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Short-term melatonin treatment improved diabetic nephropathy but did not affect hemorheological changes in diabetic rats

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Increased oxidative stress and hemorheological disturbances may play very important roles in the development of microangiopathies in diabetes mellitus. This study was designed to determine the healing effect of melatonin on hemorheological parameters and diabetic nephropathy in streptozotocin (STZ)-induced diabetic rats. Wistar male rats were divided into four groups as control, untreated-diabetic, melatonin-treated control and melatonin-treated diabetic rats. Diabetes was induced by injecting streptozotocin (45 mg/kg, i.p.). Fourteen weeks after inducement of diabetes, melatonin (10 mg/kg) was administered intraperitoneally for 5 days to the rats. Erythrocyte deformability and aggregation were measured by laser differaction analysis (LORCA). Diabetic nephropathy was assessed by histopathologic evaluation and TUNEL stain in the diabetic kidney. Decreased erythrocyte deformability and increased erythrocyte aggregation indices were determined in the diabetic group. Melatonin treatment did not improve these hemorheological abnormalities. However, renal injuries were diminished in the melatonin-treated diabetic group compared to the untreated diabetic group. Also, melatonin had an antiapoptotic effect on the diabetic kidney. It was concluded that i.p. administration of melatonin for 5 days improved renal injury in diabetic rats, probably by decreasing oxidative stress, but did not affect hemorheological changes.

1. Introduction

The high morbidity and excess mortality of diabetic patients are predominantly determined by vascular dysfunctions. Diabetic microangiopathy not only manifests as nephropathy, retinopathy and delayed wound healing, but also predisposes the patient to the development of coronary artery, cerebrovascular and peripheral arterial diseases (Calles-Escandon and Cipolla 2001; Creager et al. 2003). The pathophysiology of diabetic vasculopathy involves the dysfunction of the endothelium, which is caused by increased production of reactive oxygen species as a consequence of hyperglycemia (Tesfaramiam and Cohen 1992; Cınar et al. 2001; Paskaloglu et al. 2004).

Blood rheology alterations have been implicated in vascular complications both in diabetic animals (Diamantopoulos et al. 2004; Kipiani et al. 2006) and in patients (Solerte et al. 1997; Le Devehat et al. 2004). In diabetic patients, long term hyperglycemia may result in an increase in both lipid peroxidation in erythrocyte membranes (Jain et al. 1989; Chung et al. 1998) and oxidation of membrane spectrin (Schwartz et al. 1991) and the oxidative stress may cause abnormalities in erythrocyte membranes and blood rheological properties (Huang et al. 2004). The main factors that determine hemorheology are whole blood viscosity, hematocrit, erythrocyte deformability, erythrocyte aggregation and fibrinogen concentration. Erythrocyte deformability (the ability to change shape and turn back to the previous form) depends on erythrocyte geometry, membrane flexibility and intracellular viscosities. Anomalies in blood rheology and increased erythrocyte aggregation are prominent in patients with poor glycemic control and some studies have suggested that the rheological alterations and vascular complications are associated with the HbA1c level (Schwartz et al. 1991; Le Devehat et al. 2004).

Melatonin is a powerful antioxidant that is secreted from the pineal gland. Previous studies have shown that melatonin may have some effects on blood cells. It has been shown that melatonin regulates the rhythm of erythrocyte production (Karimungi and Joshi 1996) and has an effect on the membrane skeleton that has a significant role in erythrocyte deformability (Medina-Navarro et al. 1999; Tesoriere et al.1999; Di Mascio et al. 2000). Furthermore, the studies have shown the protective effect of melatonin against the emergence of vascular complications resulting from diabetes in kidneys (Cam et al. 2003; Oktem et al. 2006), but its healing effect on the complications that occurred was not studied.

The purposes of this study were: 1) to investigate the healing effect of melatonin on the hemorheological abnormalities and vascular histopathological changes that occur due to chronic diabetes 2) to determine the effect

of melatonin on apoptosis in kidney tissues of diabetic rats 3) to investigate the relationship between HbA_{1c} and hemorrheological alterations in chronic diabetes, and 4) to compare postmortem HbA_{1c} levels in diabetic and control groups which is a preliminary study that investigates the correlation between postmortem HbA_{1c} levels and diabetes mellitus.

2. Investigations, results and discussion

Increased blood glucose concentration in diabetes results in systemic oxidative stress. Although many studies propose a relationship between diabetic complications and glucose level, its actual mechanism has not yet been clearly defined. Hyperglycemia causes glycosylation of circulating and cellular protein and changes in intracellular metabolism (activation of the polyol pathway and increased diacylglycerol and Ca^{2+} and activation of the mitogen-activated protein kinase (MAPK) pathway) and the production of advanced glycation end products, leading to cellular dysfunction and an increase in free radical levels (Yerer et al. 2003; Paskaloglu et al. 2004). Long term hyperglycemia may cause an increase in both lipid peroxidation in erythrocyte membranes and oxidation of membrane spectrin. This may cause abnormalities in erythrocyte membranes and alter the hemorheological properties of erythrocytes (Huang et al. 2004). Merely increasing glucose concentration at moderate levels may alter the rheological properties of erythrocytes *in vitro* (Riquelme et al. 2005). In our study, there was a significant decrease in the elongation index (EI) of erythrocytes in diabetic rats indicating a loss of deformability.

Oxidative stress is one of the major factors that affect the hemorheological properties of erythrocytes. Previous studies have shown that excess oxidative stress may result in erythrocyte membrane peroxidation and decrease red blood cell deformability which is believed to be highly critical for an effective microcirculatory function (Yerer et al. 2003; Aydogan et al. 2004). There are several sources of reactive oxygen species (ROS) in diabetes, including defective mitochondrial metabolism (Nishikawa et al. 2000), glucose autooxidation (Beckman et al. 2001), NADPH oxidase activation (Inoguchi et al. 2000) and synthesis of advanced glycation end products (Tan et al. 2002). Increased production of oxygen-derived free radicals and decreased free radical scavenger systems have been described in diabetes (Wolff and Dean 1987; Cınar et al. 2001). In both types of diabetes mellitus (I and II), impaired erythrocyte deformability and aggregation which may be related to excess ROS have been shown (Solerte et al. 1997; Huang et al. 2004; Le Devehat et al. 2004). Several potentially beneficial actions of antioxidants such as α-tocopherol nicotinate (Chung et al. 1998), vitamin E, vitamin C and selenium on hemorheologic abnormalities (Naziroglu et al. 2004) and vascular dysfunction (Gocmen et al. 2000; Cınar et al. 2001; Paskaloglu et al. 2004) have been reported in experimental and human diabetes. The protective effect of melatonin on hemorheological abnormalities has been demonstrated in septic rats (Yerer et al. 2004).

In the current study, we investigated a potential healing effect of short term melatonin treatment on hemorheological abnormalities in diabetic rats. The erythrocyte deformability index was significantly decreased in the diabetic group versus the control and melatonin-treated control groups at a shear stress force of 3.0 Pa ($p = 0.042$ and $p = 0.045$ respectively, Fig. 1A). Also, the erythrocyte aggregation index was increased in diabetic rats compared to the control and melatonin-treated control groups ($p = 0.006$ and $p = 0.0001$ respectively, Fig. 1B).

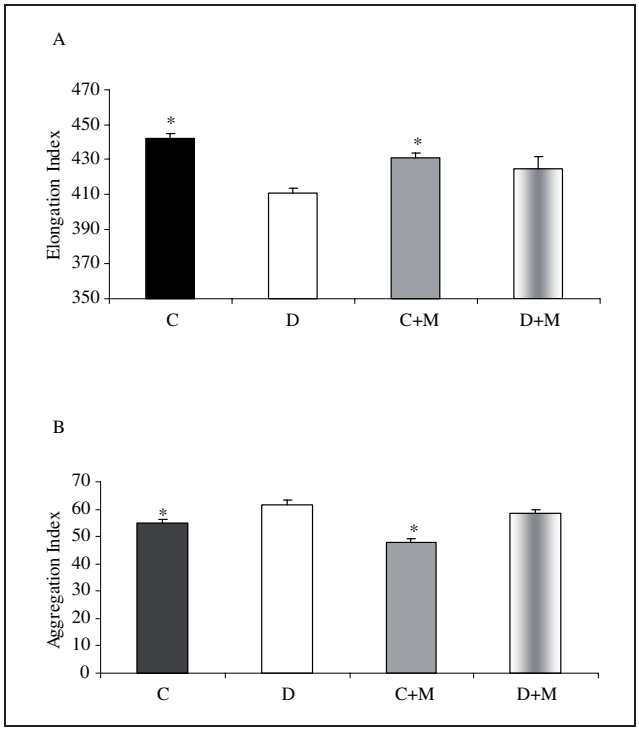

Fig. 1: Erythrocytes elongation (A) and aggregation (B) indices in the control group (C), diabetic group (D), melatonin-treated control group (C + M) and melatonin-treated diabetic group (D + M). Results are mean (± S.E.M.). * significantly different from diabetic rats, $p < 0.05$

In addition, melatonin treatment did not reverse the elongation and aggregation indices of diabetic rats to the control levels.

Melatonin is a powerful hydroxyl radical scavenger and antioxidant (Tan et al. 1993). The studies which have shown that melatonin might have an effect on blood cells are still controversial. Some authors have suggested that melatonin contributed to the reduction of oxidative damage in both the erythrocyte lipid membrane and aqueuos environment of the cell (Medina-Navarro et al. 1999; Yerer et al. 2003). It has also been shown that melatonin regulated the rhythm of erythrocyte production (Karimungi and Joshi 1996) and has an effect on the membrane skeleton that has a significant role in erythrocyte deformability (Medina-Navarro et al. 1999; Di Mascio et al. 2000). On the other hand, Tesoriere et al. (1999) showed that melatonin prevented protein peroxidation of membrane proteins and hemoglobin but the membrane lipid peroxidation in the erythrocytes. Berker et al. (2004) showed that the administration of 200 mg/kg melatonin after pinealectomy reduced erythrocyte deformability and concluded that this result implied a relationship between melatonin and erythrocyte deformability and that the inconsistency with the other studies could result from the circadian rhythm. Conflicting results in the literature may result from differences in experimental conditions or dosage and duration of melatonin treatment, circadian variations and methods used to determine the hemorheologic parameters. Although the preventive effects of melatonin on hemorheological abnormalities in different diseases have been described previously (Tesoriere et al. 1999; Aydogan et al. 2004; Yerer et al. 2004), melatonin treatment did not affect the hemorheological parameters in diabetic rats in the present study.

Table 1: Biochemical and hematological parameters in all groups. C, control; D, diabetic, C + M, melatonin-treated control and D + M, melatonin-treated diabetic groups

Groups	Number of rats	Blood glucose level (mg/dL)	Hb (g/dL)	HbA_{1c} %	Htc %
C	5	170.5 ± 5.9	13.8 ± 0.3	4.2 ± 0.1	37.1 ± 0.8
D	5	$444.6 \pm 26.3^*$	14.5 ± 0.4	$8.9 \pm 0.4^*$	40.9 ± 1.3
C + M	5	166.8 ± 4.0	13.4 ± 0.3	4.2 ± 0.7	36.4 ± 1.0
D + M	5	$456.5 \pm 43.5^*$	14.3 ± 0.5	$9.3 \pm 0.3^*$	40.0 ± 1.7

* $p < 0.05$, compared with C and C + M group. Data presented as mean ± S.E.M

Alterations in blood rheology in diabetic patients might be related to the quality of glycemic control. Le Devehat et al. (2004) determined a positive correlation between HbA_{1c} level and fibrinogen quantity, erythrocyte aggregation and blood viscosity in diabetic patients. It has been stated that high HbA_{1c} level is inversely correlated with erythrocyte deformability (Schwartz et al. 1991; Huang et al. 2004). However, Jay et al. (1991) and Demiroglu et al. (1999) have reported that rheological measurements were not related to metabolic control. In the current study, we also found a positive correlation ($r = 0.79$) between HbA_{1c} levels and erythrocyte aggregation indices between groups (Table 1). Our results support the idea that poor glycemic control may contribute to hemorheological ab-

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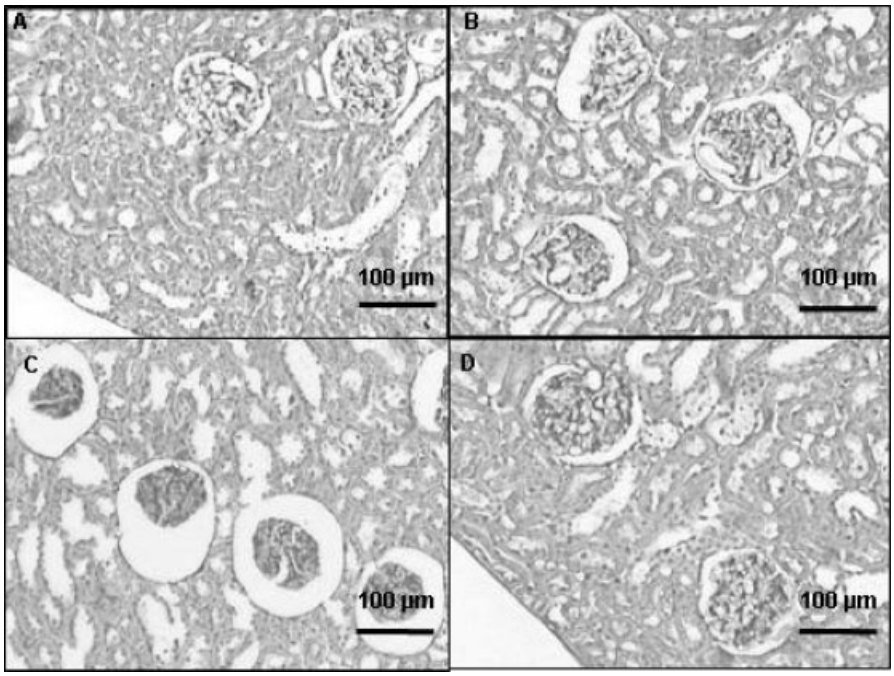

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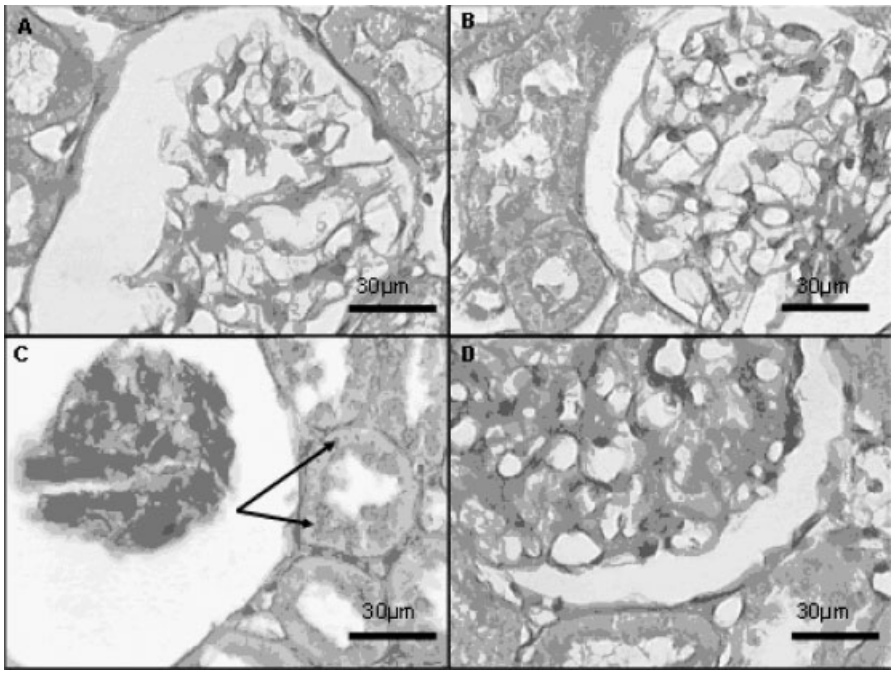

Figs. 2 and 3:
Photomicrograph kidney sections from control (A), melatonin-treated control (B), diabetic (C) and melatonin-treated diabetic rats (D). PAS stain. Diffuse glomerulosclerosis was present in the sections from the diabetic kidney (2C and 3C). Stained glycogen deposition (Armanni-Ebstein lesions) observed in the proximal tubular epithelial cells (3C-arrows) in diabetic groups. PAS (+) glycoprotein deposition in the glomerular tuft was considerably lower in melatonin-treated diabetic group compared to that of the diabetic group (3C and 3D)

normalities in diabetic animals or human subjects. However, in our study, the treatment of the diabetic rats with melatonin did not affect hyperglycemia and high HbA_{1c} levels as reported in previous studies.

HbA_{1c} has been investigated in a limited number of postmortem studies in diabetes mellitus, as HbA_{1c} is stable, is not influenced by postmortem haemolysis and reflects the antemortem glucose level between 2 to 3 months (Goulle et al. 2002). It is reported that diabetes mellitus can cause sudden death because of its microvascular, macrovascular and neuropathic complications. It is important to determine the cause of a sudden death in medicolegal investigations. In Turkey, diabetes mellitus prevalence is reported as 7.2% (Satman et al. 1998). The existence of undiagnosed cases of diabetes mellitus despite its high prevalence, inadequate screening and treatment of cases, and difficulties in postmortem diagnosis are important problems. The present study, which is also a preliminary report of continuing research, supported the fact that HbA_{1c} is a useful marker for diagnosis of diabetes mellitus.

Increased oxidative stress due to oxygen free radical production induced by diabetes plays an important role in the development and progression of diabetic nephropathy (Ha et al. 1999; Cam et al. 2003; Oktem et al. 2006). In previous studies, the significant role of oxidative stress on diabetic nephropathy has been reported and the beneficial effects of melatonin have been demonstrated (Ha et al. 1999; Oktem et al. 2006). Thus, we aimed to evaluate the effect of short term melatonin administration on healing effects in diabetic nephropathy in rats. When histological sections stained with PAS were investigated, the appearance of kidney sections relating to the control (Figs. 2A and 3A) and melatonin-treated control (Figs. 2B and 3B) groups were found to be normal. Glomerular capillaries, Bowman's capsule and tubular structures in the sections dissected from the cortex were evaluated as normal. On the other hand, diffuse glomerulosclerosis was pervasively present in the sections belonging to the diabetic group (Figs. 2C and 3C). PAS (+) substance deposition in the basal membrane and mesangium, constriction in the glomerular capillary lumen, and stained glycogen deposition (Armanni-Ebstein lesions, Fig. 3C-arrows) in the proximal tubular epithelial cells were observed in the diabetic group (Figs. 2C and 3C). Although glomerulosclerosis was rarely observed in the melatonin-treated diabetic group, in general the glomerular structures were normal (Figs. 2D and 3D). PAS (+) glycoprotein deposition in the glomerular tuft was considerably lower in the melatonin-treated diabetic group compared to that of the diabetic group. These histopathological findings confirmed diabetic nephropathy in STZ-induced diabetic rats. On the other hand, in melatonin-treated diabetic rats, the glomerular structures were generally normal and glomerulosclerosis was rare. Our results clearly showed that short-term melatonin administration reduced diabetic nephropathy in STZ-induced diabetic rats without an effect on blood glucose levels (Table 1).

In some studies (Ha et al. 1999; Oktem et al. 2006), melatonin was administered subsequent to STZ injection for 8 weeks and its preventive effects on diabetic complications were evaluated. However, in our study, melatonin was administered for 5 days starting at the 14^{th} week after STZ injection when diabetic complications had developed. Moreover, diabetic nephropathy and the healing effect of melatonin on nephropathy were investigated histopathologically while the previous studies used injury markers in the kidney.

The excessive production of ROS exerts an oxidative effect on membrane lipids, proteins, enzymes and DNA, leading to cellular dysfunction and cell death in the form of necrosis or apoptosis (Yerer et al. 2004). ROS can induce release of pro-apoptotic factors including cytochrome c and AIF (Kowaltowski et al. 2001). In the present study, localization of DNA fragmented cells in the kidney of diabetic rats were examined by the terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP-biotin nick end labeling (TUNEL) method. We observed several TUNEL-positive cells in the glomerular mesangium and tubular epithelial cells in the kidneys of diabetic rats. A decrease in the number of TUNEL-positive cells was observed after melatonin treatment in the diabetic group (Fig. 4, Table 2). In our study, the observed melatonin-induced anti-apoptotic effect was consistent with the results of other studies showing that melatonin inhibits apoptosis in the ischemic kidney (Kunduzova et al. 2003) and in hippocampal neu-

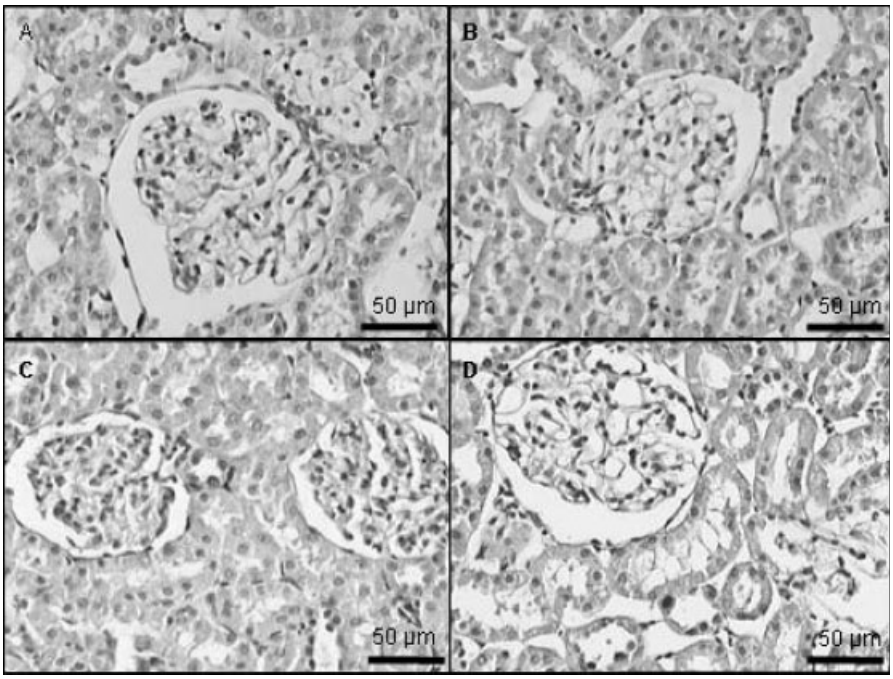

Fig. 4:
Apoptotic cells in the kidney from control (A), melatonin-treated control (B), diabetic (C) and melatonin-treated diabetic rats (D) stained by TUNEL method. TUNEL positive cells in glomerular mesangium and tubular epithelial cells in diabetic group much more than in control group

Table 2: TUNEL positive cells ratio (%) in the glomerular mesangium and tubular epithelial cells of kidney from four groups of rats. C, control; D, diabetic, C + M, melatonin-treated control and D + M, melatonin-treated diabetic groups

Groups	Number of rats	TUNEL positive cells %
C	5	9.3 ± 0.2
D	5	$5.2 \pm 0.3^{*}$
C + M	5	10.7 ± 0.1
D + M	5	$2.6 \pm 0.1^{*,a}$

* $p < 0.01$, compared with C and C + M groups, [a] $p < 0.001$ compared with D group. Data presented as mean ± S.E.M.

rons (Tugyan et al. 2006) and immortalized pineal cells (Yoo et al. 2002).

In conclusion, we found that erythrocyte deformability was decreased and erythrocyte aggregation index was increased in diabetic rats. Melatonin treatment did not affect these hemorheologic properties. We also detected diffuse glomerulosclerosis, and observed several TUNEL-positive cells in the glomerular mesangium and tubular epithelial cells of the kidney. Melatonin treatment reversed microvascular histopathologic changes due to diabetes and had an antiapoptotic effect in the diabetic kidney. The results of this study suggest that besides its protective effects, melatonin may ameliorate the diabetic nephropathy developed in STZ-induced diabetic rats. While melatonin significantly reversed histopathological changes, it did not improve hemorheological abnormalities in melatonin-treated diabetic animals. Such findings may demonstrate that the control of glycemia is as important as the antioxidant status in the treatment of hemorheologic changes in diabetic rats. The positive correlation between HbA_{1c} levels and erythrocyte aggregation index supports this hypothesis. Further studies using combined therapy with insulin may elucidate the treatment of hemorrheological abnormalities in diabetes.

3. Experimental

3.1. Drugs

Streptozotocin (Sigma Chemical Co, St. Louis, USA) was dissolved in citrate buffer, immediately before use. Melatonin (Sigma, St. Louis, MO, USA) was dissolved in absolute ethanol and diluted with physiological saline. The ethanol concentration in the final solution was 5%. The melatonin solution was administered at 10 mg/kg (i.p.), while control (C) and diabetic rats (D) received vehicle (5% ethanol/saline, i.p.) only.

3.2. Animals

Adult male Wistar rats weighing 170–300 g were used throughout the study. The study was approved by the Ethics Committee of Research of Laboratory Animals of Dokuz Eylül University Medical School, and all procedures were performed according to the "Guide for the Care and Use of Laboratory Animals of the National Institutes of Health". All animals were kept under standardized conditions of temperature (21–22 °C) and illumination (12 : 12 L/D) in cages with mesh bottoms and free access to tap water and pelleted food. The animals were fasted for 12 h before the experiment, but had free access to water until the beginning of the experiment.

3.3. Experimental study design

Twenty rats were divided into four groups as follows: streptozotocin-induced diabetic rats (D, n = 5), age-matched control rats (C, n = 5), melatonin-treated diabetic rats (D + M, n = 5) and melatonin-treated control rats (C + M, n = 5). Melatonin (10 mg/kg, i.p.) was administered for five days after 14 weeks inducement of diabetes. All drug administrations were performed between 9.00 a.m. and 11.00 a.m. to reduce the effects of circadian rhythm. The whole blood of the rats was collected from the heart via heparin-rinsed syringes after ether anesthesia and then the rats were sacrificed by cardiac puncture and kidney tissues were removed.

3.4. Induction of diabetes mellitus

Freshly prepared streptozotocin was dissolved in 0.02 M citrate buffer at pH 4.5 and administered intraperitoneally to the rats at 45 mg/kg. The age-matched control group received citrate buffer only. On the third or fourth day after streptozotocin administration, serum glucose levels were measured using a Medisense Optium glucometer (Abbott Laboratoires, Bedford MA). Rats with blood glucose levels higher than 300 mg/dL were considered diabetic. Fourteen weeks after streptozotocin administration, it was assumed that chronic diabetes had been developed.

3.5. Determination of biochemical and hematological parameters

Glycosylated haemoglobin (HbA_{1c}) was measured at the end of study. HbA_{1c} was determined quantitatively by immunoturbidimetry using Cobas Integra 400 Plus. At the same time, blood samples were taken in EDTA coated tubes for haematological parameters and measured within 4 h by hemocounter (Beckman-counter LU 750, USA).

3.6. Determination of erythrocyte deformability and aggregation

The erythrocyte deformability and aggregation measurements were done by Laser-assisted Optical Rotational Cell Analyzer (LORCA, R & R Mechatronics, Hoorn, The Netherlands). To determine the deformability, erythrocytes were suspended in a standardized viscous solution at a fluid shear stress of 3.00 Pa by laser diffraction analyses. Elongation indices (EI) were analyzed and calculated by a computer.

Aggregation measurements by LORCA aggregometer were based on the detection of laser backscattering from the sheared (disaggregated) and then un-sheared (aggregating) blood, using a computer-assisted system (Hardeman et al. 2001).

3.7. Histopathological examination

After study, the rats were sacrificed by cardiac puncture and left nephrectomy was performed. Kidney tissues were fixed in buffered 10% formalin and embedded in paraffin wax. Five-micron-thick sections were stained with haematoxylin and eosin (H & E) and periodic acid-Schiff (PAS). Histopathological findings were recorded in detail.

3.8. In situ terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay

In situ labeling of fragmented DNA was performed with TUNEL with a commercial kit (In Situ Cell Death Detection Kit® Roche, Mannheim, Germany) according to the manufacturer's instructions.

3.9. Statistical analysis

All data are expressed as mean ± S.E.M. The differences among groups were verified by one-way analysis of variance (ANOVA) followed by a post hoc Bonferroni test. The relationship between HbA_{1c} levels and agregation index was assessed by Pearson correlation analysis. Differences among groups were considered significant if $p < 0.05$.

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