group had a decrease in the STLr, a significant increase in the TDC, indicating Aβ-induced deficits of memory retention [36]. On the other hand, we found an increase of the STLr but a decrease of the TDC in both AD+MEL10 and AD+MEL 20 groups compared with the AD group. These results elucidate the possible facilitatory effects of both dosages of MEL on memory retention [36]. However, the results of both STLr and TDC tests showed no considerable differences in the AD+MEL20 group compared to the AD+MEL10 group, which means no possible difference in using the two dosages of MEL for targeting learning deficit in rats under exposure to Aβ.

MWM was also tested in the studying groups, and the results revealed that animals in the AD group spent less time in the target quadrant indicating spatial memory deficit induced by Aβ injection in this group. On the other hand, upon MEL treatment, the animals showed significantly more time spent in the target quadrant. The effect of MEL against scopolamine has been reported in rats [38]. The preventive role of MEL has been shown in rats who received an intrahippocampal injection of Aβ followed by an application of melatonin for 10 days [39]. This means that MEL administration can reverse memory impairment induced by Aβ [19-21], which was in line with the work performed by Rudnitskaya et al. on OXYS-injured rats that they found MEL-treated rats spent more time in the target quadrant, suggesting the positive effect of MEL at dosage 0.04 mg/kg on the improvement of memory deficit. In their study, they also noticed that oral administration of MEL could reduce the accumulation of Aβ in the hippocampus [14]. Similarly, Zhang et al. found that application of 500 mg/kg MEL at 24 h before Aβ1–42 injection could improve impairment of spatial learning and memory [40]. Here, we found beneficial effects of MEL dosages 10 mg/kg and 20 mg/kg spatial learning and memory after injection of the final dosage of the Aβ1–42.

There is evidence that Aβ can induce oxidative damage in mitochondrial DNA [5, 6, 8]. Sharif et al. assessed the effects of 50 and 100 µg/kg of MEL on H89-induced memory deficit, and they found that MEL, a powerful scavenger of ROS, exert its neuroprotection through reduction of oxidative stress [9, 15] and increases mitochondrial function [7, 18, 41]. The pineal hormone melatonin [10] stimulates the nonamyloidogenic processing and inhibits the amyloidogenic processing of β-amyloid precursor protein [22, 23]. Melatonin has a neuroprotective effect in the treatment of AD [20, 42]. We may have assumed in our study that protective roles of 10 mg/kg and 20 mg/kg of MEL against Aβ were probably applied through targeting oxidative stress. Our immunohistochemical findings demonstrate that the groups that received melatonin had the clearance ability of APP deposits, resulting in cognitive performance improving AD rat models. The present study is concurrent with another study indicating that melatonin protects neurons from the toxicity of the amyloid-β (Aβ) peptide (the main neurotoxin involved in AD) through GABA receptors [43]. Melatonin proteolytic cleavage of APP through the α-secretase pathway is controlled by many physiological and pathological stimuli, especially through Protein Kinase (PK) C and secretase-mediated cleavage APP [43, 44].

Interestingly, we found no significant difference between 10 mg/kg and 20 mg/kg of MEL on spatial memory. Considering this point along with the mentioned works by others, we speculate that lower doses of MEL administration could be neuroprotective against Aβ-induced memory deficit as much as the higher dosages of this hormone do, and possibly there is no need to use high doses aiming to exert better results [30]. Our findings showed that the application of dosages 10

mg/kg and 20 mg/kg of MEL did not have any significant difference in exerting protective roles against learning and spatial memory deficit.

Overall, the present study results showed no significant difference between melatonin doses (10 and 20 mg/kg) in the behavioral and histological assessment. Besides the dose, the duration of treatment with melatonin is also a factor that affects various studies of AD animal models. For example, a study indicated that melatonin injection (0.5 mg/d in drinking water) for 8–11 months significantly reduced cognitive deficits in Barnes maze and MWM and novel object recognition tests [45], while in another study, intraperitoneal injection of melatonin at a dose of 0.1, 1, and 10 mg/kg for 10 days induced spatial memory impairment [28]. Also, the results of another study showed that intraperitoneal injection of melatonin (30 mg/kg/d) for 10 days did not affect passive avoidance memory in AD rats [30].

One of our study limitations is that we only assessed the melatonin effect at doses of 10 and 20 mg/kg. Assessment of other doses of melatonin would be useful to understand the optimal dose of melatonin on the memory defect. Although it is cumbersome, we suggest the measuring of MWM, PAL, and histological assessment several times because it would be useful to understand the temporal profile of the melatonin effects on memory deficits.

Conclusion

Our findings declared that 10 and 20 mg/kg doses of melatonin have similar results on learning and memory in the AD model. But 20 mg/kg of melatonin has significantly more effect on the clearance of APP deposition.

Ethical Considerations

Compliance with ethical guidelines

The present study was approved by the Ethics Committee of Hamadan University of Medical Sciences (Code: IR.UMSHA.REC.1394.278). All study procedures were done in compliance with the ethical guidelines of the 2013 version of the Declaration of Helsinki.

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Authors contributions

Conceptualization: Arman Keymoradzadeh, Ali Reza Komaki, Nafise Faraji, Zoleikha Golipoor; Methodology: Arman Keymoradzadeh, Arash Bakhshi, Nafise Faraji, Zoleikha Golipoor; Investigation, writing the original draft, review, and editing: All authors. Funding, acquisition: Zoleikha Golipoor; Resources: Arman Keymoradzadeh, Arash Bakhshi, Zoleikha Golipoor; Supervision: Arman Keymoradzadeh, Zoleikha Golipoor.

Conflict of interest

The authors declared no conflict of interest.

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