

# Evaluation of the Role of Melatonin in Dietary Restriction Effects on Spatial Memory Impairment Induced by Streptozotocin (STZ) in Male Rats

Mohammad Mehdipour <sup>1,5</sup>, Asadollah Zarifkar <sup>1, 2,5\*</sup>, Mohammad Reza Namavar <sup>3, 4,5</sup>

<sup>1</sup>Department of Physiology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

<sup>2</sup>Shiraz Neuroscience Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

<sup>3</sup>Histomorphometry and Stereology research center, Shiraz University of Medical Sciences, Shiraz, Iran

<sup>4</sup>Clinical Neurology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

<sup>5</sup>Department of Neuroscience, School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz, Iran

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\*Corresponding Author:

Assadollah Zarifkar,  
Department of Physiology,  
School of Medicine, Shiraz  
University of Medical Sciences,  
Shiraz, Iran. E-mail:  
zarifkara@sums.ac.ir

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## Abstract

**Background:** It has been shown that dietary restriction in the form of every other day fasting (EODF) has neuroprotective effects. The mechanisms of these effects are unknown yet. Recently, it has claimed that dietary restriction can reset circadian and regulate irregular rhythms. Previous studies have demonstrated that dietary restriction can change melatonin production and secretion. Since melatonin has important role in circadian rhythms and its neuroprotective effect has been proved, it is reasonable that at least some dietary restriction effects may be due to the melatonin level changes. To investigate this hypothesis, melatonin receptors antagonist (Luzindole) was used in the rat model of Alzheimer's disease. **Material and Methods:** Sixty four male rats were assigned into 8 groups which 4 groups had Ad libitum and 4 groups had every other day fasting (EODF) diet. Each group category comprised: 1-control group receiving only solvent of drugs, 2-group which received streptozotocin (STZ) at 3 mg/kg/icv, 3-group which received STZ at 3 mg/kg/icv and luzindole 50 µgr/kg/icv and 4-group which received STZ 3 mg/kg/icv and melatonin 10 mg/kg/ip. Dietary regimen continued for 10 days from the day cannulation was done in lateral ventricles. Spatial memory was evaluated by Morris water maze test. To evaluate the histological changes in the brain tissue, hematoxylin and eosin staining was used. **Results:** Based on Morris water maze test results, STZ could impair spatial memory in rats while dietary restriction did not improve memory impairment significantly. Likewise, melatonin and luzindole could not restore memory impairment. Histological study indicated that STZ destroyed the brain tissue in different parts including paraventricular zone, fornix, cortex and hippocampus which could be attributed to memory impairment. Counting dead neurons in CA1 part of the hippocampus showed that dietary restriction accompanied by exogenous melatonin with supra physiological dose, could partially improve STZ-induced impact in brain tissue. **Conclusion:** Melatonin appears not to retain an important role in the effects of dietary restriction on spatial memory impairment. Meanwhile, dietary restriction associated with exogenous melatonin seem to exert neuroprotective effects.

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## **Introduction**

It has been well established that dietary restriction (DR) in the form of every other day fasting (EODF) with adequate nutrition increases lifespan in a diverse set of species (1) and has neuroprotective effects (2). Recent studies suggest that DR may enhance brain functions including learning and memory (3-4), synaptic plasticity (5), and neurogenesis (6). The precise mechanisms mediating such beneficial effects of DR are still unresolved. In previous studies, different mechanisms have been discussed. These mechanisms include decreased rate of apoptosis (6-7), decreased oxidative stress (2), increased insulin sensitivity (2), increased heat shock proteins (HSPs) (8), increased BDNF expression (7), change in secretion of hormones (e.g. corticosterone (9), adiponectin (10), ghrelin (11), IGF (12), GH (13) as well as increased sirt1 and its activity (14). Resetting the circadian clock is another intervention which can lead to increased life span and wellbeing, while clock disruption is associated with aging and morbidity. In fact resetting the circadian clock leads to synchrony in metabolism and physiology (15-16). Intermittent fasting can entrain the master clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus in brain (17). Melatonin is known to play an important role in circadian system (17). The rhythmicity of pineal melatonin in mammals can be controlled by daily feeding cues when the SCN clock is lacking (18). Restricted feeding restores not only a rhythmic transcription of the rate-limiting enzyme for melatonin biosynthesis [aryl-alkylamine-N-acetyl-transferase (AANAT)] but also a rhythmic synthesis of melatonin in the pineal gland (18). Neuroprotective effects of melatonin have been shown in several studies (19-23). Many studies have reported disrupted circadian rhythms and melatonin production in aging and in Alzheimer's disease. Reactivation of the circadian system by means melatonin supplementation has shown encouraging results (24).

Having considered these, this study was designed to investigate the effect of dietary restriction on memory impairment in streptozotocin (STZ) model of sporadic Alzheimer's disease and the role of endogenous melatonin in dietary restriction effects on memory impairment induced by STZ as well as the effects of exogenous melatonin on memory impairment in this model of Alzheimer's disease.

## **Materials and methods:**

### **Animals:**

In this study sixty four male Sprague-Dawley rats weighing 240-280 grams, bred in the Laboratory Animal Center, Shiraz University of Medical Sciences (SUMS) were selected and randomly placed into eight groups. Each experimental group comprised 8 rats.

Animals were kept at the standard condition of 12-hour light/dark cycle and the temperature of 21-27°C. All study phases were conducted in full compliance with the NIH guideline as well as the ethical principles laid down by the Shiraz University of Medical Sciences.

### **Surgery**

Animals were anesthetized by intraperitoneal injection (i.p.) of Ketamine (100 mg/kg) and xylazine (10mg/kg). The head of anesthetized animal was fixed in the stereotaxic frame through which guide cannulae were inserted into the lateral ventricles according to atlas of Paxinos coordinates (0.8 mm posterior to bregma, 1.5 mm lateral to sagittal suture, and 3.5 mm ventral from the surface of the brain). Then, the surgical area was covered by dental cement to ensure the cannula is completely fixed in place.

### **Drugs**

#### **ICV injection of STZ**

To provide rat model of Alzheimer's disease, streptozotocin (STZ), purchased from Sigma (USA), was dissolved in normal saline and injected at 3 mg/kg/icv bilaterally on day 1 and day 3 following cannulation.

#### **ICV injection of luzindole**

Luzindole, by Sigma Aldrich, was dissolved in DMSO solution and administered at the dose of 50 µgr/kg/icv for ten days from first STZ dose during the initial hours of dark cycles.

#### **Intraperitoneal (i.p.) administration of melatonin**

Melatonin, by Sigma (USA), was firstly dissolved in absolute ethanol and then diluted with 0.9% saline. The final ethanol concentration was <0.5% dissolved in normal saline. It was administered i.p. at the dose of 10 mg/kg for ten days from the first STZ dose during the initial hours of dark cycles.

## Grouping

Sixty four male rats were assigned into 8 groups of which 4 groups were on *Ad libitum* and 4 groups were on every other day fasting (EODF) diet. Each group category comprised 1-control group which received only solvent of drugs, 2- group which received streptozotocin (STZ) at 3 mg/kg/icv, 3- group which received STZ at 3 mg/kg/icv and luzindole 50 µgr/kg/icv, 4- group which received STZ at 3 mg/kg/icv and melatonin 10 mg/kg/ip. Dietary regimen continued for 10 days from the day after cannulation. Spatial memory evaluating was performed by Morris water maze test was done for 4 days, from day12 to day15 after surgery.

## Morris water maze test

A circular tank (diameter 140 cm, height 70 cm, painted dark) was filled with water ( $25\pm 1$  °C) to a depth of 30 cm. Spatial visual clues were provided in the form of different shaped objects on the walls of each quadrant. A circular glassy escape platform (diameter 10 cm) was submerged approximately 1 cm below the surface of the water, 10 cm off the edge of the tank at a position designated as quadrant 3 (target quadrant). A video camera was mounted on the ceiling in the center of the pool while was connected to computer. Acquisition trials (4 trials per day for 3 days) were started by placing the rat in the pool facing the wall of the tank from different randomly chosen start positions, and the time required to find the invisible platform was recorded by ethovision software.

A trial lasted until the rat found the platform or until 90 second had elapsed. If the rat did not find the platform within 90 s, it was guided to the platform and placed on it for 20 s. After the completion of the fourth trial on each day, the rat was dried and returned to its home cage. Twenty four hours after the final acquisition trial, the platform was removed from the pool and a probe trial lasting 60 s was performed; the time spent in the target quadrant were recorded. In the probe trial the rat was started facing the wall of the tank from the position opposite to the removed platform. Then after the probe trial, rats were trained for visible platform test (4 trials) to confirm that each rat had no visual deficits.

## Histopathological study

Following the behavioral test (MWM), four rats from each groups were randomly selected for histopathological study. Rats were anesthetized deeply by intraperitoneal (i.p.) injection of Ketamine (100 mg/kg) and xylazine (10mg/kg). The brain was fixed by transcardial perfusion with 250 ml of 4%

paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4 for pre-fixation of the tissue. Then the brain tissue was dissected out carefully and was kept in 4% paraformaldehyde overnight for post-fixation. After post-fixation the tissue was dehydrated by sucrose and paraformaldehyde solution. Then the brain tissue was kept in -70 °C in freezer until sectioning day. Coronal sections (30µm) were taken using the frozen microtome (HM 550 Germany) from optic chiasm down to interpeduncular fossa (hippocampus area), 1.72-6.96 posterior to bregma. Followed by that, one section out of sixteen was selected, picked up and mounted on gelatin-coated slide. Section from the rostral to the caudal portion of the brain were stained with hematoxylin and eosin (HE). Five identical microscopic fields of CA1 area from each hippocampus on each side of the brain were selected for the purpose of counting dead neurons by SUMS StereoLite software. Dead cells were morphologically identified by diffused pallor of eosinophilic background, alteration in size and shape of cells, chromatin condensation and condensed nucleus (22).

## Statistical analysis

Results were expressed as Mean±S.E.M. The statistical analyses of Morris water maze test and histological data were performed by one way ANOVA followed by Tukey's test and level of significance was  $P<0.05$  in all statistical evaluations.

## Results:

### Morris water maze test

Figure1 shows the time to reach the hidden platform on days 1 to 3 in all groups. One-way ANOVA was used to compare animal behaviors on different days of training. Results indicated significant differences of mean escape latency values between the groups on the second and third days (day 2:  $P<0.001$ , day 3:  $P<0.001$ ).

Post-hoc Tukey's tests followed by one-way ANOVA showed that STZ significantly increased the time to reach the platform in the second day of learning.

Post-hoc Tukey's tests followed by one-way ANOVA showed no significant difference between STZ-treated groups on day 3. On the other hand, EODF groups treated with STZ showed better function on finding hidden platform as compared to *Ad libitum* STZ-treated rats.

The distances traveled to reach the hidden platform on days 1-3 in all groups have been shown in Figure 2. One-way ANOVA test demonstrated a

significance difference between groups on day 3 ( $P < 0.001$ ).

Post-hoc Tukey's tests followed by one-way ANOVA showed that on day 3, EODF STZ-treated groups had less distance traveled to reach the platform as compare to Ad libitum STZ-treated groups (except EODF STZ group which received melatonin). Results were obtained through comparison of the STZ-treated groups with control groups on day 3.

Figure 3 indicates mean swimming velocity of rats in all groups on days 1, 2 and 3 of Morris water maze test. One-way ANOVA test showed no significant differences between groups in terms of mean swimming velocity.

Frequency of entrance into platform and its proximity during probe trial have been shown in figure 4. One-way ANOVA test showed significant differences between groups in latency to proximity on day 4 in probe trial ( $P < 0.001$ ).

Post-hoc Tukey's tests followed by one-way ANOVA showed significant differences on day 4, in probe test between STZ-treated and control groups. However, no significant differences were observed among STZ-treated groups. According to such

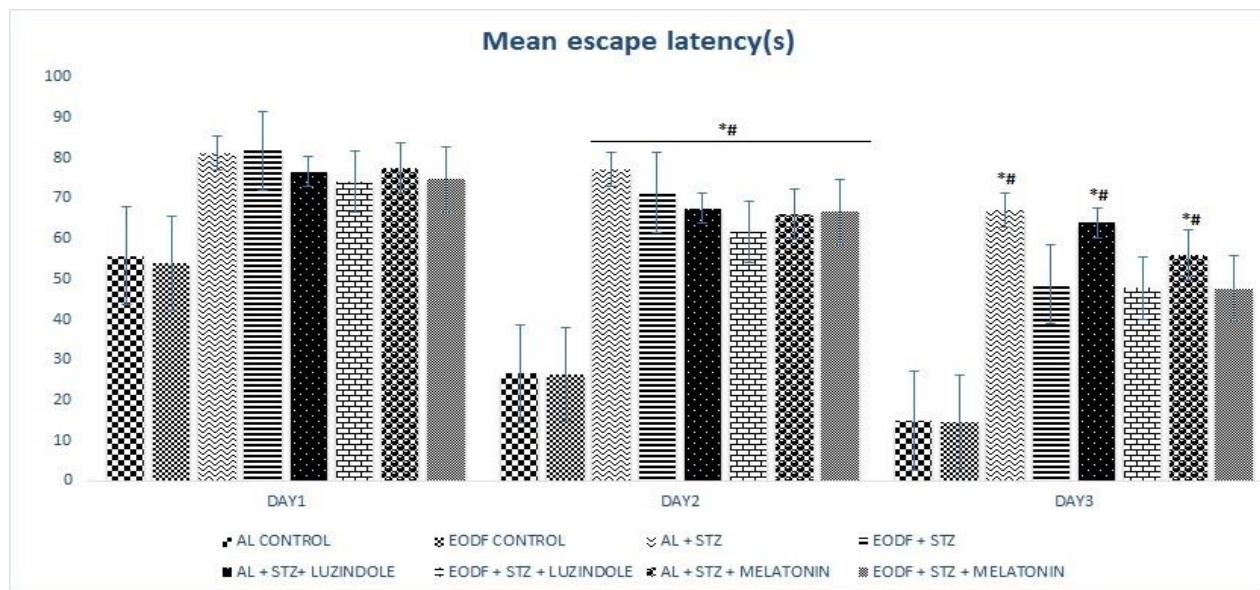
findings, EODF diet, luzindole and melatonin did not appear to have any effect on improvement STZ-induced memory impairment.

### Histopathological changes

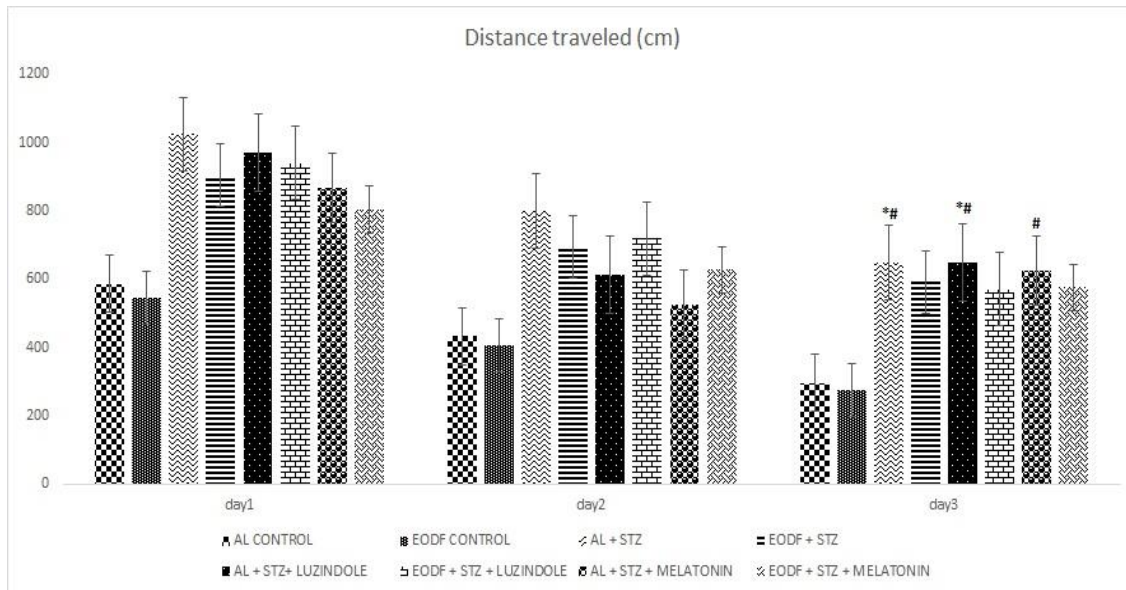
Counting dead neurons in CA1 part of hippocampus indicated that EODF diet accompanied by melatonin may reduce destructive effects of STZ. Figure 5 shows percentage of dead neurons in CA1 area in all groups. One-way ANOVA test showed a significant difference between groups in terms of percentage of dead neurons ( $P < 0.001$ ).

Post-hoc Tukey's after one-way ANOVA test showed a significant differences between STZ-treated groups and also between STZ-treated and control groups.

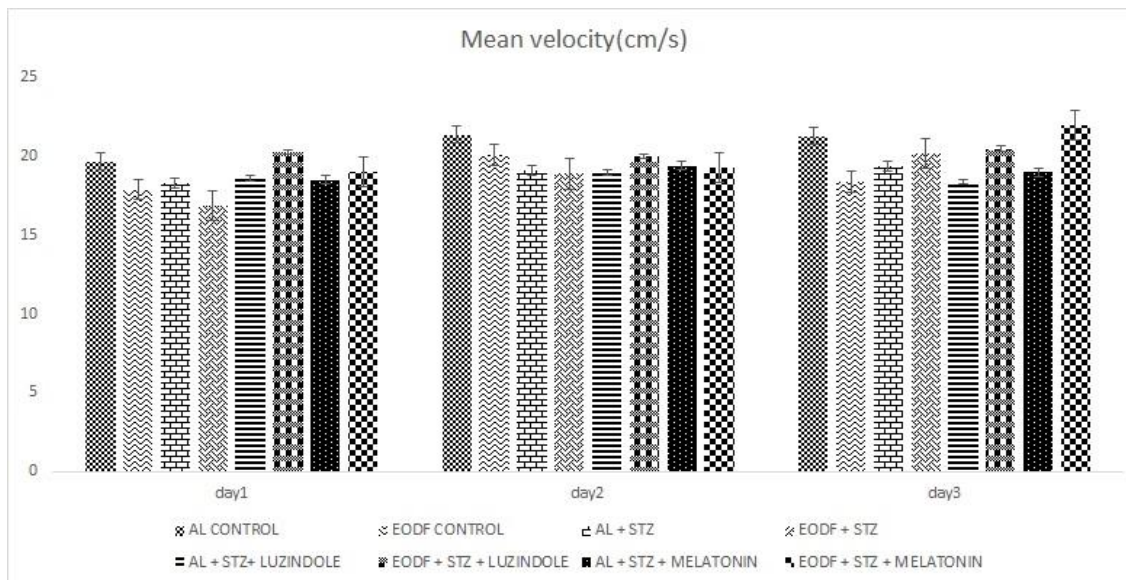
Histological study showed that streptozotocin can destroy diffuse parts of brain tissue including paraventricular area, fornix, cortex and hippocampus (Figures 6 and 7).



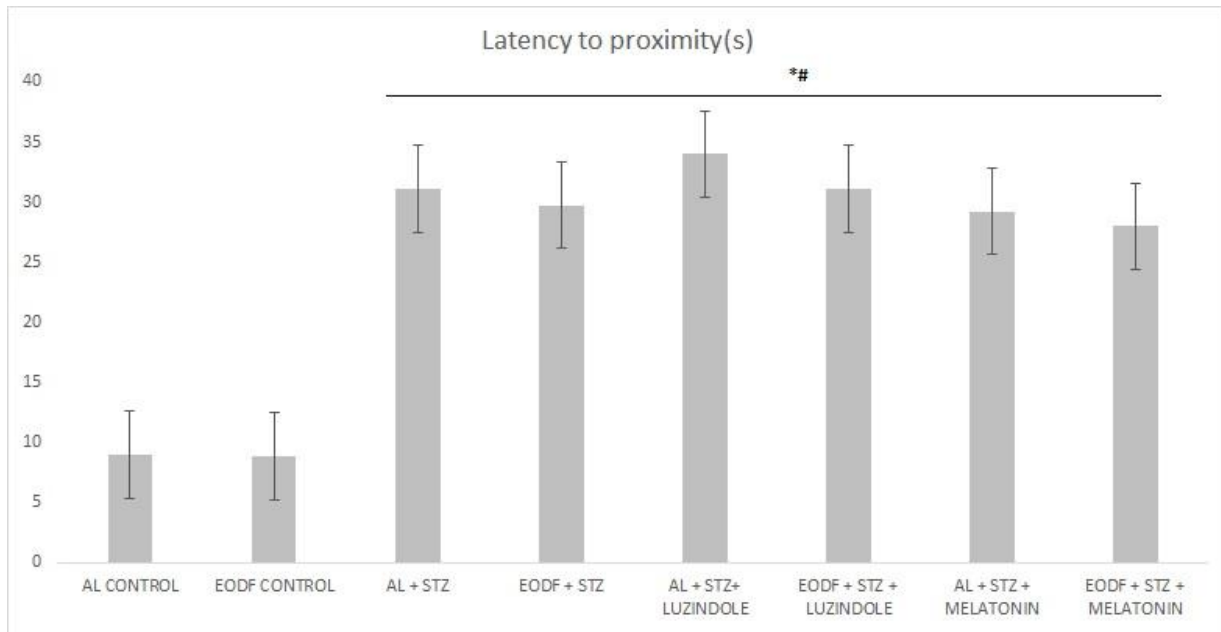
**Figure 1.** The escape latency to the hidden platform during days 1–3 of training. Data are represented as mean  $\pm$  S.E.M. \* represents the difference between STZ-treated groups and Ad libitum control group (AC)  $P < 0.05$ . # represents the difference between STZ-treated groups and EODF control group (EC)  $P < 0.05$ .



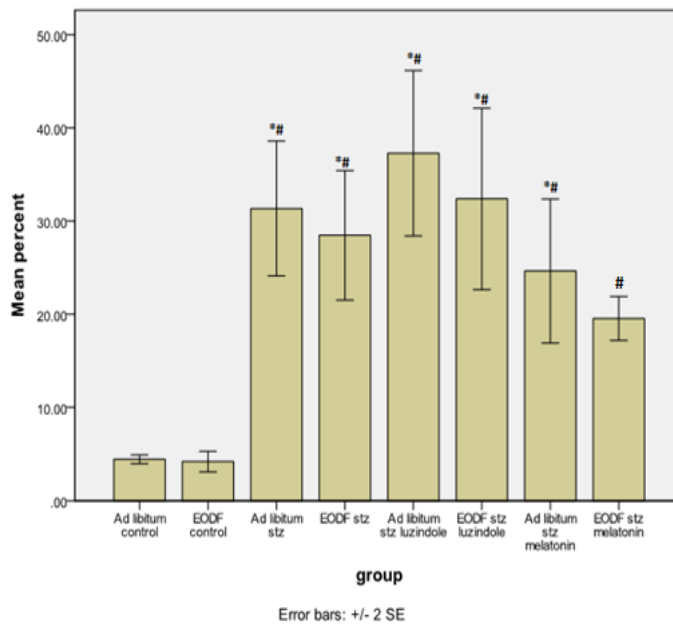
**Figure 2.** Distance traveled to reach the hidden platform within 1 to 3 days of training. Data are represented as mean  $\pm$  S.E.M. \* represents the difference between STZ-treated groups and Ad libitum control group (AC)  $P < 0.05$ . # represents the difference between STZ-treated groups and EODF control group (EC)  $P < 0.05$ .



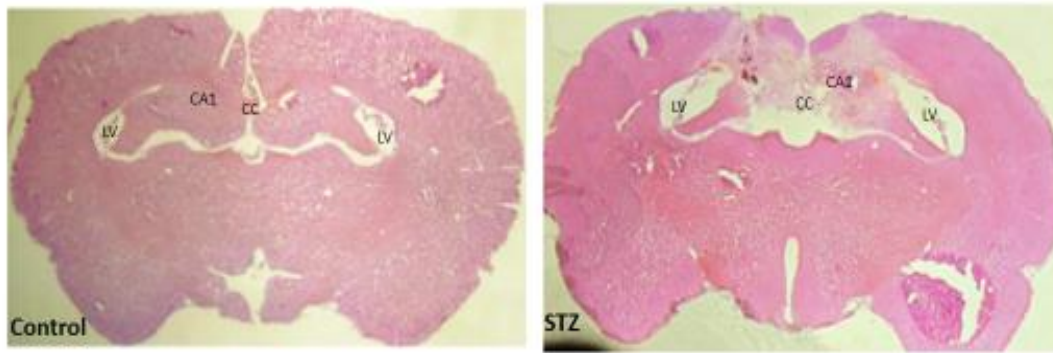
**Figure 3.** Mean swimming velocity in all groups. Data are represented as mean  $\pm$  S.E.M. Results did not show any significant difference between groups.



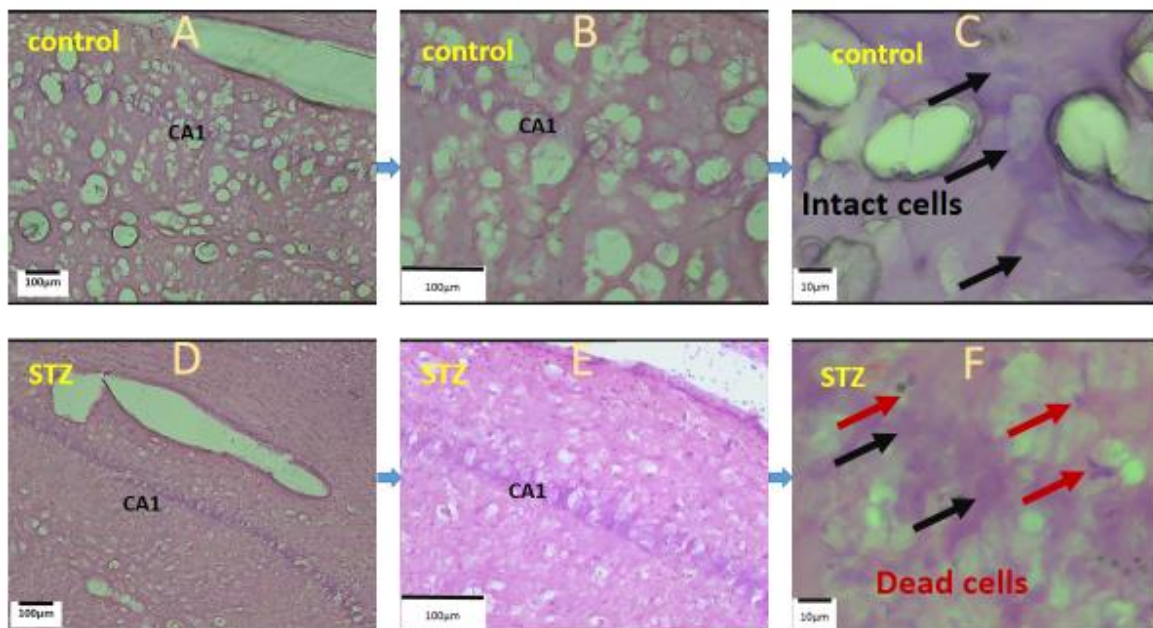
**Figure 4.** The frequency of entrance into platform and its proximity during probe trial in all groups. Data are represented as mean  $\pm$  S.E.M. \* represents the difference between STZ-treated groups and Ad libitum control group (AC)  $P < 0.05$ . # represents the difference between STZ-treated groups and EODF control group (EC)  $P < 0.05$ .



**Figure 5.** The percent of dead neurons in CA1 part of the hippocampus in all groups. Data are represented as mean  $\pm$  S.E.M. \* represents the difference between STZ treated groups and Ad libitum control group (AC)  $P < 0.05$ . # represents the difference between STZ-treated groups and EODF control group (EC)  $P < 0.05$ . the difference between Ad libitum STZ luzindole group (ASL) and EODF STZ melatonin group (ESM) was significant ( $P = 0.015$ ).



**Figure 6.** The comparison coronal sections of the brain. In macroscopic view of the brain, destructive effects of STZ are evident. Accordingly, STZ can destroy wide brain areas including periventricular zone, cortex, fornix, corpus callosum and the hippocampus in particular. LV (lateral ventricle), CC (corpus callosum).



**Figure 7.** The comparison of dead vs. normal neurons in the CA1 part of the hippocampus. Black arrows show normal neurons while red arrows point at dead neurons in control (A, B, and C) and STZ-treated groups (D, E, and F).

## Discussion

In this study, results of Morris water maze test demonstrated that intra cerebroventricular administration of STZ can impair spatial memory and create a proper model of sporadic Alzheimer's disease. Histological evaluation also demonstrated destructive effects of STZ on different parts of the brain, especially CA1 region of the hippocampus. The ICV injection of STZ disintegrates the tissue in the paraventricular regions and causes inflammation and cell death in wide areas of the brain. These effects can impair learning and

memory. Such effects had been similarly observed in other studies (25).

The first objective of this study was to investigate the effect of 10 days every-other-day fasting (EODF) on spatial memory of rat model of Alzheimer's disease. Morris water maze test results, on day 3, indicated a tendency to improve the spatial memory in EODF STZ- treated groups, although these changes was not significant compared to Ad libitum STZ- treated groups. In probe test on day 4, no significant difference between STZ-treated groups was noted. Results from our behavioral study indicated that 10 days EODF had no significant

effect on spatial memory of control and STZ-treated rats.

The second objective of this study was to evaluate the role of endogenous melatonin in dietary restriction effects on memory impairment induced by STZ. Since measurement of melatonin level changes was hardly practical, we decided to use luzindole, an antagonist of melatonin receptors to pursue this objective. According to our findings, ICV administration of luzindole 50 µgr/kg in initial hours of dark cycles (this dose had been shown can disrupt circadian rhythms or vanish neuroprotective effect of melatonin (23, 26-27) did not have any effect on spatial memory of STZ-treated rats. Likewise, the tendency toward improvement of spatial memory in EODF-STZ treated rats did not change by luzindole. In summary, it might be concluded that endogenous melatonin have no significant role in dietary restriction effects at least at melatonin receptors level.

The third objective of this study was to evaluate of effect of exogenous melatonin on spatial memory in STZ induced memory impairment rats. Melatonin 10 mg/kg/i.p were given 10 days from one day after initial dose of STZ (This dose had been shown can improve function of STZ treated rats in Morris water maze test) (22). In this study, it was observed that administration of melatonin did not have significant effect on spatial memory impairment in STZ-treated rats. This observation was against a previous study claiming that exogenous melatonin can improve spatial memory in STZ-treated rats (20-22).

The fourth objective of this study was to investigate the effects of simultaneous administration of two presumably neuroprotective interventions (EODF diet and exogenous melatonin) on spatial memory impairment in a rat model of Alzheimer's disease. This intervention did not significantly change the spatial memory in STZ-treated rats in Morris water maze test. However, histological studies demonstrated some neuroprotective effects of the same. The statistical analysis showed a significant difference in CA1 area dead neurons between the EODF STZ + melatonin group (ESM) and Ad libitum STZ+ luzindole group (ASL).

On the other hand, studies have shown that EODF diet can reduce escape latency in Morris water maze test bearing neuroprotective effects in transgenic mouse model of Alzheimer's disease, (3). Such neuroprotective effects have been demonstrated in some earlier reports (1, 2, 4, 5, 7, 28-30]. The precise mechanisms mediating these beneficial effects of diet restriction are still unresolved though many hypotheses have been considered so far. Recently, it has been said that dietary restriction can reset circadian rhythms and

regulate irregular rhythms, hence regulate the physiological function and thereby cause wellbeing (15-16).

Melatonin plays an important role in circadian system (17). Previous studies have shown that dietary restriction may change melatonin production and secretion and can also shift the production and secretion time of melatonin when the SCN clock is lacking (18). Owing to the well-described neuroprotective effects of melatonin in some studies (19-23) and the fact that melatonin level is declined in aging and specially in Alzheimer's (17), there seem to be at least some dietary restriction effects related to melatonin level changes. To investigate such a hypothesis, melatonin receptors (MT1, MT2) antagonist (luzindole) was used. As such, we investigated the effect of intermittent fasting in STZ induced model of Alzheimer's disease. Meanwhile, the role of melatonin and melatonin accompanied by EODF diet in dietary restriction effects in this model of Alzheimer's were investigated.

In summary, results of this study showed that 10 days EODF diet was unable to make any significant effect on spatial memory in STZ model of Alzheimer's disease. Morris water maze test data showed that STZ can impair spatial memory in rats and dietary restriction could not improve memory impairment significantly. Similarly, melatonin and luzindole failed to change memory impairment. Histological study indicated that STZ destroys the brain tissue in different areas including paraventricular zone, fornix, cortex and hippocampus which are attributed to memory function. The numerical density of dead neurons in CA1 part of the hippocampus showed that dietary restriction accompanied by exogenous melatonin with supra physiological dose, may partially restore destructive effects of STZ in the brain.

## **Conclusion**

Melatonin seems to bear no important role in the effects of dietary restriction on spatial memory impairment. Meanwhile, dietary restriction plus exogenous melatonin might exert some neuroprotective effects.

## **Suggestions**

Although in the STZ model of Alzheimer's disease, the role of endogenous melatonin in dietary restriction effects on spatial memory impairment was insignificant, the use of other neurodegenerative models, other behavioral tests, different molecular and genetic evaluations, or



increasing the duration of diet limitation may provide further insights into this question.

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