·Clinical Research·

Increased melatonin levels in aqueous humor of patients with proliferative retinopathy in type 2 diabetes mellitus

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Abstract

• AIM: To report the association between melatonin levels in aqueous humor and serum, and diabetic retinopathy (DR) grade in type 2 diabetic patients.

• METHODS: Aqueous humor and plasma samples from 26 patients with DR (in nonproliferative and proliferative stages) and 14 control subjects were collected during cataract surgery after 6 p.m. Melatonin concentrations were determined using an enzyme-linked immunosorbent assay (ELISA).

• RESULTS: Melatonin levels were significantly higher in the aqueous humor of patients with proliferative diabetic retinopathy (PDR) [18.57±2.67 pg/mL (range 15.20-23.06) *vs* 13.63±2.71 pg/mL (range 10.20-20.20), *P*=0.0001], but not in those with nonproliferative retinopathy (NPDR) [13.79±2.56 pg/mL (range 9.80-20.10) *vs* 13.63±2.71 pg/mL (range 10.20-20.20), *P*=0.961] compared to controls. There was decrement in the plasma melatonin level of patients with PDR, but no significant differences between the plasma melatonin levels of the study groups [5.37±1.74 pg/mL (range 2.85-8.65) *vs* 6.11±1.90 pg/mL (range 3.13-9.41), *P* =0.293], or between control and DR groups [NPDR 6.11±1.90 pg/mL (range 3.13-9.41) *vs* control 6.15±1.91 pg/mL (range 2.18-9.86); PDR (5.37±1.74 pg/mL (range 2.85-8.65) *vs* control 6.15±1.91 pg/mL (range 2.18-9.86), *P* =0.808, *P* =0.264].

• CONCLUSION: Elevated melatonin levels in aqueous humor in PDR may indicate the level to be associated with DR severity.

• KEYWORDS: aqueous humor; diabetes mellitus; diabetic retinopathy; melatonin
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INTRODUCTION

Diabetic retinopathy (DR) is one of the three major causes for visual impairment in Western countries. A recent assessment of DR in the United States showed a high prevalence of 28.5% among those with diabetes over 40 years old [1]. Diabetes mellitus (DM) activates pro-inflammatory pathogenetic pathways *i.e.*, the polyol, protein Kinase C, hexosamine, and renin angiotensin system (RAS) pathways, as well as advanced glycation end products formation. Molecular changes result in blood flow alterations, formation of reactive oxygen species (ROS) and oxidative stress, induction of inflammatory signaling systems and inflow of leukocytes, and ultimately altered gene transcription, in turn promoting the biomolecular DR characteristics. Formation of ROS is increased in DM and hyperglycemia, and is directly related to vascular dysfunction and other complications in diabetes [2]. The primary source of ROS is considered to be overproduction of superoxide anions by the mitochondrial electron transport chain (ETC)[3]. This increase in superoxide formation leads to oxidative damage of mitochondrial and cellular lipids, proteins, and nucleic acids.

Melatonin (N-acetyl-5-methoxytryptamine) is a neurohormone found in all mammals, including humans, and is mainly synthesized in pinealocytes. Its synthesis also occurs in other tissues, such as bone marrow, gut, gastrointestinal tract, lymphocytes, and in various parts of the eye including retina, ciliary body and lachrymal gland [4-6]. In the eye, melatonin may contribute to the regulation of retinomotor movements, rod outer segment disc shedding, dopamine synthesis and release, and intraocular pressure, and as an effective antioxidant and free radical scavenger [7-9]. It also protects the photoreceptor outer segment and other ocular tissues from oxidative damage[10-13].

The aim of our current study was to investigate the association between melatonin levels in aqueous humor and serum, and DR grade in type 2 diabetic patients.

SUBJECTS AND METHODS

Subjects All procedures were conducted in accordance with the Declaration of Helsinki, and informed consent was obtained from all patients after approval from the

Table 1 Clinical and laboratory characteristics of the diabetic and control groups

Parameters	NPDR (n=13)	PDR (n=13)	Control (n=14)	P
Age (a)	67.5±6.5	63.1±9.9	66.6±8.9	[1]0.564
Sex (M/F)	5/8	5/8	3/11	[1]0.556
DM (a)	12.21±3.98	18.0±6.56		[2]0.009
HbA1C (%)	6.9±0.6	7.4±1.1		[2]0.980
Fakia/pseudofakia (fellow eye)	7/6	5/8	7/7	[1]0.834
MLTN (aqueous humor)	13.79±2.56	18.57±2.67	13.63±2.71	[1]0.0001
MLTN (plasma)	6.11±1.90	5.37±1.74	6.15±1.91	[1]0.447

[1]Kruskal Wallis test analysis; [2]Mann-Whitney U test analysis. MLTN: Melatonin.

Institutional Review Board. The subjects were 40 patients (a total of 40 eyes) who had undergone cataract surgery in the night-time (6 p.m. and later). DR was documented during a standard fundus examination in all patients, who underwent fluorescein angiography (FA) if necessary to evaluate the presence of retinal neovascularization. No patients who had hypertension, cardiac arrhythmia, and concomitant drugs affecting the autonomic nervous system, including α and β adrenergic receptor blockers and other sympatholytic agents, antidepressants, anorectics, cigarette smoking, sleep disorders, clinically significant macular edema, glaucoma, were recruited in the study. The patients with non proliferative retinopathy had mild or no retinopathy without macular edema. The characteristics of patients with nonproliferative diabetic retinopathy (NPDR), proliferative diabetic retinopathy (PDR) and non diabetic subjects (controls) are summarized in Table 1. In literature, the severity of diabetic retinopathy was graded according to the modified Early Treatment Diabetic Retinopathy Study (ETDRS) retinopathy severity scale [14] and International Clinical Diabetic Retinopathy disease severity scale[15].

All of the patients with type 2 DM were evaluated by careful biomicroscopic examination using a fundus non contact lens (78 D). Fundus findings were confirmed preoperatively by standardized fundus color photography and FA, which was performed with a Topcon TRC-50IA fundus camera, an image-net system (Tokyo Optical Co. Ltd., Japan), and a preset lens with a slit-lamp.

Sample Collection and Measurement After obtaining patient consent, the aqueous humor fluid (0.1-0.2 mL) was collected into sterile tubes during cataract surgery and then was rapidly frozen to -40℃. Aqueous humor and plasma samples were used for measurement of melatonin activity.

Melatonin levels were determined with a commercially supplied ELISA kit (RE 54021; IBL, Hamburg, Germany). The serum samples have to be extracted in advance. For column preparation, 1 mL methanol was added to the columns and passed through the column by centrifugation for 1min at 200 g, and then 1 mL double distilled water was added to the columns in the same procedures.

For serum extraction, 0.5 mL standard, control or plasma samples were added to the columns and centrifuged for 1min at 200 g. After washing the columns with 1 mL, 10% methanol and then centrifuging for 1min at 500 g, the extracts were eluted with 1 mL methanol by centrifugation for 1min at 200 g. The methanol was then evaporated to dryness, using the evaporator centrifuge and the dried extracts were reconstituted in 150 μL double distilled water. After this, 50 μL extracted standard, control or serum samples were transferred into the appropriate wells of the microtitre plate. To these wells, 50 μL melatonin biotin and 50 μL melatonin antiserum were added. Following sealing with adhesive foil, the plate was carefully shaken and incubated for 20h at 4℃. Each well was then washed three times with assay buffer and added with 150 μL enzyme conjugate. The plate was sealed again and incubated for 120 min at room temperature on an orbital shaker at 500 rpm. After incubation and washing three times with assay buffer, each well was then added with 200 μL freshly prepared paranitrophenyl phosphate (PNPP) substrate solution. The plate was further incubated for 30min at room temperature on an orbital shaker. The substrate reaction was stopped by adding 50 μL of PNPP stop solution into the wells. The absorbance values were measured by a spectrophotometer. Finally, 50 μL of PNPP stop solution were added to each well, and the optical density was measured with a photometer at 405 nm (reference wavelength: 600-650 nm)[16].

Statistical Analysis For statistical analysis, variables were determined to have abnormal distribution by the Shapiro-Wilk Tests, and Kruskal-Wallis Tests were used in the comparisons of groups. For the comparison of differences within groups the Mann-Whitney U test was used. For categorical variable (gender), Chi-square tests were used to find out differences between groups. A P<0.05 was defined as statistically significant. Statistical analyses were performed using the Statistical Package for Social Science's software (SPSS version 15, SPSS Inc., Chicago, IL, USA).

RESULTS

The demographic and clinical data of the diabetic patients and controls are summarized in Table 1. No statistically significant differences were noted among the groups with regard to age and sex distribution (P=0.564, P=0.556, respectively). There were significant differences between the DM duration (P=0.009), but no significant differences in

HbA1c levels of NPDR and PDR groups (P=0.980).

Aqueous humor and plasma melatonin concentrations, shown as mean±standard deviation (range, pg/mL), for the NPDR group were 13.79±2.56 pg/mL (range 9.80-20.10), and 6.11± 1.90 pg/mL (range 3.13-9.41), respectively, and for the PDR group were 18.57±2.67 pg/mL (range 15.20-23.06) and 5.37± 1.74 pg/mL (range 2.85-8.65), respectively. The aqueous humor and plasma melatonin concentrations for the control group were 13.63±2.71 pg/mL (range 10.20-20.20) and 6.15± 1.91 pg/mL (range 2.18-9.86), respectively (Table 1). When the study groups were compared with the control group, melatonin levels were found to be significantly increased in the aqueous humor of the patients with PDR (P=0.0001), but no remarkable increased in the aqueous humor of the patients with NPDR (P=0.981). There was decrement in the plasma melatonin level of patients with PDR, but no significant differences between the plasma melatonin levels of the study groups (P=0.293), or between control and study groups (NPDR *vs* control ;PDR *vs* control, respectively) (P=0.808; P=0.264).

The significant correlations were detected between the duration of DM and melatonin levels in the aqueous humor of diabetic patients, between the levels of HbA1c and melatonin levels in the aqueous humor of diabetic patients (Spearman's rho: 0.572, P=0.002: Spearman's rho: 0.438; P=0.025).

DISCUSSION

Oxidative stress is implicated in the etiology of many ocular diseases such as glaucoma, retinal degeneration, ocular inflammation, cataracts, and diabetic complications [13,17]. Diabetes has been shown to be a state of increased free radical production [18]. Various mechanisms contribute to the formation of free radicals in diabetes, and may include not only increased non-enzymatic and auto-oxidative glycosylation, but also metabolic stress resulting from changes in energy metabolism, levels of inflammatory mediators, and the status of antioxidant defense[19]. Moreover, elevated levels of various reactive oxygen and nitrogen species have been identified in diseased ocular structures. These reactants damage the structure and deplete the eye of natural defense systems, such as antioxidants, glutathione, and the antioxidant enzyme, superoxide dismutase (SOD). Oxidative damage in the eye leads to the apoptotic degeneration of retinal neurons and fluid accumulation.

Melatonin continuously regenerates and offers a frontier anti-oxidative defense for both the anterior and posterior eye against conditions such as photo-keratitis, cataract, glaucoma, retinopathy of prematurity, and ischemia/reperfusion injury[10]. Immunocytochemical analysis of ocular tissues obtained from various animals like chickens, rats, and humans has shown that melatonin receptors (MT1, MT2) are distributed in the cornea, choroid, sclera, photoreceptors, RGCs and retinal blood vessels [20-22]. Additionally, the existence of melatonin receptors in the iris and ciliary processes has led to the proposal that they are involved in aqueous humor secretion and the circadian rhythm of intraocular pressure [23]. The existence of melatonin biosynthetic pathway in the mammalian retina was initially supported by the discovery of retinal hydroxyindole-O-methyl transferase (HIOMT) activity. The gene encoding HIOMT is selectively expressed in retinal photoreceptor cells that rhythmically secrete melatonin suggests that photoreceptors contain an endogenous "clock" that regulates melatonin biosynthesis [24]. This has been confirmed in the mammalian retina, photoreceptors, either rods or cones, contain circadian oscillators[25].

Melatonin treatment increased SOD and glutathione S-transferase (GST) activities in plasma, erythrocyte lysate and liver and kidney tissue [26-27]. Reiter *et al* [28] reported that melatonin led to a dramatic decrease in free radical production. It has been demonstrated in some studies that melatonin and anti-oxidant (SOD, GST) concentrations are elevated in both the vitreous fluid and aqueous humor of patients with active proliferative diabetic retinopathy [29-30]. Recent studies have demonstrated a close correlation between oxidative stress and morphological changes in the trabecular meshwork, highlighting that anterior chamber involvement in patients with PDR may be caused partly by redox-state imbalances [31-32]. In some human studies, decreased plasma melatonin levels were reported in type 2 DM [33-34]. Hikichi *et al* [35] demonstrated that serum melatonin levels were significantly lower in the diabetic group than in the non-diabetic group, and were lower in the PDR group than in the non-diabetic and NPDR groups, but no significant difference was found between the non-diabetic and NPDR groups. In our study, the melatonin levels of patients with PDR were found to be lower in plasma, but significantly higher in aqueous humor compared to NPDR patients and controls. These findings showed accordance with the results of plasma melatonin studies in Literature. Furthermore, there was a significant correlation between the duration of DM and aqueous humor melatonin levels of diabetic patients in our study. All patients in the PDR group of our study underwent panretinal photocoagulation. Retinal dysfunction induced not only by advanced diabetic retinopathy but also by panretinal photocoagulation should be associated with diminished retinal light perception. Bughi *et al* [36] reported that bilateral photocoagulation for PDR may be associated with loss of normal circadian cortisol variation.

Consequently, we speculated that the advanced retinal dysfunction alters serum melatonin secretion in the patients with PDR due to light perception loss. On the other hand, elevated melatonin levels in aqueous humor may be linked to actively anti-oxidative, anti-angiogenic processes and morphological changes of the trabecular meshwork in type 2 diabetics. We propose that melatonin level in aqueous humor may increase with the severity of DR, and that the levels of melatonin in aqueous samples reflect the intraocular

concentrations. Main limitations of this study were its small sample size and high standard deviations of the mean concentrations of melatonin. Further studies with larger patient cohorts are required, however, it looks like that melatonin may be promising for the treatment of DR in the future.

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REFERENCES

1 Zhang X, Saaddine JB, Chou CF, Cotch MF, Cheng YJ, Geiss LS, Gregg EW, Albright AL, Klein BE, Klein R. Prevalence of diabetic retinopathy in the United States, 2005-2008. *JAMA* 2010;304(6):649-656.

2 Giacco F, Brownlee M. Oxidative stress and diabetic complications. *Circ Res* 2010;107(9):1058-1070.

3 Naudi A, Jove M, Ayala V, Cassanye A, Serrano J, Gonzalo H, Boada J, Prat J, Portero-Otin M, Pamplona R. Cellular dysfunction in diabetes as maladaptive response to mitochondrial oxidative stress. *Exp Diabetes Res* 2012;2012:696215.

4 Tosini G, Pozdeyev N, Sakamoto K, Iuvone PM. The circadian clock system in mammalian retina. *Bioessays*2008;30:624-633.

5 Martin XD, Malina HZ, Brennan MC, Hendrickson PH, Lichter PR. The ciliary body-the third organ found to synthesize indoleamines in humans. *Eur J Ophthalmol* 1992;2(2):67-72.

6 Mhatre MC, van Jaarsveld AS, Reiter RJ. Melatonin in the lacrimal gland: first demonstration and experimental manipulation. *Biochem Biophy Res Commun* 1988;153(3):1186-1192.

7 Besharse JC, Dunis DA. Methoxyindoles and photoreceptor metabolism: activation of rod shedding. *Science*1983;219(4590):1341-1343.

8 Sengupta A, Baba K, Mazzoni F, Pozdeyev NV, Strettoi E, Iuvone PM, Tosini G. Localization of melatonin receptor 1 in mouse retina and its role in the circadian regulation of the electroretinogram and dopamine levels. *PLoS One* 2011;6(9):e24483.

9 Agorastos A, Huber CG. The role of melatonin in glaucoma: implications concerning pathophysiological relevance and therapeutic potential. *J Pineal Res* 2011;50(1):1-7.

10 Siu AW, Maldonado M, Sanchez-Hidalgo M, Tan DX, Reiter RJ. Protective effects of melatonin in experimental free radical-related ocular diseases. *J Pineal Res* 2006;40(2):101-109.

11 Lundmark PO, Pandi-Perumal SR, Srinivasan V, Cardinali DP. Role of melatonin in the eye and ocular dysfunctions. *Vis Neurosci* 2006;23(6):853-862.

12 Rosen RB, Hu D,Chen M, McCormick SA, Walsh J, RobertsJE. Effects of melatonin and its receptor antagonist on retinal pigment epithelial cells against hydrogen peroxide damage. *Mol Vis* 2012;18:1640-1648.

13 Mirunalini S, Karthishwaran K, Dhamodharan G, Mohan S. Melatonin attenuates lipid peroxidation and enhances circulatory antioxidants during mammary carcinogenesis in rats. *J Biochem Tech* 2010;2(3):171-174.

14 Grading diabetic retinopathy from stereoscopic color fundus photographs-an extension of the modified Airlie House classification. ETDRS report number 10. Early Treatment Diabetic Retinopathy Study Research Group. *Ophthalmology* 1991;98 (Suppl 5):786-806.

15 Wilkinson CP, Ferris FL 3rd, Klein RE, Lee PP, Agardh CD, Davis M, Dills D, Kampik A, Pararajasegaram R, Verdaguer JT; Global Diabetic Retinopathy Project Group. Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. *Ophthalmology* 2003;110(9):1677-1682.

16 Khaleghipour S, Masjedi M, Ahade H, Enayate M, Pasha G, Nadery F, Ahmadzade G. Morning and nocturnal serum melatonin rhythm levels in patients with major depressive disorder: an analytical cross-sectional study. *Sao Paulo Med J* 2012;130(3):167-172.

17 Ohia SE, Opere CA, Leday AM. Pharmacological consequences of oxidative stress in ocular tissues. *Mutat Res* 2005;579:22-36.

18 Yang H, Jin X, Kei Lam CW, Yan SK. Oxidative stress and diabetes mellitus. *Clin Chem Lab Med* 2011;49(11):1773-1782.

19 Griesmacher A, Kindhauser M, Andert SE, *et al* Enhanced serum levels of thiobarbituric-acid-reactive substances in diabetes mellitus. *Am J Med* 1995;98(5):469-475.

20 Scher J, Wankiewicz E, Brown GM, Fujieda H. MT (1) melatonin receptor in the human retina: expression and localization. *Invest Ophthalmol Vis Sci* 2002;43(3):889-897.

21 Wiechmann AF, Rada JA. Melatonin receptor expression in the cornea and sclera. *Exp Eye Res* 2003;77(2):219-225.

22 Rada JA, Wiechmann AF. Melatonin receptors in chick ocular tissues: implications for a role of melatonin in ocular growth regulation. *Invest Ophthalmol Vis Sci* 2006;47(1):25-33.

23 Alarma-Estrany P, Pintor J. Melatonin receptors in the eye: location, second messengers and role in ocular physiology. *Pharmacol Ther* 2007;113(3):507-522.

24 Cahill GM, Besharse JC. Circadian clock functions localized in xenopus retinal photoreceptors. *Neuron*1993;10(4):573-577

25 Tosini G, Iuvone PM (Eds.). Chapter 4, Role of Melatonin and Dopamine in the Regulation of Retinal Circadian Rhythms. *The Retina and Circadian Rhythms*Germany: Springer; 2014;49-68.

26 Anwar MM, Meki AR. Oxidative stress in streptozotocin-induced diabetic rats: effects of garlic oil and melatonin. *Comp Biochem Physiol A Mol Integr Physiol* 2003;135(4):539-547.

27 Tan DX, Reiter RJ, Manchester LC, Yan MT, El-Sawi M, Sainz RM, Mayo JC, Kohen R, Allegra M, Hardeland R. Chemical and physical properties and potential mechanism: melatonin as a broad spectrum antioxidant and free radical scavenger. *Curr Top Med Chem* 2002;2(2):181-198.

28 Reiter RJ, Tan DX, Mayo JC, Sainz RM, Lopez-Burillo S. Melatonin, longevity and health in the aged: an assessment. *Free Radic Res* 2002;36(12):1323-1329.

29 Chiquet C, Claustrat B, Thuret G, Brun J, Cooper HM, Denis P. Melatonin concentrations in aqueous humor of glaucoma patients. *Am J Ophthalmol* 2006;142(2):325-327.

30 Izzotti A, Bagnis A, Saccà SC. The role of oxidative stress in glaucoma. *Mutat Res* 2006;612(2):105-114.

31 Saccà SC, Izzotti A, Rossi P, Traverso C. Glaucomatous outflow pathway and oxidative stress. *Exp Eye Res* 2007;84(3):389-399.

32 Mancino R, Pierro DD, Varesi C, Cerulli A, Feraco A, Cedrone C, Pinazo-Duran MD, Coletta M, Nucci C. Lipid peroxidation and total antioxidant capacity in vitreous, aqueous humor, and blood samples from patients with diabetic retinopathy *Mol Vis* 2011;17:1298-1304.

33 Tutuncu NB, Batur MK, Yildirir A, Tutuncu T, Deger A, Koray Z, Erbas B, Kabakci G, Aksoyek S, Erbas T. Melatonin levels decrease in type 2 diabetic patients with cardiac autonomic neuropathy. *J Pineal Res* 2005;39(1):43-49.

34 Peschke E, Frese T, Chankiewitz E, Peschke D, Preiss U, Schneyer U, Spessert R, Mühlbauer E. Diabetic Goto Kakizaki rats as well as type 2 diabetic patients show a decreased diurnal serum melatonin level and an increased pancreatic melatonin-receptor status. *J Pineal Res* 2006;40(2):135-143.

35 Hikichi T, Tateda N, Miura T. Alteration of melatonin secretion in patients with type 2 diabetes and proliferative diabetic retinopathy. *Clin Ophthalmology* 2011;5:655-660.

36 Bughi S, Shaw S, Bessman A. Laser damage to retinal ganglion cells: the effect on circadian rhythms. *J Diabetes Complication* 2006;20(3):184-187.