The Protective Effect of Melatonin on the Retinas of Rats with Streptozotocin-Induced Diabetes Mellitus

Melatoninin Streptozosin ile Uyarılmış Diabetes Mellitus'lu Farelerin Retinaları Üzerindeki Koruyucu Etkileri

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ABSTRACT Objective: To investigate the protective effect of melatonin on the retinas of rats with streptozotocin (STZ)-induced diabetes mellitus (DM). **Material and Methods:** Three groups of seven male Wistar rats were enrolled in this study: (1) a diabetic group, (2) a diabetic group treated with melatonin, and (3) control group. Diabetes was induced with a single dose of 60 mg/kg intraperitoneal (i.p). STZ. In addition melatonin (10 mg/kg/day) was injected i.p. to rats in the treatment group. All animals were euthanized 30 days later. Oxidative damage was examined by measuring retinal malondialdehyde (MDA) levels. **Results:** The mean MDA levels in tissue weight of retinas were 55.68 ± 6.04 per 100 mg in diabetic rats induced with STZ, 36.13 ± 7.48 per 100 mg in diabetic rats treated with melatonin, and 23.04 ± 4.03 per 100 mg tissue in control group. Retinal MDA levels were found different significantly in both diabetic group and melatonin-treated diabetic group compared to the control group ($p< 0.001$). There was also a difference between the diabetic and melatonin-treated diabetic groups for retinal MDA levels ($p< 0.01$). MDA levels of diabetic animals treated with melatonin were not different from control group ($p> 0.05$). **Conclusion:** Our study revealed that the melatonin improved the retinal damage in diabetic male rats induced with STZ and melatonin had a protective effect against the oxidative damage of DM.

Key Words: Diabetes mellitus, experimental; melatonin; mitochondrial oxidative damage endonuclease; retina

ÖZET Amaç: Melatoninin streptozotosin (STZ) ile uyarılmış Diabetes Mellitus'lu (DM) farelerin retinaları üzerindeki koruyucu etkisini incelemektir. **Gereç ve Yöntemler:** Bu çalışmaya diyabetik grup, melatonin verilen diyabetik grup ve kontrol grubu olmak üzere üç gruba ayırmak suretiyle 21 Wistar cinsi fare alındı. Diyabeti uyarmak için 60 mg/kg STZ tek doz periton içine uygulandı. Ayrıca tedavi grubuna melatonin 10 mg/kg/gün olarak periton içine uygulandı. Tüm farelere 30. gün ötenazi uygulandı. Oksidatif hasar, retinada ölçülen malondialdehid (MDA) seviyesi ile belirlendi. **Bulgular:** Retinaların her 100 mg ıslak ağırlıklarındaki ortalama MDA seviyeleri sırasıyla, STZ ile uyarılmış diyabetli farelerde 55.68 ± 6.04 100 mg, melatonin verilen diyabetli farelerde 36.13 ± 7.48 ve kontrol grubunda 23.04 ± 4.03 bulunmuştur. Retina MDA seviyeleri diyabetik farelerde ve melatonin verilen diyabetik farelerde kontrol grubuna göre anlamlı olarak farklı bulunmuştur ($p< 0.001$). Diyabetli ve melatonin verilen diyabetli fareler arasında da retina MDA seviyeleri arasında farklılık bulunmuştur ($p< 0.01$). Melatonin ile tedavi edilen diyabetik hayvanların MDA seviyeleri ile kontrol grubu arasında farklılık saptanmadı ($p> 0.05$). **Sonuç:** Çalışmamızın sonuçları, melatoninin STZ ile uyarılmış diyabetik erkek farelerde retinal hasarı düzelttiği ve diyabetes mellitusa bağlı oksidatif hasara karşı koruyucu etkisi olduğunu göstermektedir.

Anahtar Kelimeler: Diabetes mellitus, deneysel; melatonin; mitokondri oksidatif hasar endonükleazı; retina

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Diabetes mellitus (DM) is a common disorder of glucose metabolism that has potential of damage in each organ of the body. The most serious complications of diabetes are retinopathy, nephropathy and neuropathy. Diabetic retinopathy is the most common retinal vascular disease. The progression of diabetic retinopathy is related to many factors.[1] Membrane lipid peroxidation in the retina is an early pathological finding of oxidative stress associated with DM.[2] High production rate of reactive molecules and high concentration of unsaturated fatty acids in the retina render these cells sensitive to lipid peroxidation.[3] Lipid peroxidation is known to cause gradual changes in membrane structure leading to the loss of membrane function and several pathologies such as diabetic retinopathy, cataracts and glaucoma.[4] Malondialdehyde (MDA) level is widely used as a marker of lipid peroxidation during increased oxidative stress.[5] MDA can be measured spectrofluorometrically to determine the level of oxidative damage in the retina.[6]

Streptozotocin (STZ) is the major preferred chemical for induction of diabetes in experimental animals and it acts by destroying pancreatic beta cells.[7] The cytotoxic action of STZ is mediated by reactive oxygen species.[7] Many studies have been performed to describe the devastating effect of diabetes in the different organs and tissues including ocular tissues. Several molecules such as aminoguanidine, melatonin, caffeic acid, and ramipril have been investigated.[8] Melatonin has the potential effect to decrease the oxidative stress on the cornea in STZ-induced diabetic rats.[9]

The aim of this study was to investigate the protective effects of exogenous melatonin on retinal damage in STZ-induced diabetic rats.

MATERIAL AND METHODS

This study was performed in compliance with the ARVO (The Association for Research in Vision and Ophthalmology) resolution for the use of animals in Ophthalmic and Vision Research. The study was approved by the Ethical Committee of Firat University School of Medicine; the tenets of the 2nd Declaration of Helsinki were followed.

One eye of 21 male Wistar rats at 4 to 5 months of age, each weighting 200-230 g, was used in all experiments. Standard rat chow and water were given ad libitum, and animals were housed in groups of 3–4 rats under standard conditions (22 ± 2°C and 55% ± 5% relative humidity) with 12-hour light-dark cycles. The animals were classified randomly into three groups, including seven animals in each group. The groups were as follows: (1) diabetic group, (2) melatonin-treated diabetic group, and (3) control group.

In two groups, diabetes was induced with one dose of 60 mg/kg of intraperitoneal (i.p.) STZ, dissolved in citrate buffer (pH 4.5). The glucose level was measured as a parameter of DM with a glucometer in a tail vein four days after the beginning of the experiment (Companion 2, Medisense, Birmingham, UK). All animals that were given STZ developed hyperglycemia within 24 hours. The rats with serum glucose levels >250 mg/dL were accepted as diabetic. The last group, sham-operated control animals (Group 3), was injected with isotonic saline solution. One of the STZ-induced groups was not further treated. The other group was injected with 10 mg/kg/day of melatonin (Sigma, St. Louis, USA) i.p. dissolved in a solution of 20% ethanol in 0.8% saline, following STZ treatment, and it continued until rats were killed. All animals were euthanized at the end of 30th day. The rats were sacrificed by cardiac puncture under ether anesthesia and transcardially perfused with heparinized saline followed by 10% formalin in phosphate buffer. The eyes were enucleated, and dissected quickly under the stereo-microscope in the light. The eyeball was cut posteriorly to the limbus, and then anterior segment was discarded. The vitreous was removed, and the retina was carefully dissected from the sclera/choroid/pigment epithelium and was used for the MDA fluorometric assay. The wet weight of the isolated retina was measured as soon as possible. Then retinal tissue was kept frozen at -80 °C until the subsequent biochemical analyses were performed. Retina samples from each rat in all experimental groups were used for measurement of total MDA concentrations. Levels of MDA for each retina were measured spectrofluorimetrically, using the modified thiobarbitu-

ric acid-reactive substance (TBARS) method.[10] TBAR is recognized as the end product of polyunsaturated fatty acid peroxidation and it is also formed during injury of DNA, proteins and carbohydrates.

Retinal samples were homogenized in a volume of one-tenth retina weight of ice-cold 6% perchloric acid at 4 °C with a glass homogenizer. Approximately 200 ml of supernatant was collected after centrifugation at 4000 g for 10 minutes at 4 °C. Standards were prepared from different dilutions of 1,1,3,3,-tetraethoxypropane. Tetraethoxypropane was hydrolyzed in acid (0.125 M in 0.125 M HCI, total volume 80 ml) for 24 hours at room temperature; pH was adjusted to 6.9 with 2 M NaOH. The volume was filled up to 100 ml with distilled water.

The reaction mixture contained 100 µl of each sample (standards, retina samples and blank), 200 ml of 8.1% sodium dodecyl sulfate (SDS, Sigma Chem., Co., USA), 200 µl of acetic acid solution (pH: 3.5), and 200 µl of 0.8% thiobarbituric acid (dissolved in 10% acetic acid, pH 3.5) and water was added to a final volume of 1 ml. The mixtures were then placed into a heating block at 95 °C for 45 minutes, and then cooled to room temperature with tap water. Samples were then centrifuged at 4000 g for 10 minutes. The supernatant was measured by spectrofluorimetry, with excitation at 520 nm and emission at 555 nm. Calculations were performed using a linear regression from tetraethoxypropane for the MDA standard curve. Readings were expressed in nmol/100 mg tissue wet weight using the regression equation.

Differences among the three groups for MDA levels were evaluated using one-way ANOVA analysis, where applicable. The Tukey's test was used as post hoc test after one-way ANOVA. The level of significance was set at <0.05. All statistical analyses of the study were performed by using SPSS for Windows (SPSS Inc., Chicago, IL, USA) software.

RESULTS

The wet weight range of the isolated retinas was between 15-23 mg. Diabetic animals exhibited many symptoms commonly associated with diabetes (e.g. polyuria, polydipsia and diarrhea). Final mean plasma glucose levels in the diabetic group, melatonin-treated diabetic group, and control groups were 378.25 ± 59.01 mg/dl, 363.08 ± 69.13 mg/dl, and 112.30 ± 21.2 mg/dl, respectively. The STZ-induced diabetic animals exhibited hyperglycemia. Melatonin treatment did not significantly change blood glucose levels of diabetic animals and did not affect blood glucose levels of the control group.

The mean MDA levels in tissue wet-weight of retinas were 55.68 ± 6.04 per 100 mg in diabetic rats induced with STZ, 36.13 ± 7.48 per 100 mg in diabetic rats treated with melatonin, and 23.04 ± 4.03 per 100 mg tissue in control group. The mean values of the MDA for three groups are shown in Table 1. One-way ANOVA showed statistically significant differences among the three groups ($p= 0.0001$; $F= 84.82$; $df= 2$; one-way ANOVA). The MDA levels, as a marker of lipid peroxidation, were higher in the retinal homogenates of diabetic animals than either control animals or diabetic animals treated with melatonin ($p< 0.001$, $p< 0.01$, respectively). There was also difference between the diabetic and melatonin-treated diabetic groups for retinal MDA levels ($p< 0.01$). MDA levels of diabetic animals treated with melatonin were not different from control group ($p> 0.05$).

TABLE 1: The malondialdehyde (MDA) concentrations of the three groups.

Groups	MDA Level* (nmol per 100 mg tissue wet-weight)	P Value
Streptozotocin-induced diabetic group	55.68 ± 6.04	p= 0.0001
Melatonin-treated diabetic group	36.13 ± 7.48	p= 0.0001
Control group	23.04 ± 4.03	n.a.**

* Values are given as mean ± standard deviation.

** n.a.: not applicable.

DISCUSSION

DM is the most common endocrine disease that leads to metabolic disorders involving regulation of carbohydrate metabolism. These abnormalities produce pathologies including vasculopathies, neuropathies, ophthalmopathies and nephropathies, among many other medical disorders.[1] An excessive and/or sustained increase in reactive oxygen species (ROS) production has been implicated in the pathogenesis of cancer, diabetes mellitus, atherosclerosis, neurodegenerative diseases, rheumatoid arthritis, ischemia/reperfusion injury, and other diseases.[11] Oxidative stress plays a role in the development of diabetic complications.[12] Elevated glucose levels are associated with increased production of ROS by several different mechanisms.[11] In cultured bovine aortic endothelial cells, hyperglycemia was shown to cause increased ROS production at the mitochondrial complex. In addition, superoxide is generated by the process of glucose auto-oxidation that is associated with the formation of glycated proteins in the plasma of diabetic patients. The interaction of advanced glycation end-products with corresponding cell surface receptors stimulates ROS production and decreases intracellular glutathione levels. In diabetes mellitus, lipid peroxidation can be induced by protein glycation and glucose auto-oxidation that leads to the formation of free radicals.[13] These free radicals all might play a role in DNA damage, glycation and protein modification reactions, and in lipid oxidative modification in diabetes. The increase in ROS production contributes to the development of diabetic complications such as atherosclerosis and other vascular complications. In addition, hyperglycemia enhances cell-mediated low-density lipoprotein peroxidation in endothelial cells. Treatment with antioxidants ameliorates diabetic complications including the dysfunction of endothelial cells or increased platelet aggregation.[11]

The damage caused by these radicals on the cells might be quantitatively determined by measurement of levels of MDA, a product of lipid peroxidation. MDA is a degraded product of lipid peroxidation that can produce cytotoxicity by reacting with the amino terminals of nucleic acid.[13] Changes of MDA levels are an indicator of degree of lipid peroxidation and therefore reflect the severity of tissue damage.[14] In our study, severe retinal damage was measured by increased levels of MDA.

Melatonin is an important component of the antioxidant profile of many tissues. It is a major scavenger of both oxygen- and nitrogen-based reactive molecules.[15] It has scavenging actions at both physiologic and pharmacologic concentrations. Not only melatonin but also several of its metabolites can detoxify free radicals and their derivatives.[15] Moreover, melatonin induces the activity of γ-glutamylcysteine synthetase, stimulating the production of another intracellular antioxidant, glutathione. Melatonin also supports several intracellular enzymatic antioxidant enzymes, including superoxide dismutases and glutathione peroxidase.[16] It possesses genomic actions and regulates the expression of several genes, including those for superoxide dismutases and glutathione peroxidase. Melatonin influences both antioxidant enzyme activity and cellular mRNA levels for these enzymes under physiological conditions and during elevated oxidative stress.[16] It enhances ability of cells to resist oxidative damage by inhibiting the pro-oxidant nitric oxide synthase.[17] Antioxidant capacity of melatonin on plasma has been well established in the literature.[9] Hussein et al.[18] showed that a single and high dose of melatonin provided protection in X-ray-induced skin damage in rats. Anwar and Moustafa[19] have documented the preventive and treatment role of systemically applied melatonin against ultraviolet-induced oxidative stress in the rat lens. Vardı et al.[20] investigated the improving effects of melatonin on the histological alterations of the liver in the STZ-induced diabetic rat model. In histological investigations, hydropic and nuclear changes were observed in the hepatocytes in the diabetic group. In addition, both glycogen granules in the hepatocyte cytoplasm and mast cell granules had decreased compared to the control and DM groups, and this improvement has been ascribed to melatonin. Vardı et al. suggest that melatonin may be used to prevent diabetic liver damage.

The influence of melatonin supplementation on the oxidative stress parameters in the elderly non-insulin dependent diabetes mellitus (NIDDM) patients was investigated.[21] Improvements of antioxidative defense were observed after melatonin supplementation in NIDDM individuals and melatonin supplementation was suggested as an additional treatment for the control of diabetic complications.[21] In our study, results of melatonin treatment were similar with those studies. Melatonin reduced oxidative impacts of hyperglycemia on the retina. STZ increased MDA levels ($p< 0.05$) in retinal tissue. Treatment of diabetic rats with melatonin markedly reduced MDA production ($p< 0.05$). Melatonin did not inhibit development of diabetes induced by STZ treatment, as reported earlier.[22] These results suggest that oxidative stress may contribute to retinal damage in diabetics and moreover melatonin as an antioxidant can improve the injury in STZ-induced diabetes.

Retinal toxicity of drugs can be evaluated by histopathologic changes, electron micrographic changes, electrophysiologic responses, and degraded product of cellular injury. Here we report the results of our investigations, which assessed the effect of melatonin on the retinas of STZ-induced diabetic rats. We have chosen MDA as the indicator of retinal toxicity to measure the level of oxidative damage in this study. In conclusion, melatonin improves the retinal damage in diabetic male rats. Melatonin can contribute to a balanced oxidant-antioxidant status and provides a useful therapeutic option to reduce the associated retinal injury in patients with diabetes mellitus.

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