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Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Therapeutic efficacy of melatonin in reducing retinal damage in an experimental model of early type 2 diabetes in rats

Abstract: Diabetic retinopathy (DR) is a leading cause of acquired blindness in adults, mostly affected by type 2 diabetes mellitus (T2DM). We have developed an experimental model of early T2DM in adult rats which mimics some features of human T2DM at its initial stages and provokes significant retinal alterations. The aim of this work was to analyze the effect of melatonin on retinal changes induced by the moderate metabolic derangement. For this purpose, adult male *Wistar* rats received a control diet or 30% sucrose in the drinking water. Three weeks after this treatment, animals were injected with vehicle or streptozotocin (STZ, 25 mg/kg). One day or 3 wk after vehicle or STZ injection, animals were subcutaneously implanted with a pellet of melatonin. Fasting and postprandial glycemia, and glucose, and insulin tolerance tests were analyzed. At 12 wk of treatment, animals which received a sucrose-enriched diet and STZ showed significant differences in metabolic tests, as compared with control groups. Melatonin, which did not affect glucose metabolism in control or diabetic rats, prevented the decrease in the electroretinogram a-wave, b-wave, and oscillatory potential amplitude, and the increase in retinal lipid peroxidation, NOS activity, TNFα, Müller cells glial fibrillary acidic protein, and vascular endothelial growth factor levels. In addition, melatonin prevented the decrease in retinal catalase activity. These results indicate that melatonin protected the retina from the alterations observed in an experimental model of DR associated with type 2 diabetes.

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Key words: diabetic retinopathy, experimental type 2 diabetes mellitus, melatonin

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Introduction

About 350 million people across the globe are estimated to have diabetes [1], and type 2 diabetes mellitus (T2DM) accounts for roughly 90% of all diagnosed cases [2]. Chronic hyperglycemia of diabetes leads to microvascular complications that severely impact life quality. Diabetic retinopathy (DR) may be the most common of these complications and is a leading cause of visual impairment and blindness. In inadequately controlled patients with type 1 or type 2 diabetes mellitus, the retinal microvasculature is constantly exposed to high glucose levels, and this insult results in many structural and functional alterations [3]. Current treatments for DR such as laser photocoagulation, corticosteroids, or antivascular endothelial growth factor agents are indicated for DR but have significant adverse effects. Therefore, the development of resources to protect the retina against diabetic damage is a goal of vast clinical importance.

Understanding the molecular mechanisms of retinal damage associated with T2DM should help identify therapies to treat/postpone this sight-threatening complication of diabetes. For this purpose, a large number of genetically modified animal models including transgenic, generalized knockout, and tissue-specific knockout mice have been employed. However, a typical diabetic profile is not always seen in these genetically induced models, nor do they absolutely mimic the pathogenesis of human T2DM [4, 5] because these gene mutations are extremely rare in human populations. In a similar way, animal models of T2DM induced by removal of a portion of the pancreas [6] are not representative of T2DM etiology in humans, which is typically preceded by obesity [7, 8]. To better understand the events which precede and precipitate the onset of T2DM, some nutritional animal models have been also developed [9, 10].

Initially, the natural history of T2DM includes a period of normal or near-normal fasting plasma glucose levels and marked postprandial glycemic excursions. Historically, it was believed that microvascular complications of diabetes including T2DM develop only after *c*. 10–15 yr of active disease [11, 12]. However, it is increasingly clear that complications may begin at lower glucose concentrations or during sporadic increases in glucose levels, rather than after current thresholds for the diagnosis of T2DM are consistently reached [13]. Recently, we have shown that a combination of diet-induced insulin resistance and a slight secretory impairment resulting from a low-dose STZ treatment in adult rats mimics some features of human T2DM at its initial stages, such as slight fasting hyperglycemia, hyperinsulinemia, and elevated postprandial (nocturnal) glycemic levels [14]. Noteworthy, only animals exposed to this combined treatment develop significant retinal alterations, whereas each one of these maneuvers per se (a sucrose-enriched diet or the injection of a low dose of STZ) do not induce significant retinal changes [14]. Thus, this model could offer the opportunity to investigate DR at an early stage in a setting of moderately altered glucose metabolism.

Oxidative and nitrosative stress and inflammation play critical roles in the development of DR [15–17]. Thus, any beneficial treatment that limits oxidative and inflammatory effects of diabetes could greatly reduce its consequences at retinal level. Melatonin is one of the most powerful natural free radical scavengers [18] preventing oxidative damage to macromolecules, including lipids, proteins, and nucleic acids [19, 20]. In addition to the potent antioxidant property of melatonin by itself, some of its metabolites are themselves direct free radical scavengers [21] increasing melatonin's antioxidant capacity. Besides that melatonin stimulates several antioxidant enzymes [20, 22] and significantly decreases retinal NOS activity [23]. It has been demonstrated that melatonin reduces diabetes-induced corneal injury [24] in rats, and oxidative stress in photoreceptors from Syrian hamsters [25]. Moreover, it was recently demonstrated that melatonin improves some retinal histopathological changes including apoptosis and oxidative stress in STZ-induced diabetes in rats [26]. In this context, the aim of this work was to analyze the effect of melatonin on retinal alterations observed in an experimental model in rats which mimics early stages of human T2DM.

Materials and methods

Animals

Three-month-old male *Wistar* rats (400 ± 50 g) were housed in a standard animal room with food and water ad libitum under controlled conditions of humidity, temperature (21 ± 2°C), and luminosity (200 lux), under a 12-hr light/12-hr dark lighting schedule (lights on at 7:00 AM). All the experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Feeding and treatments

The following four groups of rats were included in this study: control animals receiving a standard commercial rat chow and tap water (group 1, control); animals receiving the same treatment than group 1 and were treated with melatonin (group 2, melatonin), animals receiving a standard commercial rat chow and 30% sucrose in the drinking water, which were i.p. injected with STZ (25 mg/kg in 0.1 M citrate buffer, pH 4.5), 3 wk after receiving 30% sucrose in the drinking water (group 3, T2DM), and a group of animals which received the same treatment as animals from group 3 and were treated with melatonin (group 4, T2DM + melatonin). Groups 1 and 2 were i.p. injected with vehicle instead of STZ. Groups 2 and 4 were subcutaneously implanted with a pellet of melatonin (20 mg with 3% w/v vegetable oil) compressed in a cylinder of 2.5 mm diameter and 1 mm length), while groups 1 and 3 were sham-operated without pellet implanting. Melatonin was obtained from Sigma Chemical Co. (St Louis, MO, USA). The pellet of melatonin was implanted under the skin of the neck, 48 hr or 3 wk after vehicle or STZ injection, and it was replaced every 15 days. Sucrose solution was replaced every 2 days.

Glucose level assessment

Plasma glucose levels were determined in tail vein blood samples using the Accu-Check Performa blood glucose meter (Roche Diagnostic, Mannheim, Germany). In some experiments, animals were fasted for 4 hr before the measurements, whereas in other experiments, plasma glucose levels were assessed at 11.00 or 23.00 hr in nonfasted animals.

Intraperitoneal glucose tolerance and intraperitoneal insulin tolerance test

Intraperitoneal glucose tolerance (IPGTT) and intraperitoneal insulin tolerance test (IPITT) were performed at wk 6 and 12, as previously described [14]. Animals were restricted of food and water 4 hr before the experiments. For the IPGTT, rats received an i.p. injection of glucose (1 g/kg body weight). Blood samples for plasma glucose measurement were collected from the tail vein at time 0 (before glucose injection) and 30, 60, 90, and 120 min after glucose administration. The area under the curve (AUC) was calculated using the trapezoidal rule estimation, as previously described [14].

For the IPITT, rats were intraperitoneally injected with regular human insulin (0.75 IU/kg body weight). Blood samples were taken from the tail vein at time 0 (before insulin injection) and 15, 30, 45, and 60 min after insulin administration. Insulin sensitivity was estimated from the slope of the 0–60 min glucose disappearance rate. This slope was determined by linear regression as previously described [14].

Electroretinography

The electroretinographic activity was assessed as previously described [27]. Briefly, after 6 hr of dark adaptation, rats were anesthetized under dim red illumination. Phenylephrine hydrochloride and tropicamide were used to dilate the pupils, and the cornea was intermittently irrigated with balanced salt solution to maintain the baseline recording and to prevent keratopathy. Rats were placed facing the stimulus at a distance of 20 cm. All recordings were completed within 20 min and animals were kept warm during and after the procedure. A reference electrode was placed through the ear, a grounding electrode was attached to the tail, and a gold electrode was placed in contact with the central cornea. A 15-W red light was used to enable accurate electrode placement. This maneuver did not significantly affect dark adaptation and was switched off during the electrophysiological recordings. Electroretinograms (ERGs) were recorded from both eyes simultaneously and ten responses to flashes of unattenuated white light (5 ms, 0.2 Hz) from a photic stimulator (light-emitting diodes) set at maximum brightness (9 cd s/m^2 without filter) were amplified, filtered (1.5-Hz low-pass filter, 1000 high-pass filter, notch activated), and averaged (Akonic BIO-PC, Buenos Aires, Argentina). The a-wave was measured as the difference in amplitude between the recording at onset and the trough of the negative deflection, and the b-wave amplitude was measured from the trough of the a-wave to

the peak of the b-wave. Runs were repeated three times with 5-min intervals to confirm consistency. Mean values from each eye were averaged, and the resultant mean value was used to compute the group means a- and b-wave amplitude ± S.E.M. The mean peak latencies and peak-to-peak amplitudes of the responses from each group of rats were compared.

Oscillatory potentials (OPs) were assessed as previously described [27]. Briefly, the same photic stimulator with a 0.2 Hz frequency and filters of high (300 Hz) or low (100 Hz) frequency were used. The OP amplitude was estimated by measuring the heights from the baseline drawn between the troughs of successive wavelets to their peaks. The sum of three OPs was used for statistical analysis.

Histological examination

Rats were killed and their eyes were immediately enucleated, immersed for 24 hr in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2), and embedded in paraffin. Eyes were sectioned (5 μm) along the vertical meridian through the optic nerve head. Microscopic images were digitally captured with a Nikon Eclipse E400 microscope (illumination: 6-V halogen lamp, 20 W, equipped with a stabilized light source) via a Nikon Coolpix s10 camera (Nikon, Abingdon, VA, USA). Sections were stained with hematoxylin and eosin (H&E) and analyzed by masked observers. Measurements (×400) were obtained at 1 mm dorsal and ventral from the optic disk.

Immunohistochemical studies

Antigen retrieval was performed by heating (90°C) slices for 30 min in citrate buffer (pH 6.3) and then preincubated with 2% normal horse serum, 0.1% bovine serum albumin, and 0.4% Triton X-100 in 0.01 M PBS for 1 hr. For immunodetection of glial cells, sections were incubated overnight at 4°C with a mouse monoclonal antiglial fibrillary acidic protein (GFAP) antibody conjugated to Cy3 (1:1200; Sigma Chemical Co.). For vascular endothelial growth factor (VEGF) immunodetection, paraffin sections were treated with 0.3% H_2O_2 in PBS for 20 min (for blocking endogenous peroxidase activity) and incubated overnight at 4°C with a rabbit polyclonal anti-VEGF antibody (1:800; Calbiochem, La Jolla, CA, USA). Immunohistochemical detection was performed using the LSAB2 System-HRP (Dako, Carpinteria, CA, USA), based on biotin–streptavidin–peroxidase and visualized using 3,3′-diaminobenzidine as chromogen. An Olympus BX50 microscope (Olympus, Tokyo, Japan) was used for microscopic observations.

Measurement of thiobarbituric acid reactive substances levels

Thiobarbituric acid reactive substances (TBARS) levels in retinal tissue were analyzed as described [28]. Retinas were homogenized in 15 mM potassium buffer plus 60 mM KCl, pH 7.2. The homogenate (300 μL) was mixed with 75 μL 10% SDS and 1.4 mL 0.8% thiobarbituric acid dissolved in 10% acetic acid (pH 3.5). This solution was heated to

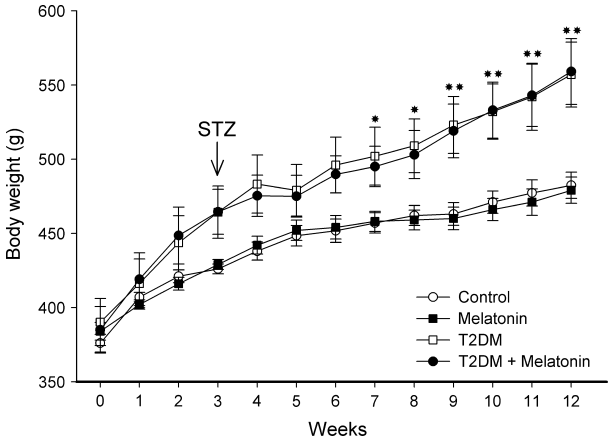

Fig. 1. Temporal course of body weight for all the experimental groups. Body weight significantly increased throughout the experiment in all groups, but from week 7 to 12, it was significantly higher in groups receiving sucrose and streptozotocin (STZ; groups 3 and 4) than in those which did not (groups 1 and 2). A pellet of melatonin implanted 48 hr after vehicle or STZ injection did not affect this parameter. Data are mean ± S.E.M. (n = 10). $*P < 0.05$, $**P < 0.01$ for groups 3 and 4 versus groups 1 and 2, by Tukey's test.

100°C for 60 min. After cooling, the flocculent precipitate was removed by centrifugation at 3200 g for 10 min. After addition of 1.0 mL water and 5.0 mL of n-butanol-pyridine mixture (15:1, vol/vol), the mixture was vigorously shaken and centrifuged at 2000 g for 15 min. The absorbance of the organic layer was measured at an emission wavelength of 553 nm by using an excitation wavelength of 515 nm with a Jasco FP 770 fluorescence spectrophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan). The range of the standard curves of malondialdehyde bis-dimethyl acetal (MDA) was 10–2000 pmol. Results were expressed as nanomoles MDA per milligram protein.

Catalase activity

Retinal catalase activity was assessed as previously described [29]. Individual retinas were homogenized in 50 mM phosphate buffer, pH 7.4. Two hundred microliters of the homogenate was mixed with 25 μL of 20% Triton X-100, and the mixture was incubated for 5 min at 4°C and centrifuged at 10,000 g for 1 min. The supernatant (50 μL) was diluted with 900 μL of 50 mM phosphate buffer, and the reaction was started by adding 50 μL of 200 mM H_2O_2 in 50 mM phosphate buffer. Absorbance at 240 nm was recorded at 37°C for 100 s every 10 s. The enzymatic activity was calculated by using the extinction coefficient of H_2O_2 at 240 nm, 0.0394/mM/cm, and results were expressed as nmol H_2O_2/min milligram of protein.

NOS activity assessment

Retinal NOS activity was assessed as previously described [28]. Each retina was homogenized in 100 μL of buffer solution containing 0.32 m sucrose and 0.1 mm EDTA (adjusted to pH 7.4 with Tris base). Reaction mixtures contained 50 μL of the enzyme source, 50 μL of a buffer stock solution (final concentrations: 10 mM HEPES, 3 mM

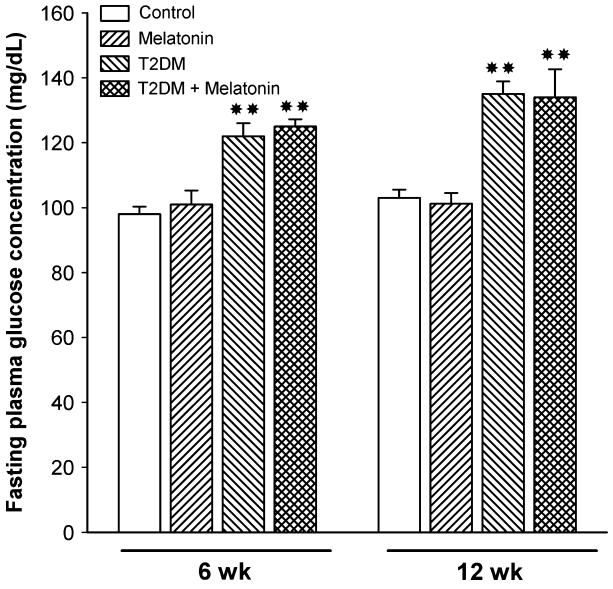

Fig. 2. Fasting glycemia in control and diabetic animals untreated or treated with melatonin. At 6 and 12 wk of treatment, fasting plasma glucose levels were significantly higher in group 3 and 4 than in groups 1 and 2. Melatonin did not affect this parameter in control or diabetic rats. Data are the mean ± S.E.M. (n = 10); $^{**}P < 0.01$ versus groups 1 and 2, by Tukey's test.

$CaCl_2$, 1 mM NADPH, 5 μM FAD, 1 mM β-mercaptoethanol, L-^{3}H-arginine 5 μCi/mL, purity >98%), and 1 μM L-arginine. After incubation at 37°C for 30 min, the reaction was stopped by adding 200 μL of stop buffer (50 mm HEPES, 10 mm EDTA, and 10 mm EGTA, pH 5.5) and cooling the tubes for 5 min. The solution was mixed with 600 μL of resin Dowex AG50W-X8 (Na^+ form) to remove L-arginine and centrifuged at 10,000 g for 5 min. L-^{3}H-citrulline in the supernatant was quantified by liquid scintillation counting. Nonenzymatic conversion of L-^{3}H-arginine to L-^{3}H-citrulline was tested by adding buffer instead of the enzyme source.

Assay for TNFα level assessment

Two retinas were homogenized in 150 μL of phosphate saline buffer (PBS) pH 7.0 supplemented with 10% fetal bovine serum heat inactivated and a cocktail of protease inhibitors. Samples were cleared by centrifugation for 10 min at 15,700 g. TNFα levels were determined as previously described [14], using specific rat enzyme-linked immunosorbent assays (ELISA) using antibodies and standards obtained from BD Biosciences, Pharmingen (San Diego, CA, USA), according to the manufacturer's instructions. The reaction was stopped and absorbance was read immediately at 450 nm on a microplate reader (Model 3550; Bio-Rad Laboratories, Hercules, CA, USA).

Protein level assessment

Protein content was determined by the method of Lowry et al. [30] using bovine serum albumin as the standard.

Statistical analysis

Statistical analysis was performed by a two-way ANOVA, followed by Tukey's post hoc test.

Results

Fig. 1 depicts the temporal course of the body weight from group 1 (control), group 2 (melatonin), group 3 (T2DM), and group 4 (T2DM + melatonin). In groups 2 and 4, a pellet of melatonin was implanted 48 hr after vehicle or STZ injection. The body weight significantly increased throughout the experiment in all groups, but

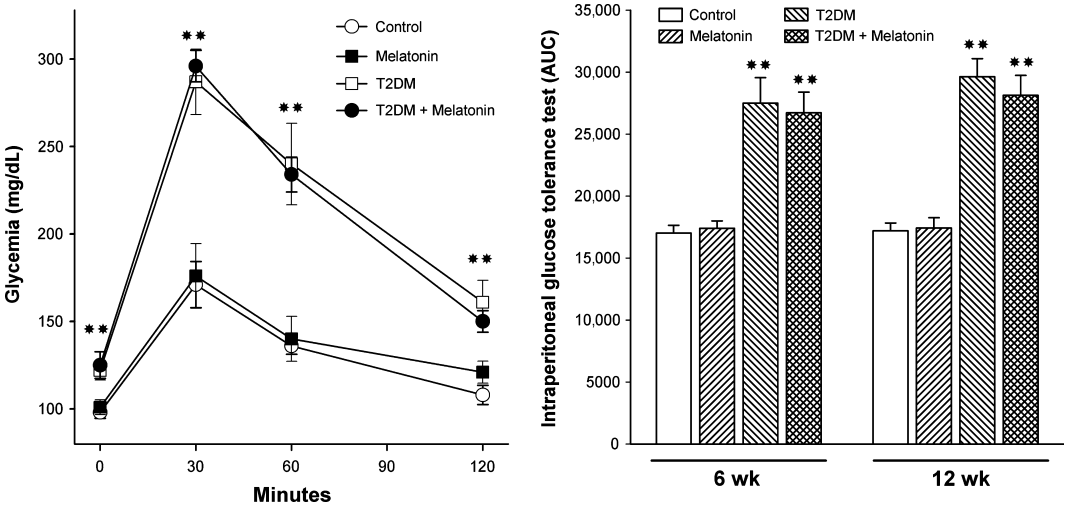

Fig. 3. Left panel: Mean plasma glucose concentrations in response to an intraperitoneal glucose challenge assessed before and 30, 60, and 120 min after 1 g/kg glucose injection for all the experimental groups at 12 wk of treatment. A similar profile was observed at 6 wk of treatment (not shown). Right panel: Average area under the curve (AUC) of the intraperitoneal glucose tolerance (IPGTT) at 6 or 12 wk of treatment. At both time points, higher values of the AUC were observed in groups 3 and 4 than in groups 1 and 2. Melatonin did not affect the IPGTT in control or diabetic rats. Data are the mean ± S.E.M. (n = 10); $^{**}P < 0.01$ versus groups 1 and 2, by Tukey's test.

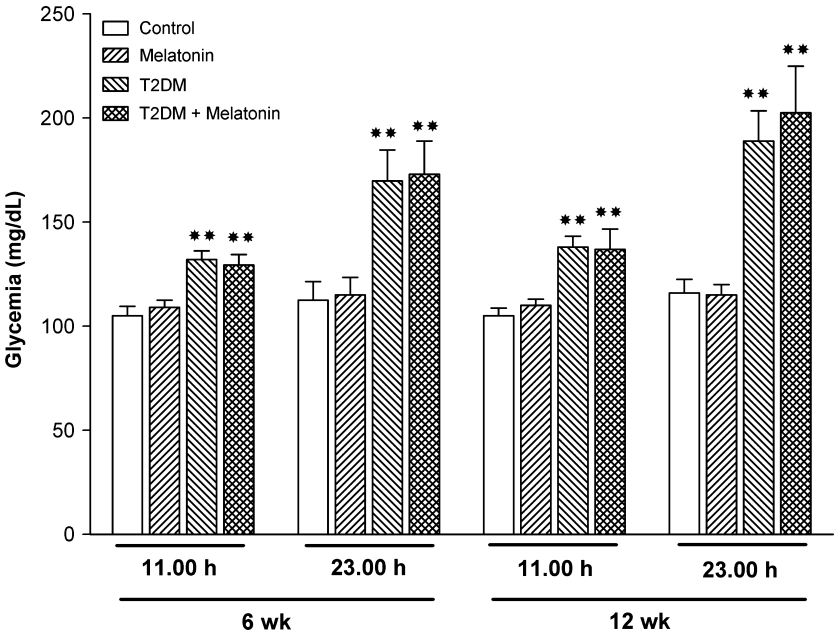

Fig. 4. Nonfasting glycemia assessed at 11.00 and 23.00 hr. At 6 and 12 wk of treatment, diurnal (i.e., at 11.00 hr) and nocturnal (i.e., at 23.00 hr) plasma glucose levels were significantly higher in groups 3 and 4 as compared with groups 1 and 2. Melatonin did not affect diurnal or nocturnal nonfasting plasma glucose levels in control or diabetic animals. Data are the mean ± S.E.M. (n = 10 animals per group); $^{**}P < 0.01$ versus groups 1 and 2, by Tukey's test.

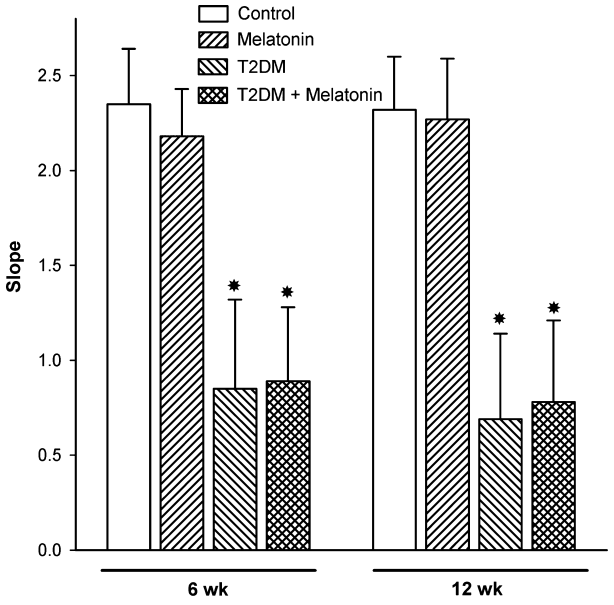

Fig. 5. Rats were fasted for 4 hr and then intraperitoneally injected with insulin (0.75 U/kg of body mass). Blood glucose levels were assessed before and 15, 30, 45 and 60 min after insulin administration. Insulin sensitivity was measured by the glucose disappearance rate within 60 min, evident from the average slope in the fitting curve. Higher insulin resistance was observed in groups 3 and 4 than in groups 1 and 2. Melatonin did not affect this parameter in control or diabetic rats. Data are means ± S.E.M. (n = 10), $^{*}P < 0.05$ versus groups 1 and 2, by Tukey's test.

from week 7 to 12, it was significantly higher in animals from groups 3 and 4, than in control animals untreated (group 1) or treated with melatonin (group 2). Melatonin did not affect the body weight in control or diabetic rats at any of the time points examined. Fasting plasma glucose levels were assessed at 6 and 12 wk of treatment (Fig. 2). At both time points, this parameter was significantly higher in animals receiving sucrose + streptozotocin (groups 3 and 4) than in control animals (groups 1 and 2), while melatonin did not affect this parameter in control or diabetic animals. Results of the IPGTT are shown in Fig. 3. At 6 or 12 wk of treatment, glucose levels assessed before and 30, 60, 90, and 120 min after an i.p. administration of 1 g/kg glucose and the average AUC were calculated for all the experimental groups. At both time points, higher values of AUC were observed for diabetic groups as compared with control groups, while melatonin did not affect this parameter. At 6 and 12 wk of treatment, plasma glucose levels were assessed at 11.00 and 23.00 hr in nonfasted animals (Fig. 4). At 6 and 12 wk, diurnal (i.e., at 11.00 hr) and nocturnal (i.e., at 23.00 hr) glycemia was significantly higher in diabetic animals as compared with control animals. Melatonin did not modify diurnal or nocturnal glycemia in control or diabetic animals. Insulin resistance was significantly higher in diabetic animals untreated or treated with melatonin than in control animals with or without melatonin, as shown in Fig. 5.

The functional state of the retinas was analyzed by scotopic electroretinography. The average amplitude of scotopic ERG a-wave, b-wave, and OP amplitudes at 12 wk of treatment and representative ERG traces are depicted in Fig. 6. These parameters were significantly reduced in diabetic animals as compared with control animals. Melatonin significantly prevented the effect of experimental T2DM on these parameters. No differences in the ERG a-wave, b-wave, and OP latencies were observed among groups (data not shown).

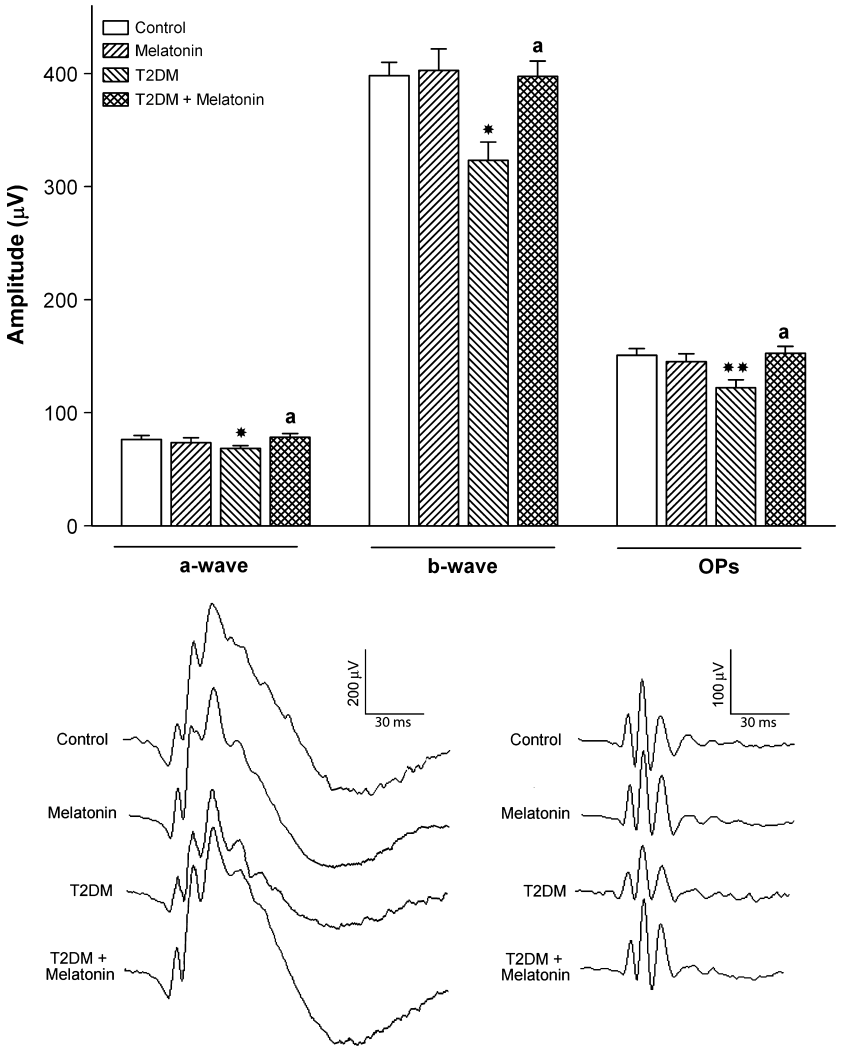

Fig. 6. Effect of melatonin on retinal function. Upper panel: Average amplitudes of scotopic electroretinogram (ERG) a-wave, b-wave and oscillatory potentials (OPs). At 12 wk of treatment, a significant decrease in the amplitude (but not their latencies) of ERG a-wave, b-wave, and OP amplitude was observed in group 3 as compared with groups 1 and 2. Melatonin significantly prevented the retinal dysfunction induced by the combination of a sucrose-enriched diet and a low dose of streptozotocin (STZ). Lower panel: representative scotopic ERG traces from all experimental groups. Data are the mean ± S.E.M. (n = 15), $*P < 0.05$, $**P < 0.01$ versus groups 1 and 2; a: $P < 0.05$ versus group 3, by Tukey's test.

At 12 wk of treatment, the retinal structure was examined by light microscopy. The integrity of the laminar structure of the retina was intact in all groups (Fig. 7, upper panel). The immunohistochemical assessment of GFAP is shown in the middle panel of Fig. 7. In retinas from the groups 1, 2, and 4, astrocytes localized in the nerve fiber layer and ganglion cell layer (GCL) were GFAP-immunopositive, whereas in retinas from group 3, an increase in retinal GFAP levels in glial processes from the inner limiting membrane to the outer retina associated with activated Müller cells was observed. VEGF levels in the GCL from animals receiving sucrose + STZ in the absence of melatonin (group 3) increased in comparison with retinas from the control, melatonin, and melatonin + T2DM groups (Fig. 7, lower panel).

Retinal TBARS levels (an index of lipid peroxidation) and catalase activity were assessed at 12 wk of treatment. Experimental T2DM increased lipid peroxidation and decreased catalase activity, whereas melatonin prevented retinal oxidative stress in diabetic animals (Fig. 8). Retinal NOS activity and TNFα levels were significantly higher in diabetic than in control animals, whereas melatonin, which showed no effect per se, prevented the increase in these parameters, as shown in Fig. 8.

To analyze the effect of a delayed treatment with melatonin on experimental DR, the pellet of melatonin was implanted 3 wk after STZ injection, and functional studies were performed at 12 wk of treatment. As shown in Fig. 9, melatonin significantly avoided the decrease in the scotopic ERG a-wave, b-wave, and OP amplitude induced by experimental T2DM.

Discussion

The present results indicate that melatonin significantly prevented retinal alterations observed in an experimental

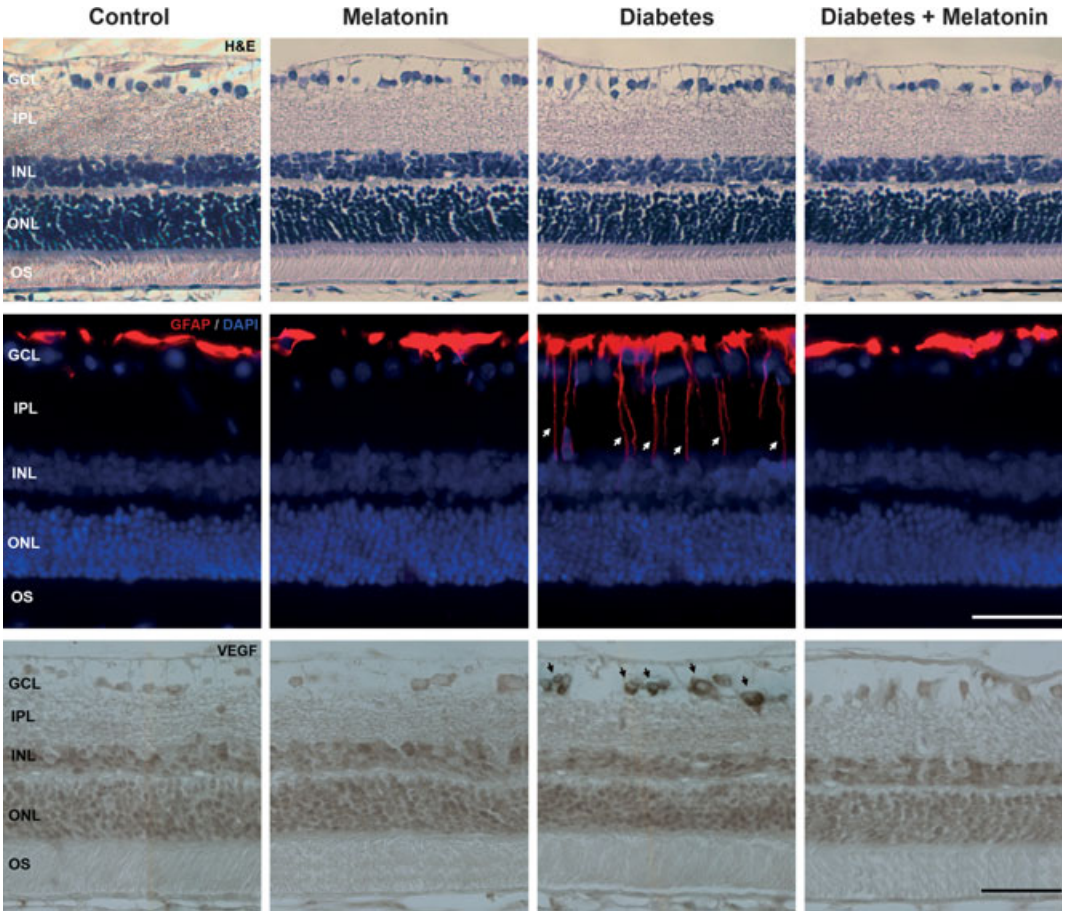

Fig. 7. Effect of experimental T2DM and melatonin on retinal histology. Upper panel: Representative photomicrographs of retinal sections from all the experimental groups stained with H and E. No evident changes in retinal morphology were observed among groups. Middle panel: Immunohistochemical detection of antiglial fibrillary acidic protein (GFAP). In retinas from group 3, an intense GFAP (+) immunoreactivity was observed in astrocytes and glial processes from the inner limiting membrane to the outer retina associated with activated Müller cells (white arrows), whereas in the other groups, GFAP immunostaining was restricted to astrocytes localized in the nerve fiber layer and GCL layer. Lower panel: vascular endothelial growth factor (VEGF) in retinal sections. In retinas from groups 1, 2, and 4, a weak VEGF-immunoreactivity was diffusely observed throughout the inner retina. In retinas from group 3, intense immunoreactivity in ganglion cell layer (GCL) cells (black arrows) was observed. Scale bar: 50 μm. IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segments of photoreceptors. Shown are photomicrographs representative of 6 retinas/group.

model in rats which mimics early stages of human T2DM. The treatment of melatonin was unable to ameliorate the metabolic abnormalities in this particular diabetes model, including body weight gain, fasting hyperglycemia, diurnal and nocturnal hyperglycemia in nonfasted animals, glucose tolerance, and insulin resistance, which suggest a direct effect of melatonin at retinal level. In this regard, we have previously shown that subcutaneously administered, melatonin reaches the retina, increasing the local levels of the methoxyindole [28].

The effect of melatonin on glucose metabolism in a diabetic background is still controversial. Agil et al. [31] show that melatonin reduces fasting hyperglycemia and improves insulin action in Zucker diabetic fatty rats (an experimental model of metabolic syndrome and T2DM), while Shieh et al. [32] have demonstrated that intraperitoneal injection of melatonin ameliorates glucose utilization and insulin sensitivity in high fat diet-induced diabetic mice. Moreover, it was demonstrated that long-term melatonin administration to noninsulin-dependent diabetic Otsuka Long-Evans Tokushima Fatty rats results in reduced hyperinsulinemia, and decreased insulin resistance [33], and similar results were reported in studies on patients with type 2 diabetes [34]. In contrast, Peschke et al. [35] show that glucose levels and several parameters of glucose sensing were unchanged after melatonin administration in both *Wistar* and type 2-diabetic Goto-Kakizaki rats [35], while Sudnikovich et al. [36] do not find effects of melatonin on hyperglycemia or glycated hemoglobin levels in STZ-induced diabetes, and similar results were reported by Guven et al. [37]. In our experimental setting, a subcutaneous pellet of melatonin did not change glucose metabolism in the model of T2DM induced by a combination of a sucrose-enriched diet and a low dose of STZ. Currently, we do not have any explanation for the lack of effect of melatonin on glucose metabolism in this T2DM model. However, differences in species, experimental models, administration route, or dose could account for it.

With some exceptions, most of the antidiabetic properties of melatonin were demonstrated in the widely used

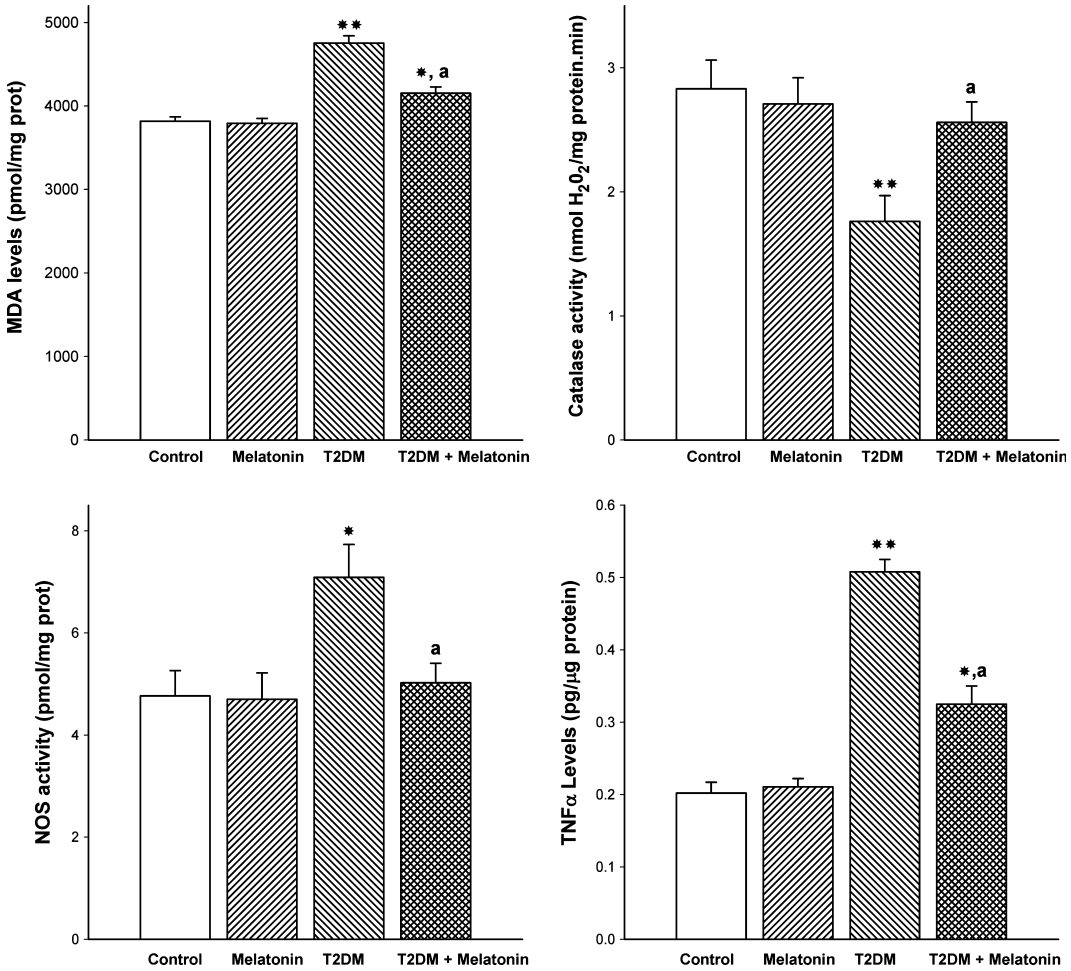

Fig. 8. Retinal thiobarbituric acid reactive substances (TBARS), catalase and NOS activity, and TNFα levels assessed at 12 wk of treatment. Lipid peroxidation, NOS activity, and TNFα levels were significantly higher, and catalase activity was significantly lower in group 3 than in groups 1, 2, and 4. Data are mean ± S.E. (n = 10), $*P < 0.05$, $**P < 0.01$ versus groups 1, 2; a: $P < 0.05$ versus group 3, by Tukey's test.

STZ-induced type 1 diabetes rat model. High doses of STZ rapidly lead to destruction of pancreatic β-cells with acute insulin deficiency, body weight loss, and a pronounced reduction in life span, which markedly differ from the clinical and metabolic features that characterize human T2DM, and from the metabolic alterations induced by this experimental model of early stages of T2DM in rats. In addition, while the evidence accumulated to date is strongly suggestive of the utility of melatonin as an agent that may reduce the multiple side effects of hyperglycemia, so far, its therapeutic effect on DR has been poorly examined, mainly considering the high incidence of this visual alteration.

The ERG is considered a sensitive marker of early neuronal abnormalities, long before DR can be clinically detected [38]. As shown herein, the combined treatment induced significant alterations in the retinal function which were prevented by melatonin. Moreover, the delayed treatment with melatonin (started 3 wk after STZ injection) resulted in a similar functional protection when compared to eyes treated from the onset of the metabolic disarrangement (i.e., 48 hr after STZ injection).

It has been postulated that Müller cells may be critical in the initial steps of retinopathy in T2DM [39]. GFAP is an intermediate filament that is normally expressed in astrocytes but not in retinal Müller cells. However, in a variety of retinal injuries, including STZ-induced DR [40], GFAP expression in retinal Müller cells became evident. Therefore, GFAP expression in Müller cells is widely used as a molecular indicator for retinal stress. The combined treatment provoked alterations in Müller cells (as shown by the increase in GFAP immunoreactivity), whereas melatonin significantly prevented the reactive expression of GFAP in Müller cells. In agreement, it was shown that melatonin decreases retinal glia reactivity in STZ-induced diabetes in rats [19].

Although the pathogenesis of DR is still far from being fully understood, VEGF is recognized as a major contributor to its development and is implicated as the initiator and mediator of nonproliferative and proliferative DR [41, 42]. In fact, clinical trials using anti-VEGF therapies are showing promising results against advanced stages of DR [43]. Our results indicate that melatonin treatment reduced retinal VEGF levels in diabetic rats.

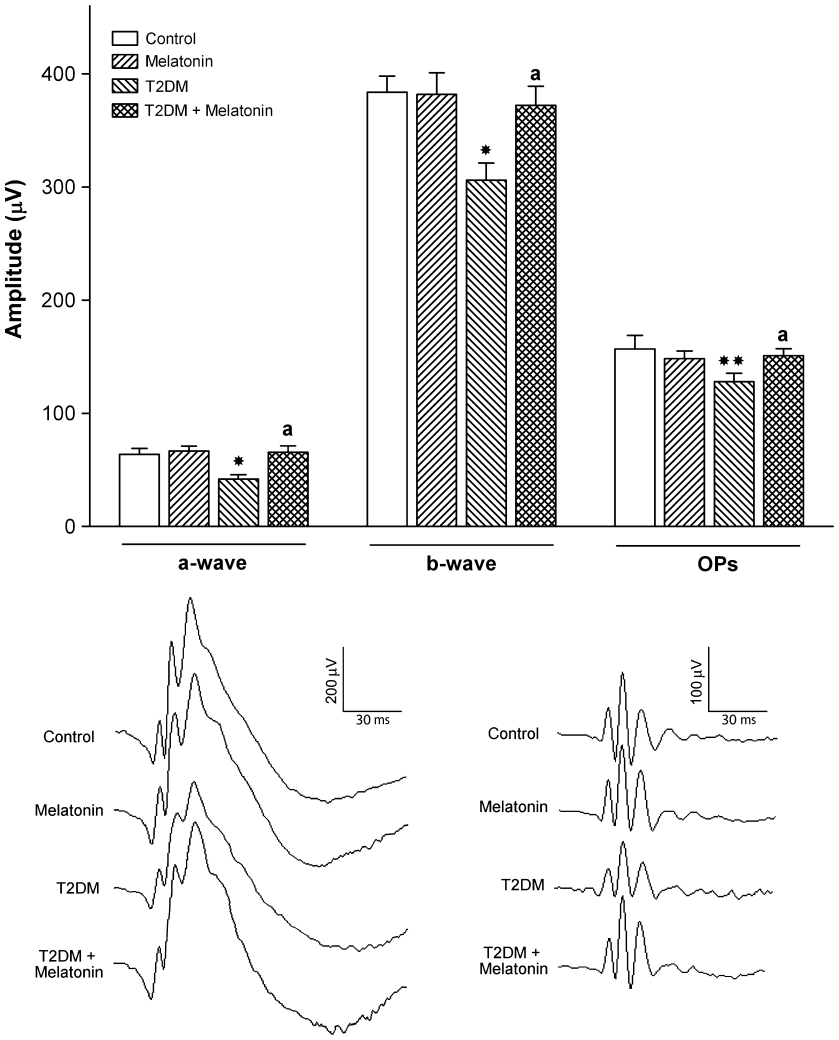

Fig. 9. Effect of a delayed treatment with melatonin on the retinal dysfunction induced by experimental T2DM. Three weeks after vehicle or streptozotocin (STZ) injection, a pellet of melatonin was implanted subcutaneously, and the scotopic electroretinogram (ERG) was assessed at 12 wk of treatment. Upper panel: average amplitudes of scotopic ERG a-wave, b-wave, and oscillatory potentials (OPs). Experimental T2DM significantly decreased ERG a-wave, b-wave and OP amplitude, whereas the treatment with melatonin significantly reversed the effect of experimental diabetes. Data are the mean ± S.E.M. (n = 15), $*P < 0.05$, $**P < 0.01$ versus groups 1 and 2; a: $P < 0.05$ versus group 3, by Tukey's test. Lower panel: representative scotopic ERG traces.

Enhanced oxidative stress and declines in antioxidant capacity are considered to play important roles in the pathogenesis of chronic diabetes mellitus and its complications [16, 44]. A significant increase in retinal lipid peroxidation and NOS activity, as well as a decrease in catalase activity was observed in diabetic animals, which were prevented by melatonin treatment. In that sense, it was shown that melatonin reduces retinal lipid peroxidation [19] and enhances the inhibited activity of catalase in the cytoplasm of liver cells and from rats with STZ-induced diabetes [45]. In DR, abnormalities in vascular and neuronal function are closely related to the local production of inflammatory mediators [46]. A significant increase in retinal TNFα levels was observed in rats submitted to the combined treatment, whereas melatonin prevented the increase in this parameter. In agreement with these results, it was demonstrated that melatonin treatment reduces the elevated levels of TNF-α and NOS in sciatic nerves of diabetic animals [47].

Oxidative, nitrosative, and inflammatory mechanisms appear as a unified pathway leading to metabolic deterioration resulting from hyperglycemia, and insulin resistance [48]. The present results suggest that melatonin could be a promissory resource in the management of DR because by itself exhibits antioxidant, antinitridergic, and anti-inflammatory properties at retinal level.

In summary, these results suggest that melatonin prevented retinal alterations observed in an experimental model which mimics early stages of human T2DM. Thus, as melatonin is a molecule with very low-toxicity profile at virtually any dose and even after long-term usage [49, 50], melatonin could be a new therapeutic tool for the treatment of retinal alterations induced by diabetes.

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