

## Effects of Melatonin in Prevention of Neuropathy in STZ-Induced Diabetic Rats

<sup>1,2</sup>Nasser Zangiabadi, <sup>1</sup>Vahid Sheibani, <sup>1</sup>Majid Asadi-Shekaari,  
<sup>1</sup>Mohammad Shabani, <sup>1</sup>Mandana Jafari, <sup>2</sup>Ali Reza Asadi,  
<sup>1</sup>Haleh Tajadini and <sup>1</sup>Morteza Jarahi  
<sup>1</sup>Neuroscience Research Center,  
Kerman University of Medical Sciences, Kerman, Iran  
<sup>2</sup>Afzal Research Center, Kerman, Iran

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**Abstract: Problem statement:** Diabetes mellitus occurs mainly with chronic polyneuropathy, and oxidative stress plays an important role in emergence of most neurologic and behavioral changes in diabetic patients. Many studies have focused on the beneficial effects of various antioxidants such as melatonin on diabetic neuropathy. The aim of this study is to evaluate the effect of melatonin in prevention of neuropathy in Streptozotocin-induced diabetic rats. After prescribing Streptozotocin (STZ), treatment rats received melatonin (10 mg kg day<sup>-1</sup>) or DMSO for a period of 6 weeks. **Approach:** At the end of the sixth week, non diabetic control group, diabetic control group (sham) and treated rats were examined by thermal pain response tests (hot plate and tail flick). The horizontal and vertical activities of rats were measured in an open field test. After that, Motor Nerve Conduction Velocity (MNCV) of sciatic-tibial nerve recorded. Also, to study morphological alterations resulting from diabetic neuropathy of sciatic nerve, Myelinated Fiber Diameter (MFD), Axon Diameter (AD) and Myelin Sheath Diameter (MSD) were evaluated by light microscope. **Results:** According to hot plate results, response time to thermal pain at the end of sixth week in sham group showed a significant decrease in comparison with the control group ( $p < 0.01$ ). In hot plate test, although melatonin approximated to the response time to control group, the significant difference was not observed among melatonin receivers and other groups. In the open field test, Total Distance Moved (TDM) and mobility duration showed significant decrease in sham and DMSO groups in comparison to the control and melatonin groups. Diabetic rats treated with melatonin showed significant increase in MNCV compared to sham and DMSO groups ( $p < 0.05$ ). In morphological study, pretreatment with Melatonin significantly reversed sciatic nerve diameters (MFD, AD, and MSD) reduction in diabetic rats. Electron microscopy showed myelin splitting and myelin sheath infolding in diabetic control group compare to non diabetic group. **Conclusion:** This study showed that melatonin can decrease the destructive progress of diabetes and causes neuroprotection against damages resulting from STZ-induced hyperglycemia.

**Key words:** Sciatic nerve, Axon Diameter (AD), Myelinated Fiber Diameter (MFD), Motor Nerve Conduction Velocity (MNCV), diabetic rats, Reactive Oxygen Species (ROS)

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### INTRODUCTION

Hyperglycemia in diabetic patients as the main factor of diabetic neuropathy induces oxidative stress through various cellular pathways such as increasing aldose reductase activity (Srivastava *et al.*, 2005), increasing glycation end-products (Sugimoto *et al.*, 2008) and altering protein kinase C activity (Yamagishi *et al.*, 2008). Long-standing hyperglycemia through producing a large amount

of Reactive Oxygen Species (ROS) can damage mitochondrial DNA in dorsal root ganglia leading to peripheral nerves dysfunction (Schmeichel *et al.*, 2003). Several studies have proposed that oxidative stress is one of the major factors impairing sensory nerves and dorsal root ganglia (Arora *et al.*, 2008; Sharma *et al.*, 2009). Today many studies have focused on the beneficial effects of various antioxidants such as melatonin on diabetic neuropathy (Zangiabadi *et al.*,

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**Corresponding Author:** Nasser Zangiabadi, Neuroscience Research Center, Kerman University of Medical Sciences, Kerman, Iran, P.O. Box: 76198-13159 Tel: +98-341-226-4198/+98-913-3978116

2007; Negi *et al.*, 2010; Srinivasan *et al.*, 2010). Melatonin is one of the tryptophan derivatives that is mainly synthesized in the pineal gland and also in retina, intestine, ovary, testis and bone marrow (Pevet *et al.*, 1980; Gutierrez-Cuesta *et al.*, 2007; Geoffriau *et al.*, 1998). The physiologic importance of melatonin has not been recognized yet and its intervention in circadian rhythms has already been proposed as the most important function of this hormone. Melatonin in addition to physiologic functions such as controlling day-night rhythm, inducing sleep, seasonal regulation of reproduction and increasing immune system activity has been known as a powerful biologic antioxidant in neutralizing free radicals (Tan *et al.*, 2007; Hardeland and Pandi-Perumal, 2005). Moreover, some psychopharmacological effects like sedative, anti-epileptic and anti-anxiety effects have been proposed for melatonin (Geoffriau *et al.*, 1998). It has been found in some behavioral studies that melatonin has a prolonged and severe antinociceptive effect in neuropathic pains via interaction with opioidergic and GABAergic system receptors (Golombek *et al.*, 1996; Mantovani *et al.*, 2003). Although, several studies have mentioned melatonin role in the treatment of neuropathy due to diabetes (Negi *et al.*, 2010; Srinivasan *et al.*, 2010; Arreola-Espino *et al.*, 2007), there are few studies about its effect on prevention of diabetic neuropathy. Therefore, the present study was designed to investigate the protective effects of melatonin in prevention of neuropathy in Streptozotocin-induced diabetic rats.

## MATERIALS AND METHODS

This study was performed on Wistar rats weighing 200-250gr. The care of laboratory animals followed the guiding principles for care and use of laboratory animals of the Neuroscience Research Center of Kerman Medical University and the study protocol was approved by the animal ethics committee of this institution (Code:EC/KNRC/88-15).

Animals were kept in standard conditions, temperature of  $22\pm 2^{\circ}\text{C}$  and 12-h dark-light cycle. They had free access to food and water. These rats were divided into four groups: First group consisted of control animals. The second, third and fourth groups consisted of diabetic animals. The second group was as diabetic control group (sham). The third and fourth groups treated with melatonin ( $10\text{ mg kg day}^{-1}$ , i.p.) and DMSO, at least 8 rats in every group. Diabetes was induced by intra-peritoneal injection of  $45\text{ mg kg}^{-1}$  Streptozotocin. In order to confirm diabetes, three days after Streptozotocin injection blood glucose was

measured using glucometer instrument (Accu-check-active, ROCHE, Germany) and animals with blood glucose of over  $200\text{ mg dL}^{-1}$  were considered as diabetics. In order to investigate the effect of melatonin in prevention of neuropathy, melatonin injection ( $10\text{mg kg day}^{-1}$ ) started 4 days after STZ injection and confirmation of diabetes and continued for 6 weeks.

**Hot plate test:** In Hot Plate Test, pain sensitivity was evaluated by using an apparatus (LE710 model, Lsi LETICA, Spain) that contained a plate with the diameter of 19cm and a Plexiglas wall with height of 30cm. Plate temperature was adjusted to  $52\pm 2^{\circ}\text{C}$ . Response time to thermal pain was considered as the time between test onset and licking front paw or jumping (maximum cut off was considered 60 sec); (Sharma *et al.*, 2007).

**Tail flick:** Tail flick test is one of the standard tests for measuring analgesia amount. In this test, thermal light was shown to the end of tail of rats with intensity of 5 using tail flick machine (made by Spanish Lsi LETICA, model LE7406) and tail flick latency was measured from the light emission to take the tail (on second). To prevent tissue damage, maximum light mission to the tail was considered 10 sec. For each animal rat, tail flick latency was measured for three times and the average was reported as tail flick latency. Between every test, rats were left free for half an hour (Liepinsh *et al.*, 2009).

**Open field test:** This test was used to investigate the effect of diabetic neuropathy on explorative behavior of diabetes-induced rats and the protective effect of melatonin. Explorative behavior of rats was studied by a video Track System (TSE) in a  $45\times 45\times 45\text{cm}$  black wood box. At the end of the 6th week following diabetes induction, animals were placed in the center of field and their explorative behavior including the distance in center and border areas and time spent in center and border areas as well as the frequency of grooming and rearing were detected for 5 min (Shabani *et al.*, 2011).

**Electrophysiological evaluation:** Six weeks after initiation of hyperglycemia, animals were anesthetized by injection of Ketamin/Xylozine solution ( $50/20\text{ mg kg}^{-1}$ , i.p). The environment temperature was maintained at  $25\pm 1^{\circ}\text{C}$  during all stages of study. After shaving the animals' back, a small incision was made in right sciatic notch and ankle. Then, by bi-polar electrodes, proximal part of sciatic notch and distal part of ankle were stimulated and after stimulation, Motor Nerve Conductivity Velocity (MNCV) of sciatic-tibial was recorded (powerlab/ML856). Immediately after each

stimulation, action potential of first interosseous muscle of the hind paw was recorded by uni-polar electrodes. The obtained records are biphasic responses with a primary M-wave produced due to the stimulation of motor fibers (Kumar *et al.*, 2007; Saini *et al.*, 2007). MNCV was calculated by dividing the distance between the two stimulated sites (mm) by the difference between proximal and distal latencies (ms).

**Morphological study of sciatic nerve:** For morphometric evaluation, sciatic nerve was isolated and divided into 2 mm segments. They were fixed immediately with glutaraldehyde solution 2.0% in 0.1 M Phosphate Buffer Solution (PBS) for 24 h. The specimens were washed with PBS for 3 times and post fixed for 1.5 h in 1% tetroxide osmium and dehydrated in graded concentration of ethanol and finally embedded in Epoxy resin. Semithin sections (350 nm) were stained with 1% toluidin blue and examined by light microscopy. Ten fields of transverse sections were morphometrically analyzed by computerized image analysis system (Motic Images China e-kup Co., Ltd). MFD, AD and MSD were measured for each section.

For ultrastructural study, ultrathin sections (80 nm) were stained with 1% uranyl acetate and 2% lead citrate before viewing in Philips TEM300 (Philips, Eindhoven, Netherlands).

**Statistical analysis:** Data are presented as mean $\pm$ SEM. One-way ANOVA was used in order to determine statistical difference and in the case of significant difference, Tukey test was used to determine the difference among the groups. LSD test was used in morphological study and  $p < 0.05$  was considered as statistical significant level.

## RESULTS

**Metabolic characteristics:** Three days after STZ injection, Plasma glucose showed significant increase in diabetic groups as compared to the control group (Control:  $106.2 \pm 4.1$  mg dL<sup>-1</sup>, Diabetic rats:  $318.9 \pm 10$ ,  $p < 0.001$ ). There was no significant difference among groups in regard to the primary weight. Weight gain of diabetic rats at the end of the 7th week was significantly less than that of the control group. That is, in the control group weight had increased at the end of the 7th week compared to the 1st week, while in diabetic groups of melatonin and DMSO it had decreased (Table 1).

**The protective effect of melatonin in response to thermal pain:** According to the results of hot plate test, response time to thermal pain in the 7th week following

diabetes induction, showed significant decrease in sham group as compared to the control ( $p < 0.001$ ), melatonin ( $p < 0.05$ ) and DMSO groups ( $p < 0.001$ ). Although in melatonin group, response time to thermal pain showed significant increase ( $p < 0.05$ ) compared to the sham group and was close to that in the control group, it was significantly lower ( $p < 0.05$ ) in comparison to the control group. It seemed that in this test, melatonin solvent (DMSO) has better protective effects than melatonin in response to thermal pain. In evaluation of response to thermal pain, there was no significant difference among groups in tail flick test (Fig. 1).

**The effect of melatonin on explorative behavior in open field test:** In open field test performed to find the effect of diabetic neuropathy and melatonin treatment on rats' explorative behavior, frequency of rearing and grooming in all diabetic groups (melatonin, sham and DMSO) showed significant decrease compared to the control group ( $p < 0.05$ ). The total distance in central and border areas (TDM) and mobility duration showed significant decrease ( $p < 0.05$ ) in diabetic sham and DMSO groups in comparison to the control and melatonin groups, while there was no significant difference between melatonin and control groups in this regard. There was no significant difference between the control group and none of the diabetic groups of sham, DMSO and melatonin in regard to the time spent in central and border areas and also immobility duration and mobility speed in central and border areas (Fig. 2).

**The effects of melatonin on MNCV in diabetic rats:** At the end of the 7th week, MNCV showed significant decrease in diabetic groups of sham and DMSO compared to the control group ( $p < 0.05$ ). Diabetic rats treated with melatonin for 6 weeks had no significant difference with the control group in MNCV, while they showed significant increase in MNCV compared to sham and DMSO groups ( $p < 0.05$ ) (Fig. 3).

**Morphometry:** Light microscopy study of sciatic nerves sections showed that normal structure and morphology of myelinated fibers in control group. In Sham and DMSO groups, some abnormalities including increased numbers of mast cells, edema and myelin sheath splitting were seen (Fig. 4). Increasing in number of abnormal myelinated fibers was observed in Sham and vehicle groups. Pretreatment of rats with Melatonin prevented all of these abnormalities in high extent. The mean of MFD and AD were  $9.75 \pm 0.45$  ( $\mu$ m) and  $5.11 \pm 0.34$ ,  $8.67 \pm 0.3$  and  $4.2 \pm 0.5$ ,  $8.25 \pm 0.41$  and  $3.73 \pm 0.22$ ,  $8.15 \pm 0.19$  and  $4.09 \pm 0.15$  in Control, Melatonin, DMO and Sham respectively.

Table 1: Body weight and blood glucose levels of all groups

Animal group	Body weight (g)		Blood glucose (mg dL <sup>-1</sup> ) Before sacrifice
	Before STZ injection	End experiment	
Control (8)	265±4.22	286.25±5.32	106.25±4.16
Sham (9)	261.25±2.95	220±5.01****	306.25±19.45****
DMSO (8)	269.7±5.3	238.5±5.6****	365±29.8****
Melatonin (11)	265±4.2	246.2±4.8***†	270.7±25.7****

Data are the mean ± SEM \*\*\*:p<0.001, \*\*\*\* p<0.0001, compared with the control group; †:p<0.05, compared with the sham group

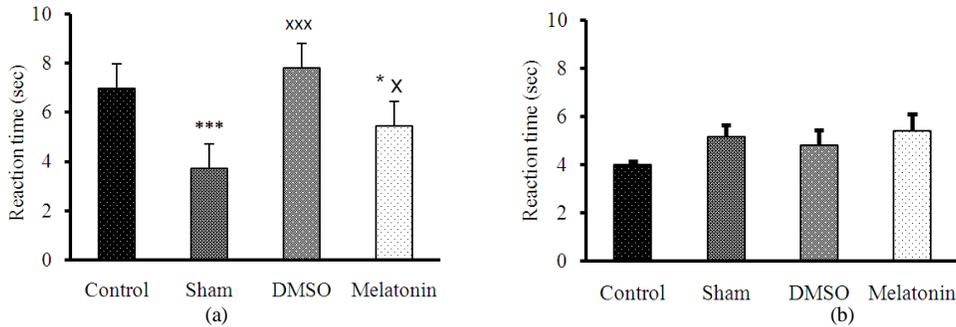


Fig. 1: Effect of Melatonin (10 mg kg day<sup>-1</sup>, ip, for 6 weeks) on the pain threshold values in streptozotocin-injected diabetic rats subjected to hot plate (A) and tail flick (B). \* P<0.05, \*\*\* P<0.001 as compared to control group, x P<0.05, xxx P<0.001 as compared to Sham group. There are no significance effects between DMSO and melatonin -treated diabetic groups. Values are expressed as mean±SEM. (n=8 rats in each group)

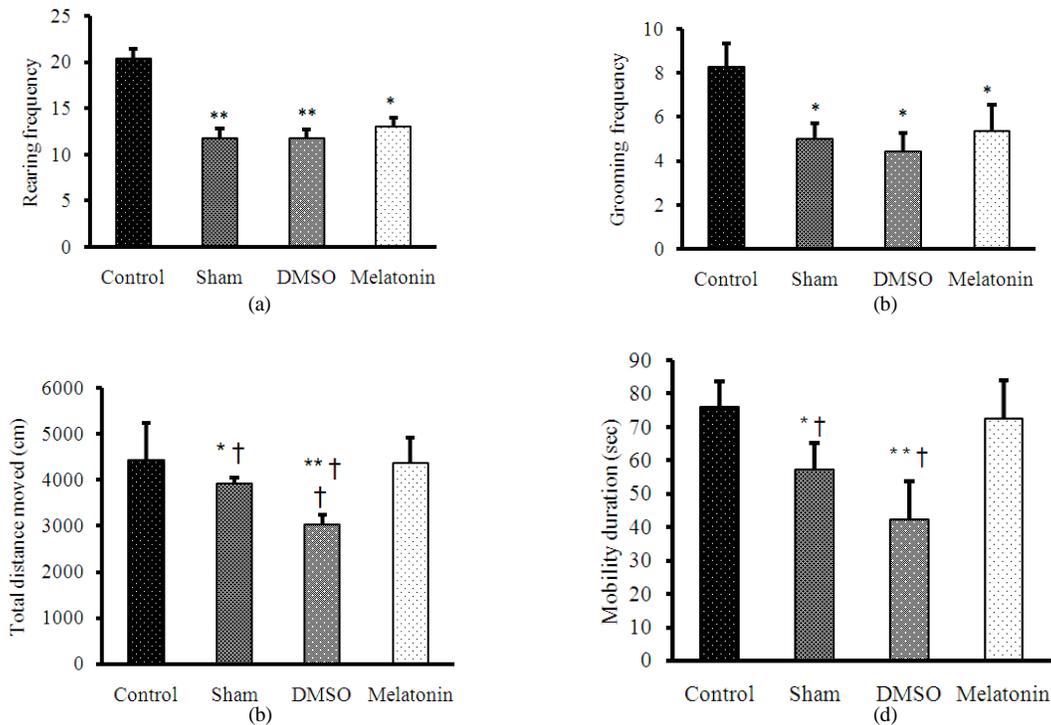


Fig. 2: Effect of Melatonin on explorative behavior of rats in open field test. Rearing frequency (a), Grooming frequency (b), TDM (c) and Mobility duration (d), \* p<0.05, \*\* p<0.01 compared with the control group, † p<0.05, †† p<0.01, compared with the Melatonin group. Values are expressed as mean ± SEM. (n=8 rats in each group)

Table 2: The effect of Melatonin on histomorphometric parameters of rat sciatic nerve

Groups	N	MFD ( $\mu\text{m}$ )	AD ( $\mu\text{m}$ )	MSD ( $\mu\text{m}$ )
Control	4	9.75 $\pm$ 0.45	5.11 $\pm$ 0.34	4.64 $\pm$ 0.35
Sham	4	8.15 $\pm$ 0.19*	4.1 $\pm$ 0.15*	4.06 $\pm$ 0.23
DMSO	4	8.25 $\pm$ 0.41*	3.73 $\pm$ 0.22*	4.52 $\pm$ 0.33
Melatonin	5	8.67 $\pm$ 0.3	4.2 $\pm$ 0.5	4.65 $\pm$ 0.30

N: number of animals. MFD: Myelinated Fiber Diameter, AD: Axon Diameter, MSD: Myelin Sheath Diameter. Data are presented as Mean $\pm$ SEM. \*p<0.05 Vs. control

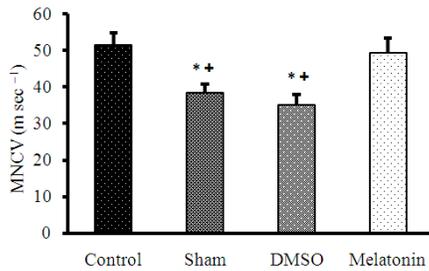


Fig. 3: Effect of melatonin on motor nerve conductive velocity (MNCV) in rats. Data are the mean  $\pm$  SEM. \*: p<0.05 compared with the control group; †: p<0.05, compared with the date extract group

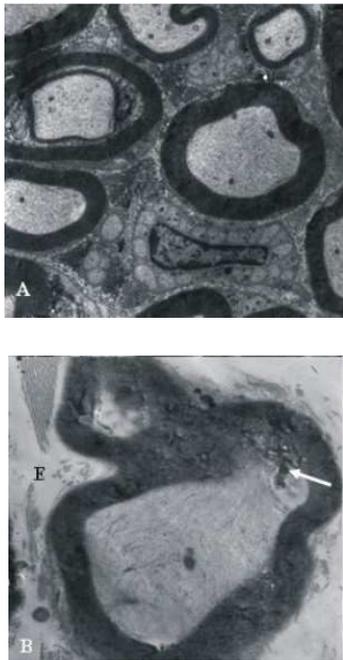


Fig. 4: Electron micrograph of rat sciatic nerves. (a) Control group: Myelinated fiber with normal structure and morphology. Some unmyelinated fibers are seen in bottom the picture. (b) Sham group: Myelinated fiber shows myelin infolding and splitting (white arrow). Edema (E) is clear in the field. Axonal degeneration was revealed with neurofilament disintegration. A $\times$ 8900 B $\times$ 11500

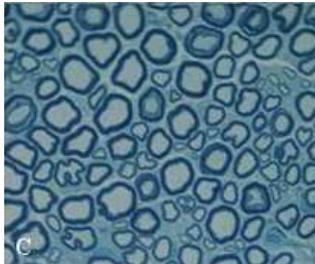
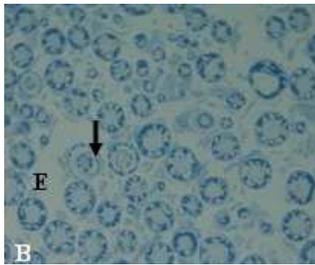
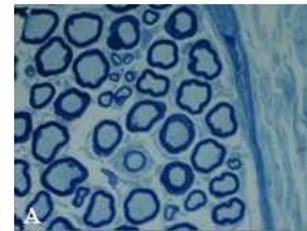


Fig. 5: Light micrograph of transverse semi-thin sections of rat sciatic nerves. (a): Control group, myelinated nerve fibers are in normal structure and morphology. (b): Sham group, nerves fibers show some abnormalities such as myelin splitting (black arrow), and Edema (E), (c): The DFE-treated group, the proportion of nerve fibers with abnormalities was reduced.  $\times$ 1000. Toluidin blue staining

According to the data, MFD and AD of DMSO and Sham groups were decreased comparison to control group. Pretreatment with Melatonin for 6 weeks significantly reversed each diameter reduction in diabetic rats (Table 2).

**Electron microscopy:** Ultrastructural examination of sciatic nerves confirmed the light microscopy findings

(Fig. 5). In addition, some evidences of axonal degeneration such as mitochondrial swelling and disintegration of neurofilaments were observed under transmission electron microscope. Degenerative, reactive and proliferative of Schwann cells were not observed in any groups.

## DISCUSSION

It was observed in the present study that intra-peritoneal injection of 10 mg kg<sup>-1</sup> melatonin has protective effects against peripheral nerves injury due to diabetic neuropathy. Decrease of motor nerve conductivity velocity in STZ-induced diabetic rats is an evidence of diabetic neuropathy development in these rats. In the present study, we observed 25.6% defect in MNCV at the 7th week following diabetes induction in the sham group compared to the control group. While, melatonin after diabetes induction prevented diabetes destructive effects on nerve conductivity in sciatic-tibia motor nerve.

Peripheral neuropathy is one of the major complains in both types I and II diabetic patients and it is associated with several problems such as cardiovascular defects, retinopathy and muscular pain or weakness (Yagihashi *et al.*, 2007; Sima, 2003; Rajbhandari and Piya, 2005). Since these defects affect the quality and quantity of life, treatment of diabetic neuropathy or prevention of its accompanying symptoms has been considered as a major goal. Although several therapeutic interventions and various herbal and chemical medications have already been suggested for the prevention of diabetes complications, their exact effectiveness as reliable preventive medications for diabetic neuropathy has not been considered seriously yet. Melatonin is one of the substances that its therapeutic effects on decreasing complications of diabetes have been reported in some studies. In the present study, preventive effects of melatonin on occurrence of complications such as muscle weakness, nerve tissue injury and decrease of MNCV in diabetic rats were observed.

MNCV at the end of the 7th week following diabetes induction had significant decrease ( $p < 0.05$ ) in the diabetic groups of sham and DMSO compared to the control group. While, i.p. injection of melatonin after diabetes induction for 6 weeks prevented diabetes destructive effects on nerve conductivity in sciatic-tibia motor nerve.

Oxidative stresses increase and mitochondrial dysfunction are among pathophysiologic causes in neurodegenerative diseases such as Alzheimer, Parkinson and diabetes-induced peripheral neuropathy and the protective effect of melatonin in some of these neurodegenerative diseases has been proved (Tunez *et al.*,

2003; Leon *et al.*, 2004; Srinivasan, *et al.*, 2006). Melatonin with regard to its various roles in relation to free radicals scavenging, regulation of oxidant and pro oxidant enzymes and also preventing the formation of mitochondrial radicals has a special importance in the treatment of neurodegenerative diseases (Srinivasan *et al.*, 2006). There are several studies showing induction of oxidative stress due to the increase of free radicals in hyperglycemic conditions (Kumar *et al.*, 2007; Saini *et al.*, 2007). Vascular defect following oxidative stress decreases blood flow and causes hypoxia and decrease of nerve function leading eventually to MNCV reduction (Saini *et al.*, 2007; Yorek *et al.*, 2004).

MNCV decrease can be resulted from the change of nerve fiber diameter or myelin layer defect (Yorek *et al.*, 2002; Azcoitia *et al.*, 2003). In line with study Azcoitia *et al.* (2003) and Yorek *et al.* (2002) study, our study showed that STZ administration increases morphological changes of myelin layer (sheath) in sciatic nerve. MFD and AD in diabetic groups of sham and DMSO showed significant decrease compared to the control group, while melatonin prevented morphological changes of sciatic nerve in diabetic rats. Sciatic nerve injury associated with morphologic changes was confirmed by explorative behavior reduction and decrease of motor activity in Open field Test, while melatonin administration following decrease of tissue changes prevented significant changes of explorative behavior in diabetic rats. Zencirci *et al.* (2010) showed that 21 days treatment with 5 and 21mg kg<sup>-1</sup> melatonin causes increase in nerve conductivity velocity and decrease of latency in injured sciatic nerve of diabetic rats (Zencirci *et al.*, 2010). Sciatic nerve injury and increase in response to thermal nociception stimulations in diabetic neuropathy reported in several studies can be the reason of rats' motor activity and explorative behavior reduction. Therefore, decrease of sciatic nerve defect following melatonin administration in previous studies and neuroprotective effects of melatonin in regard to sciatic nerve injury in our study can explain motor activity improvement in diabetic rats following melatonin administration. Some studies have reported that MNCV decrease in diabetes is due to alterations of Na-K ATPase pump activity and sodium gradient (Gerbi *et al.*, 1999; Negi *et al.*, 2010). Alteration of Na-K pump activity can be attributed to the tissue injuries and metabolism abnormality following hyperglycemia in diabetic patients. Decrease of nerve blood flow in diabetic neuropathy leads to nerve metabolic abnormality and consequently defect of ATP-sensitive ion exchanger pumps like Na-K pump. Defect of Na-k pump is finally led to the membrane disability in preserving resting potential and consequently disturbs nerve conductivity (Negi *et al.*,

2010; Gerbi *et al.*, 1999). There are several studies indicating beneficial effects of some medicines and herbal compounds in the improvement of Na-K pump activity and consequently returning MNCV into the normal rate. Oner *et al.* (2002) have reported that pretreatment with  $10\text{mg kg}^{-1}$  melatonin prior to the administration of ethanol prevents ethanol-induced Na-K ATPase and  $\text{Ca}^{2+}$ -ATPase inhibition in rat synaptosomes and causes neuroprotection against harmful effects of ethanol through this mechanism (Oner *et al.*, 2002). In human red blood cells, too, the moderating role of melatonin on Na-K ATPase activity and  $\text{Na}^{+}$ - $\text{H}^{+}$  exchanger has been recognized. This fact is related to the antioxidant effects and moderating role of melatonin on membrane fluidity (Bhatti *et al.*, 2011).

Peripheral neuropathy in primary stages is associated with elevation of nerve fiber activity and disturbs normal sensitivity of neurotic system to injuring and painful stimuli resulting in diabetic hyperalgesia (Malcangio and Tomlinson, 1998; Watson *et al.*, 2003). In the present study, too, in the sham group response time to thermal pain had significant decrease compared to the control group and caused a kind of diabetic hyperalgesia to the thermal pain, while DMSO as melatonin solution showed better protective effects in Hot Plate Test and caused an increase in response time to thermal pain compared to the sham and melatonin groups. Similar to the present study, Colucci *et al.* (2008) have asserted that DMSO has antinociceptive effects against thermal pain in Hot Plate and Tail Flick Tests. It seems that in studies that use DMSO as solution, these effects in relation to the characteristics of the administered medicines should be considered.

### CONCLUSION

Therefore, it seems that melatonin administration in early stages of diabetes induction prior to the neurotic damage and occurrence of diabetic neuropathy can decrease the destructive progress of diabetes and causes neuroprotection against damages resulting from diabetic hyperglycemia. Melatonin probably exerts its protective effects through several specified routes such as decrease of free radicals production, Cyclooxygenase inhibition, and moderating role on Na-K ATPase pump and also activating opioids receptors.

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