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Original article

Effects of Melatonin and Vitamin E on Peripheral Neuropathic Pain in Streptozotocin-Induced Diabetic Rats

*[1]Farrin Babaei-Balderlou, [1]Samad Zare, [1]Reza Heidari, [1]Farah Farrokhi

Abstract

Objective(s)
Previous studies have indicated that diabetes mellitus might be accompanied by neuropathic pain. Oxidative stress is implicated as a final common pathway in development of diabetic neuropathy. Pharmacological interventions targeted at inhibiting free radical production have shown beneficial effects in diabetic neuropathy. The aim of this study was to investigate and compare the possible analgesic effects of melatonin and vitamin E in diabetic rats.

Materials and Methods
This study was performed on 32 male Wistar rats divided into 4 groups: control, diabetic, melatonin-treated diabetic and vitamin E-treated diabetic. Experimental diabetes was induced by intraperitoneal streptozotocin (50 mg/kg) injection. Melatonin (10 mg/kg, i.p.) and vitamin E (100 mg/kg, i.p.) were injected for 2 weeks after 21st day of diabetes induction. At the end of administration period, pain-related behavior was assessed using 0.5% formalin test according to two spontaneous flinching and licking responses. The levels of lipid peroxidation as well as glutathione-peroxidase and catalase activities were evaluated in lumbosacral dorsal root ganglia.

Results
Formalin-evoked flinching and total time of licking were increased in both acute and chronic phases of pain in diabetic rats as compared to control rats, whereas treatment with melatonin or vitamin E significantly reduced the pain indices. Furthermore, lipid peroxidation levels increased and glutathione-peroxidase and catalase activities decreased in diabetic rats. Both antioxidants reversed the biochemical parameters toward their control values.

Conclusion
These results suggest that oxidative stress may contribute to induction of pain in diabetes and further suggest that antioxidants, melatonin and vitamin E, can reduce peripheral neuropathic pain in streptozotocin-induced diabetic rats.

Keywords: Diabetes, Melatonin, Oxidative stress, Pain, Peripheral neuropathy, Vitamin E

1- Department of Biology, Faculty of Sciences, Urmia University, Urmia, Iran
*Corresponding Author: Tel: +98- 441-3456642; Fax: +98- 441-3440197; email: st_f.babaei@urmia.ac.ir

Introduction

Diabetes mellitus comprises a group of chronic diseases characterized by hyperglycemia (1, 2). The long-term hyperglycemia increases glycation proteins and lipids, enhances glucose auto-oxidation and changes the activity of many cellular enzymes (1-3). Wolff and Dean (3) were the first to show that glucose auto-oxidation generates reactive oxygen species (ROS). These species lead to the chronic oxidative stress in diabetes mellitus (4). ROS also have toxic effects upon the structure and function of various organs, inducing retinopathy, nephropathy, vascular complications and neuropathy (1, 3, 4). Oxidative damage to the peripheral neurons often leads to an increased activity of spinal glial cells and nerve fibers, and to release of preinflammatory factors, cytokines and glutamate, as well as augmented sensitivity to painful stimuli (5). Abnormal sensations and pain are features of approximately 10% of all cases of diabetic neuropathy and can cause marked diminution in the quality of life for these patients (6). Like other neuropathic pain states, painful diabetic neuropathy has an unknown pathogenesis and, in many cases, is not alleviated by non-steroidal anti-inflammatory drugs or opiates (6). Also these drugs may cause intolerable side effects. Therefore, investigation of other options of treatment such as antioxidants is needed.

Melatonin (*N*-acetyl-5-methoxytriptamin) is an endogenous neurohormone produced by the pineal gland in mammals. Its synthesis is augmented in darkness and inhibited by the exposure of animals to light. This compound participates in a number of physiological processes, like the reproduction regulation and circadian rhythms (7, 8). At the same time, melatonin is an effective scavenger of different free radicals (9). It also crosses all morpho-physiological barriers and enhances the expression of antioxidant enzymes in all cells (10). Vitamin E is a lipid soluble antioxidant which especially protects biological membranes from lipid peroxidation (11). It has been shown that melatonin and vitamin E reduce several diabetes-induced complications in animals such as oxidative-antioxidative status (4, 12). There is evidence about efficacy of antioxidants such as vitamin E on neuropathic pain (13). Possible participation of melatonin MT2 and δ opioid receptors in the antinociceptive activity of melatonin in diabetic rats has also been suggested (14). However, up to now, the antioxidative role of melatonin on peripheral neuropathic pain has not been elucidated in diabetes. Since melatonin demonstrates good antioxidative property, we have hypothesized that melatonin could reduce peripheral neuropathic pain by decreasing the oxidative stress in diabetic rats. Therefore, the purpose of this study was to assess the possible analgesic effect of melatonin due to its antioxidant activity in diabetic rats and to compare it with vitamin E.

Materials and Methods

Animals and treatment

Thirty two male Wistar rats weighing 180-220 g each were used in the study. The animal room temperature was maintained at 22±2 ˚C, under a 12 hr/ 12 hr light/dark cycle with lights on from 8:30 am. Food and water were available *ad libitum*. All animals were randomly divided into two groups: control and diabetic. Animals were rendered diabetic by an intraperitoneal injection of 50 mg/kg streptozotocin only once. Streptozotocin was dissolved in 0.05 M citrate buffer at pH 4.5 immediately before administration. Control rats (n=8) were injected with the vehicle alone. Blood glucose levels as a parameter of diabetes mellitus were determined using a glucometer (ACON Laboratories, Inc., USA) and a tail vein 72 hr later. The rats with hyperglycemia (glucose higher than 220 mg/dl) were considered as diabetic. Maturing animals exposed to chronic hyperglycemia manifest pathological alterations in peripheral nerve structure and function, and are relevant models for studying the diabetic peripheral neuropathy (15). Accordingly three weeks after streptozotocin injection, glycemia was again determined and all rats with a final blood glucose levels above 220 mg/dl were randomly assigned to three groups (each were included eight rats): the first group received daily melatonin at a dose of 10 mg/kg

intraperitoneally. Melatonin was dissolved in ethanol and this solution was then diluted with saline to a final volume (final concentration of ethanol, 4%). The second group received daily vitamin E dissolved in corn oil containing 4% ethanol at a dose of 100 mg/kg intraperitoneally. The third group of diabetic rats and the control group were injected with vehicle alone (16). Study design contains the control groups and the melatonin and vitamin E doses used in this study were chosen on the basis of previously published experiments (16, 17). All solutions were intraperitoneally injected at a volume of 0.1 ml per 100 g body weight for 2 weeks. All chemicals were purchased from Sigma (St. Louis, MO, USA). The study was performed following the ethical guidelines of the International Association for the Study of Pain, and the experimental protocol was reviewed and approved by the Local Institutional Committee for the Ethical Use of Animals.

Formalin test

At the end of administration period, pain-related biphasic responses were measured by formalin test. This occurred 24 hr after the last melatonin or vitamin E injection for investigation of the efficacy of long-term treatment. All animals were acclimated to the experimental setting, by placement in an open plexiglas observation chamber for 30 min before formalin injection. Plexiglas chamber was placed on the surface of glass and beneath it, was mounted a large mirror at a 45˚ angle to allow an unobstructed view of the animal paws. Fifty microliters of 0.5% formalin (prepared by diluting a commercial solution in sterile 0.9% NaCl) were injected subcutaneously (s.c.) into the dorsal surface of the right hind paw (18) using a syringe equipped with a 30 gauge needle. After the injection, rats were immediately put back into the plexiglas chamber, and observed for 60 min. Only one animal was tested at one time. The formalin-induced pain-related responses were assessed in all rats by the same investigator, by quantifying two behaviors: licking the injected paw and flinching. Each episode of shaking or vibrating the injected paw or shudder of the back/hind quarters was recorded as one flinch, as previously described (19). The number of the flinches was quantified during 1-min periods every 5 min, up to 60 min after injection (19). The amount of time (expressed in sec), animals spent in licking the injected paw, was recorded up to 1 hr (20). For each behavioral parameter, cumulative values were calculated for each of the tow phases of the response to formalin, in agreement with previous studies (14, 18). Data collected between 0 and 10 min post-formalin injection correspond to phase 1 (acute pain) and between 15 and 60 min post-formalin injection correspond to phase 2 (chronic pain). Animals were used only once and at the end of the formalin test each rat was anesthetized by ketamine/xylazine and after a skin incision, the left and right lumbosacral dorsal root ganglia were microdissected and immediately frozen at -20 ˚C until analysis.

Biochemical measurements

The lumbosacral dorsal root ganglia were homogenized in 1:10 (W/V) cold 25 mM potassium phosphate buffer (pH 7.4) and used to determine lipid peroxidation, glutathione peroxidase and catalase activities.

Malondialdehyde (MDA) levels were estimated by the method of Esterbauer and Cheeseman (21). The degree of lipid peroxidation was assessed according to MDA formation, which is accepted as an index of lipid peroxidation. Malondialdehyde, an end-product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. The principle of the method is the spectrophotometric measurement of the color generated by the reaction of the TBA with MDA. For this purpose, 300 µl of 10% trichloroacetic acid were added to 150 µl of each sample and centrifuged at 1000×g for 10 min at 4 ˚C. Three hundred microliters of the supernatant were transferred to a test tube and incubated with 300 µl 0.67% thiobarbituric acid at 100 ˚C for 25 min. The mixture was allowed to cool on water for 5 min. The resulting pink stained TBA-RS were determined in a spectrophotometer at 535 nm. TBA-RS were quantified using an extinction

coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nmol of MDA per g wet tissue.

Glutathione peroxidase activity was measured according to the method of Lawrence and Burk (22). The enzymatic reaction was initiated in the tube that contained reduced nicotinamide adenine dinucleotide phosphate, reduced glutathione, sodium azide and glutathione reductase by the addition of cumene hydroperoxide (CuOOH) and the change in absorbance at 340 nm was monitored with a spectrophotometer. Activity is given in unit per mg protein.

Catalase activity was assayed measuring the absorbance decrease at 240 nm in a reaction medium containing 30 mM H_2O_2, 50 mM potassium phosphate buffer pH 7.0 and 50 µl of the sample, according to Aebi method (23). One unit of enzyme is defined as one µmol of H_2O_2 consumed per min and the specific activity is reported as unit per mg protein.

Protein concentrations were determined according to the method of Lowry *et al* (24).

Statistical analysis

All data were expressed as mean±SEM for 8 animals per group. For the formalin test, flinches/min curve was used for the mean number of flinches against time. The area under the number of flinches against time curve (AUC) for both phases was calculated according to trapezoidal rule. Statistical analyses were performed for each behavioral parameter and biochemical data by one-way analysis of variance (ANOVA) followed by Tukey's test, using the software package SPSS for windows (SPSS, Inc., USA).

Results

Effects of diabetes and antioxidants treatment on the pain-related activities

Behavioral responses in the experimental groups are reported in Figure 1 and 2. All rats exposed to 0.5% formalin exhibited the biphasic pattern of this test characterized by two periods of behavioral responses of the injected hind paw, separated by a period of decreased activity.

Flinching behavior was affected by streptozotocin administration (Figure 1A). The overall analyses of flinching behavior as AUC, showed a significant difference ($P< 0.05$) between the diabetic and control rats during the second phase (Figure 1C). Whereas additional analyses of AUC revealed that there was no significant difference between the control and diabetic groups during the first phase (Figure 1B).

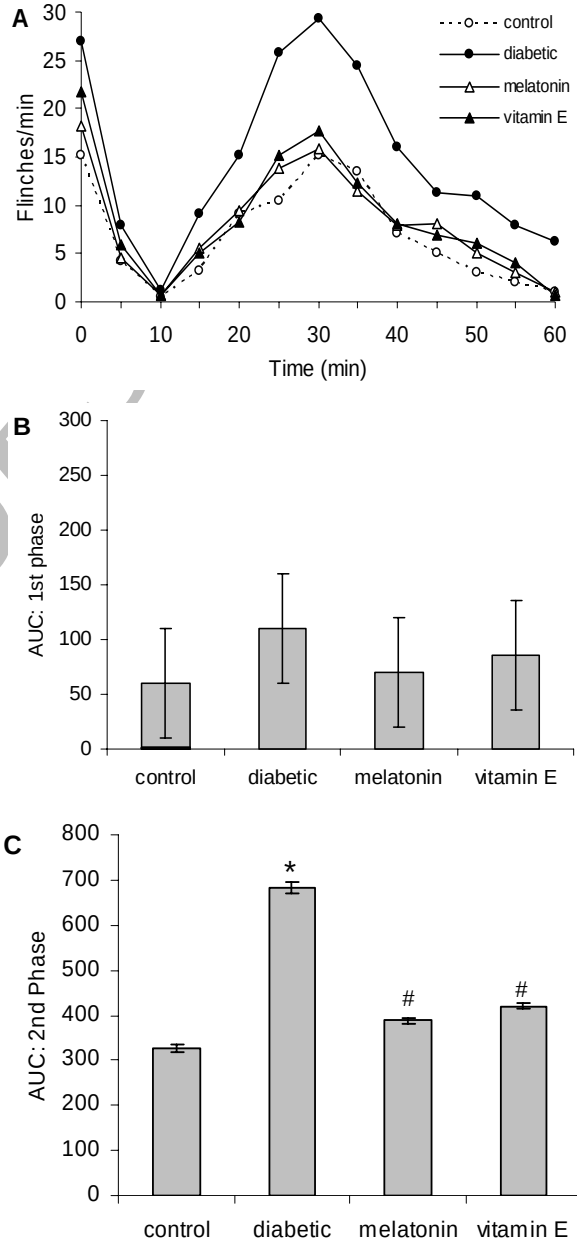

Figure 1. Formalin-evoked flinching behavior. A) Time course of the pain-related activities induced by 0.5% formalin. B) and C) The area under the number of flinches against time curve (AUC) as formalin induced pain-related activities during 1st and 2nd phases, respectively. Data are expressed as mean±SEM for 8 animals per group. *$P< 0.05$ in comparison with control values, #$P< 0.05$ in comparison with diabetic values.

The licking behavior was also increased in diabetic rats as compared to control rats (Figure 2). The time spent in licking the injected paw was significantly ($P< 0.05$) higher in diabetic rats compared to the control rats, in both first (Figure 2A) and second (Figure 2B) phases of the behavioral response.

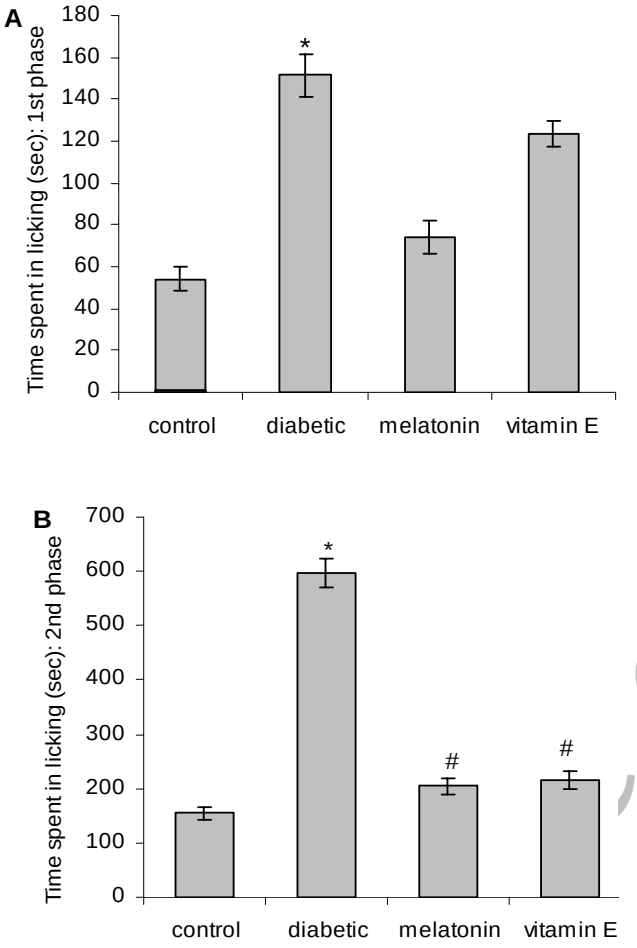

Figure 2. Time spent in licking during in A) 1st and B) 2nd phases of formalin test. Data are expressed as mean±SEM for 8 animals per group. *$P< 0.05$ in comparison with control values, #$P< 0.05$ in comparison with diabetic values.

Intraperitoneal administration of melatonin or vitamin E significantly ($P< 0.05$) reduced pain-induced flinches in diabetic rats (Figure 1A). AUC analyses revealed that treatment with melatonin or vitamin E significantly ($P< 0.05$) decreased flinching behavior during phase 2 (Figure 1C) in diabetic rats but not in phase 1 (Figure 1B).

The licking behavior was also significantly affected by melatonin and vitamin E administration (Figure 2). Treatment with melatonin or vitamin E significantly ($P< 0.05$) reduced the amount of total time spent licking the injected paw in diabetic rats in second phase (Figure 2A) but not in the first phase (Figure 2B) of pain.

Effects of diabetes and antioxidants treatment on biochemical parameters in dorsal root ganglia

In the present study we determined the levels of lipid peroxidation, and glutathione peroxidase and catalase activities in lumbosacral dorsal root ganglia. The lipid peroxidation levels in dorsal root ganglia from diabetic rats was significantly ($P< 0.05$) increased, whereas the glutathione peroxidase and catalase activities significantly ($P< 0.05$) decreased. Treatment with melatonin or vitamin E returned the levels of lipid peroxidation and glutathione peroxidase and catalase activities toward their control values (Table 1).

Table 1. The effects of melatonin and vitamin E on the activity of antioxidative enzymes and malondialdehyde levels in dorsal root ganglia (mean±SEM)

	Control	Diabetic	Melatonin	Vitamin E
Malondialdehyde nmol/g wet tissue	20.04±2.02	36.75±1.74 [a]	23.45±2.25 [b]	24.24±2.23 [b]
Glutathione peroxidase U/mg protein	0.032±0.002	0.023±0.002 [a]	0.035±0.003 [b]	0.031±0.001 [b]
Catalase U/mg protein	0.146±0.015	0.098±0.007 [a]	0.131±0.001 [b]	0.131±0.003 [b]

[a] $P<0.05$ *vs* control group.
[b] $P<0.05$ *vs* diabetic group.

Discussion

Streptozotocin-induced diabetes is accompanied by chronic oxidative stress due to the resulting hyperglycemia (25). Enhanced generation of reactive oxygen species occurred during the hyperglycemia contributes to the increased neuronal damage by oxidizing proteins, lipids, and augmented levels of lipid peroxidation products in cellular membranes (26, 27). Oxidative partial damage to the peripheral neurons often leads to an augmented sensitivity to painful stimuli and peripheral neuropathic pain (5). Maturing animals exposed to chronic hyperglycemia manifest pathological alterations in peripheral nerve structure and function, and are relevant models for studying the diabetic peripheral neuropathy (15). Different studies indicate that various ROS scavengers reduce pain behaviors predominantly through peripheral nerve protection (28, 29). In the current study we have examined the effects of treatment with melatonin or vitamin E on the peripheral neuropathic pain in diabetic rats. The treatment was aimed at reducing the oxidative stress and suppressing the hyperactivity of the abnormal nerves; and we investigated the levels of lipid peroxidation and the antioxidant enzymes activities in dorsal root ganglia. These tissues are the relevant structures for investigation of peripheral neuropathy (30).

In the present study, administration of streptozotocin significantly increased malondialdehyde levels in the dorsal root ganglia studied. One reason for the elevated lipid peroxidation in streptozotocin-induced diabetes is the reduction of antioxidant enzymes such as glutathione peroxidase and catalase activities. In this experiment we found that untreated diabetes caused reduced activities of glutathione peroxidase and catalase in dorsal root ganglia. Our findings are consistent with the previously published reports (31, 32). It has been shown that the antioxidant enzymes activities were changed in peripheral nerve tissues in chronic experimental diabetic neuropathy. These changes have been related to duration of diabetes or post-translational modifications (31).

In our study, treatment of diabetic animals with either melatonin or vitamin E significantly reduced lipid peroxidation in the dorsal root ganglia. Furthermore, we found that a decrease of glutathione peroxidase and catalase activities in peripheral nerve structures was reversed by the administration of melatonin or vitamin E. Despite considerable evidence identifying melatonin and vitamin E as potent antioxidants (12, 17, 33), their protective effects against oxidative stress in dorsal root ganglia have not been reported to date. This is the first report to show that melatonin and vitamin E protect these structures against oxidative stress. Nevertheless, beneficial effects of several antioxidants such as α-lipoic acid and dietary antioxidant supplements on structure and function of peripheral neurons have been reported previously (25, 28, 29, 34). It is important to mention that peripheral nerve antioxidant defenses are very low compared to central nerves (32).

In the current study we have further examined the effects of antioxidants, melatonin or vitamin E on suppression of pain-related activities in diabetic rats by the formalin test. We showed that diabetes could significantly lead to the hypersensitivity to formalin injection. Previously increase in peripheral nociception, as revealed by the formalin response, has been demonstrated in streptozotocin induced diabetes (14, 18). Increase in peripheral neuropathic pain in diabetic rats could be due to a peripheral release of cholecystokinin, which in turn would act on receptors located on the primary afferent neurons (18). However, oxidative stress also contributes to the peripheral neuropathic pain during hyperglycemia (5, 29). Therefore, antioxidants might be of general use in the prevention of nerve damage and peripheral neuropathic pain associated with diabetes (13, 29). Herein we found that melatonin and vitamin E could reduce the pain-related activities during the formalin test. Vissers *et al* (35) have shown the correlation between the formalin test and the neuropathic pain behavior in different species of experimental models. Therefore, it has been

speculated that melatonin and vitamin E have reduced the peripheral neuropathic pain in diabetic rats. Sayyed *et al* (36) have shown that treatment with U83836E prevented the hyperalgesia in diabetic rats; in this study U83836E acted as an antioxidant to reduce oxidative damage to the peripheral neurons and ameliorated the alterations in lipid peroxidation products levels and antioxidant enzymes in diabetic rats.

The exact mechanism of analgesic effects of melatonin in diabetes is still under debate. Arreola-Espino *et al* (14) have suggested the possible participation of melatonin MT_2 and δ opioid receptors in the antinociceptive activity of melatonin in diabetic rats. Based on our findings, melatonin and vitamin E can reverse the reduced activities of antioxidant enzymes and increased lipid peroxidation in peripheral nerve system, and thereby can improve neuropathic pain in diabetes mellitus. Therefore both oxidative stress and peripheral neuropathic pain were concomitantly prevented by the treatment with melatonin or vitamin E suggesting that oxidative stress was probably involved in the diabetes-induced peripheral neuropathic pain. Especially the analgesic effects of melatonin and vitamin E prominently appeared in chronic phase of pain indicating that ROS are critically involved in the maintenance of persistent neuropathic pain.

Herein we also compared the effects of melatonin and vitamin E on the pain-related activities. There was no significant difference between these two antioxidants on peripheral neuropathic pain, however the vitamin E dose used in the present study was ten times higher than the melatonin dose. Therefore, it is possible to postulate that melatonin is a more potent antioxidant than vitamin E to prevent development of diabetic complications.

Conclusion

In summary, this study showed the involvement of oxidative stress in peripheral neuropathic pain in diabetes mellitus by damaging the peripheral neurons; whereas the antioxidants, melatonin and vitamin E, show analgesic activities in diabetic rats by preventing the nerve oxidative damage. Our data suggest that either melatonin or vitamin E may be useful in the prevention of pain associated with diabetes. However vitamin E was found to be less effective than melatonin in alleviation of pain.

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