ORIGINAL RESEARCH

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In Memoriam

This paper is dedicated to the memory of Professor Tuncay Altuğ of the Experimental Animal-Research and Breeding Laboratory, Istanbul University, Cerrahpasa Medical Faculty, Turkey, who kindly provided experimental animals for our current research.

Manuscript submitted 4th October, 2012

Manuscript accepted 20th November, 2012

Clin Invest Med 2012; 35 (6): E370-E377.

Melatonin ameliorates oxidative damage in hyperglycemia-induced liver injury

Abstract

Purpose: Melatonin (N-acetyl-5-methoxy-tryptamine) is synthesized mainly by the pineal gland and its antioxidant properties have been demonstrated both in short and long term studies. Our aim was to clarify the effects of hyperglycemia and to administer melatonin on lipid peroxidation, protein oxidation and oxidative DNA damage in rat.

Methods: Malondialdehyde (MDA), protein carbonyl (PCO) and total thiol (T-SH) levels were determined in plasma and liver tissue, glutathione (GSH) levels in erythrocyte and liver tissue, and 8-hydroxy-2-deoxyguanosine (8-OHdG) levels in plasma and liver. Thirty-eight male Wistar rats were divided into four groups: 1 - injected with saline (n = 8), 2 - injected with melatonin (n = 10), 3 - injected with STZ (65 mg/kg, i.p.) (diabetic group) (n = 10) and 4 - injected with melatonin (10 mg/kg/day, i.p.) and STZ (65 mg/kg, i.p.) (n = 10) for 8 weeks (diabetic+ melatonin group). Colorimetric methods were used to determine the level of the oxidative stress markers. 8-OHdG was measured using ELISA.

Results: MDA, PCO and 8-OHdG levels in the plasma and the liver homogenates of diabetic rats were higher than controls and were significantly reduced after melatonin treatment. T-SH and GSH levels in samples were markedly reduced in untreated diabetic rats compared with control rats; however, these parameters were increased in diabetic rats following melatonin treatment.

Conclusion: Our findings showed that melatonin administration partially ameliorated oxidative damage in liver injury in STZ-induced diabetic rats. The present study suggests that melatonin functions as a potent antioxidant agent in diabetes. Melatonin, a nutritional supplement, may be a good therapeutic option for diabetic patients.

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Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and insufficiency of secretion or action of endogenous insulin. Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of both types of diabetes mellitus [1]. Streptozocin (STZ) and alloxan (ALX) are widely used to induce experimental diabetes in animals. STZ- and ALX-stimulated oxidative damage probably acts as a mediator of STZ- and ALX-induced DNA fragmentation [2].

Melatonin (N-acetyl-5-methoxytryptamine), a secretory product of the pineal gland, is a powerful endogenous antioxidant. Exogenous application of this hormone leads to a remarkable decline in oxidative stress by directly neutralizing the hydroxyl radicals [3]. In addition, melatonin also indirectly enhances the levels of potential antioxidants such as glutathione peroxidase, superoxide dismutase (SOD) and glutathione (GSH) [3, 4]. Numerous experimental and clinical studies suggest that daily melatonin supplementation may be beneficial for the treatment of diabetes [5-8]. Since there is insufficient knowledge on the beneficial role of melatonin in the reversal/prevention of oxidative DNA damage in diabetes, the present study has been undertaken.

In order to understand the beneficial effects of melatonin on the oxidative damage resulting from the hyperglycemic conditions of diabetes, its effects on various oxidative parameters, such as malondialdehyde (MDA), total thiol (T-SH) and protein carbonyl (PCO) levels in plasma and liver tissue, glutathione (GSH) levels in erythrocyte and liver tissue, and 8-hydroxy-2-deoxyguanosine (8-OH dG) levels in liver tissue were studied after melatonin administration under hyperglycemic conditions in diabetic rats.

Materials and Methods

Chemicals

All chemicals were analytical grade or of the highest grade available and purchased from Sigma (St. Louis, USA) and Merck (Darmstadt, Germany). Deionized water was used in all analytical procedures. All reagents were stored at +4°C. The reagents were equilibrated at room temperature for 0.5 h before use.

Apparatus

All centrifugation procedures were performed using a Jouan G 412 centrifuge. MDA, PCO, T-SH and GSH levels were measured using an Biotek H1 Hybrid Multi-Mode Microplate Reader. 8-OHdG measurements were performed using an ELISA (Human GmbH, Wiesbaden, Germany). Serum glu-

cose and glycated hemoglobin (HbA1c) levels were determined colorimetrically on a Modular Analyzer (Roche GmbH, Mannheim, Germany).

Animals

Thirty-eight male Wistar albino rats, between 12 and 14 weeks of age and weighing 190–270 g, were obtained from Experimental Animal Research and Breeding Laboratory, Cerrahpasa Medical Faculty, Istanbul University, Istanbul, Turkey. The animals were kept in the same unit at a constant temperature ($22 \pm 1^\circ\text{C}$) under a 12 h light/dark cycle. Food and fresh tap water were supplied *ad libidum* throughout the experiment. All of the experimental procedures were conducted under 3R's rule. All rats were weighed at the same time and food and water were monitored daily throughout the study period.

Study groups and design

Animals ($n = 38$) were randomly divided into four groups. Group 1: $n=8$ rats were injected (i.p.) with saline (saline controls). Group 2 ($n=10$) rats were injected (i.p.) with melatonin (10 mg/kg/day melatonin in 0.5 ml of normal saline containing 0.1 ml of 1% ethanol). The intraperitoneal injection is more widely used than intravenous or subcutaneous injection for simplicity of handling. Group 3 ($n=10$) was injected (i.p.) with streptozotocin (STZ) (65 mg/kg, in 20 mmol/L citrate buffer, pH 7.4) to induce diabetes [9]. After STZ injection, blood glucose levels were determined following an overnight fast: a blood glucose level over 200 mg/dl was considered indicative of diabetes. Group 4 ($n=10$) was injected with melatonin, as described above, and then 3 days later, was injected with STZ. Chow consumption of the all rats was monitored and recorded daily.

Blood samples were drawn from the central ear artery both at the beginning of the experimental period and 8 weeks later, at 8 a.m. At the end of the eight week study period, the animals were weighed and then anaesthetized by the administration of ketamine (50 mg/kg) and xylazine (10 mg/kg) into the marginal ear vein. Under anesthesia, the thorax was opened and blood from the right ventricle was collected into dry and heparinised tubes for determination of various analytes. Livers were immediately excised, weighed and immersed in ice-cold physiological saline. The blood samples were centrifuged for 5 min at $1000 \times g$ at 4°C and the all samples were stored at -80°C until analysis. Experimental parameters were analyzed in all samples in a single batch, after the protocol had been completed (all samples were analysed in the same batch).

Preparation of tissue samples

About 190–200 mg of each liver sample was weighed and diluted 20% w/v in 20 mM ice-cold Tris-HCl, pH 7.4, and homogenized with a Bosch Scintilla SA homogenizer (Solothurn, Switzerland). The homogenate was centrifuged at 5000xg for 10 min and biochemical analyses were performed on the supernatant fraction.

Assay of protein carbonyl

Plasma and tissue PCO levels were measured spectrophotometrically using the method of Reznick and Packer [10]. PCO groups react with 2,4-dinitrophenylhydrazine (DNPH) to generate chromophoric dinitrophenylhydrazones. DNPH was dissolved in HCl, and following the DNPH reaction proteins were precipitated with an equal volume of 20% trichloroacetic acid (w/v), and washed three times with 4 ml of an ethanol/ethyl acetate mixture (1:1). Washings were carried out by mechanical disruption of pellets in the washing solution using a small spatula, and re-pelleting by centrifugation at 6000 xg for 5 min. Finally, the precipitates were dissolved in a 6 M guanidine-HCl solution, and the absorbances were measured at 360 nm, using the molar extinction coefficient of DNPH, $\epsilon = 2.2 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$. Protein content was determined on the HCl blank pellets spectrophotometrically using a Folin kit (Sigma Diagnostics, St. Louis, USA). The coefficients of intra- and interassay variations for the carbonyl assay were 4.3% (n=10) and 8.4% (n=10), respectively.

Assay of malondialdehyde

Lipid peroxidation levels in plasma and tissue were measured with the thiobarbituric acid (TBA) reaction (method of Yagi) [11]. This method was used to obtain a spectrophotometric

measurement of the color produced during the reaction to TBA with MDA at 535 nm. The coefficients of intra- and interassay variations for the MDA assay were 3.4% (n=10) and 5.2 % (n=10), respectively.

Assay of total thiol

Plasma and tissue T-SH concentrations were determined using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as described by Hu [12]. The coefficients of intra- and interassay variations were 1.9% (n=9) and 4.8% (n=10), respectively.

Assay of glutathione

Erythrocyte and liver tissue GSH concentrations were estimated according to the Beutler et al. [13]. One milliliter of erythrocyte preparation and tissue homogenate were deproteinized and then centrifuged at 600 x g for 20 min. After addition of dithiobis-nitrobenzoate and phosphate buffer (pH 8.0) into the clear supernatants of the samples, the color that developed was read at 412 nm. The GSH concentrations of the samples were calculated using $1,36 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ as the molar absorption coefficient. The intra- and interassay coefficients of variation for GSH were 3.3% (n=10) and 3.6% (n=10), respectively.

Assay of 8-hydroxy-2-deoxyguanosine

The plasma 8-OHdG levels were determined using an enzyme-linked immunosorbent assay detection kit (Bioxytech, Oxis Health Products, Portland, USA). The coefficients of intra- and interassay variations for 8-OHdG assay were 6.7% (n=10) and 7.1% (n=10), respectively.

TABLE 1: Initial and final body weights, blood glucose and HbA1c levels measured in the current study

	Saline (n=8)	Melatonin (n=10)	Diabetic (n=10)	Diabetic+melatonin (n=10)
Initial body weight (g)	239,6±22.45	239.6±21.16	237.3±22.50	237.90±21.18
Final body weight (g)	279.4±21.06*	277.2±17.45#	225.4±23.06#	249.30±20.79#
Glucose (mg/dl)	118.75±11.00c ^Δ ,d ^Δ	117.90±9.96c ^Δ ,d ^Δ	456.10±67.54a ^Δ ,b ^Δ ,d ^Δ	369.50±28.57a ^Δ ,b ^Δ ,c ^Δ
HbA1c (%)	4.85±0.30c ^Δ ,d ^Δ	4.93±0.28c ^Δ ,d ^Δ	7.76±0.37a ^Δ ,b ^Δ ,d ^Δ	6.31±0.43a ^Δ ,b ^Δ ,c ^Δ

Values are represented as (mean ± SEM). Differences of body weight are given with respect to initial weight

a; statistically different from saline

b; statistically different from melatonin

c; statistically different from diabetic

d; statistically different from diabetic+melatonin

*; p<0.05, # p<0.01, Δ; p<0.001

FIGURE 1. A-Plasma malondialdehyde levels. B-Plasma protein carbonyl levels. C- Plasma 8-hydroxy-2-deoxyguanosine levels. Results are mean $\pm$ standard errors. * $P < 0.001$ versus other three groups.

Statistical analysis

All results are expressed as mean $\pm$ standard errors. One way analysis of variance (ANOVA), Tukey and Wilcoxon's signed rank tests compared statistical analysis of differences. The statistical analysis was made with SPSS (Statistical Package for Social Sciences 10.0 ver.). $P < 0.05$ was considered statistically significant.

Results

Allergic or gastrointestinal complications or hepatotoxicity were not observed during the experimental period. There was neither an intraoperative death nor any death in the experimental period. The initial and final body weights, blood glucose and HbA1c levels of the rats are shown in Table 1. The baseline weight at the beginning of the study was similar in all groups. As expected, The STZ-diabetic rats exhibited hyperglycaemia with blood glucose and HbA1c levels significantly higher than in the non-diabetic groups (Table 1). There was a significant weight reduction in rats following an eight week period of diabetes.

The mean plasma levels of MDA, PCO and 8-OHdG were significantly higher in the STZ-diabetic rats in comparison with the STZ-diabetic + melatonin rats (Fig. 1). Similarly, plasma T-SH and erythrocyte GSH of STZ-diabetic rats were significantly lower than STZ-diabetic+ melatonin rats (Fig. 2). The mean liver MDA and PCO levels of STZ-induced diabetic rats also were significantly higher than STZ-diabetic+melatonin rats. The mean hepatic T-SH and GSH levels in STZ-diabetic rats were significantly lower than the diabetic + melatonin animals, the rats treated with saline and melatonin only (Fig 3).

Discussion

Induction of diabetes in rats with STZ or ALX uniformly results in an increase in thiobarbituric acid reactive substances (TBARS). TBARS assay values are usually reported in equivalents of MDA, a compound that results from the decomposition of polyunsaturated fatty acid lipid peroxides. During diabetes, the generation of several types of reactive oxygen species is said to potentially affect the membrane of living cells and to produce MDA. Superoxide (O_2^-), hydroxyl anion ($OH^\cdot$), hydrogen peroxides (H_2O_2) or singlet oxygen (O_2^1) could initiate the cascade, resulting in the production of MDA. The increase in TBARS associated with diabetes is prevented by treatment with melatonin [5-8], if this treatment is given before or immediately after the diabetogen. In the present study, the hypothesis that melatonin ameliorates oxidative damage in STZ-

FIGURE 2: A-Plasma total thiol levels. B- Erythrocyte glutathione levels. Results are mean $\pm$ standard errors. *P < 0.001 versus other three groups

induced liver injury was tested. Light microscopic examination of tissue specimens of liver was not used as evidence of liver damage. Instead, markedly increased liver oxidative stress parameters were used as evidence of liver damage. In the current study, a blood glucose level over 200 mg/dl was considered indicative of diabetes. Although lipid peroxidation markers have been well studied in diabetes [5-8,14,15], there have been only a few studies of hyperglycemia-induced plasma protein, thiol and 8-OHdG levels.

Oxidative damage to proteins, lipids or DNA may all be seriously deleterious and may be concomitant. Proteins are possibly the most immediate vehicle for inflicting oxidative damage on cells because they are often catalysts rather than stoichiometric mediators; hence, the effect of damage to one molecule is greater than stoichiometric [16].

In this study, plasma MDA and PCO levels in diabetic rats were found to be significantly increased compared with those of both saline and diabetic+melatonin groups. Among the various oxidative modifications of amino acids in proteins, PCO formation may be an early marker for protein oxidation [10]. The plasma and tissue MDA and PCO levels of STZ-diabetic+melatonin mice, and saline treated mice were similar providing evidence for the anti-oxidant and free radical scavenging properties of melatonin (see Figs. 1, 2, 3) [5,8,9]. Protein modifications are elicited by direct oxidative attack on Lys, Arg, Pro, or Thr, or by secondary reaction of Cys, His or Lys residues with reactive carbonyl compounds can lead to the formation of PCO derivatives (aldehydes and ketones) in diabetes [17].

The use of injectable melatonin increases both its half-life and bioavailability, improving the pharmacological effects of the hormone. Injection prevents the first step of elimination/clearance of melatonin from liver, which is not possible with all oral preparations. The expected transient concentration of i.p. injected melatonin after uptake in the blood should be in the range of 1.0 mM, which is at least one million times higher than the known daytime concentration of that hormone; however, serum melatonin levels were not measured in this study. Melatonin did not normalize hyperglycemia and body weight in the current study, consistent with a previously published study [18]. Furthermore, melatonin treatment in STZ-induced type 1 diabetic rats caused a slight increase in the lowered serum insulin concentrations and a small partial regeneration/proliferation of β -cells [19]. In contrast, melatonin reduced the hyperinsulinemia associated with type 2 diabetes [20] and may effectively normalize the impaired antioxidant status in STZ-induced diabetes [21, 22]. Oral administration of melatonin ameliorates the pro-inflammatory and oxidative stress that underlie the development of insulin resistance and its consequences, such as metabolic syndrome, diabetes mellitus and cardiovascular disease [23]. Zavodnik et al. [24] pointed out mitochondria dysfunction in the development of liver injury during diabetes as well as the possibility of corrections of mitochondrial disorders by melatonin. Melatonin may also have a promising role in treating patients with metabolic syndrome, which is characterised by symptoms of obesity, insulin resistance, hypertension, dyslipidemia and diabetes mellitus [25].

T-SH may serve an antioxidant function by scavenging the oxidants that initiate peroxidation. In the erythrocyte, the thiol GSH is an important reducing agent and antioxidant, is involved in maintaining the cellular oxidation-reduction balance, and has been shown to protect cells from a wide variety of en-

FIGURE 3: A-Tissue malondialdehyde levels. B- Tissue malondialdehyde levels. C-Tissue total thiol levels. D-Tissue glutathione levels. Results are mean $\pm$ standard errors. *P < 0.001 versus other three groups

ogenous and exogenous insults [26]. Our study indicates that plasma and tissue thiols were lower in diabetic rats than the control animals. There is a consensus in literature that diabetes causes reduced GSH levels [9,27-29]. GSH can reduce free radicals by hydrogen donation (superoxide, hydrogen peroxide and hydroxyl radicals). The current study showed that melatonin significantly reversed the levels of T-SH and GSH in the plasma and liver tissue of diabetic rats. Melatonin treatment might affect antioxidant quantity and the cellular oxidation-reduction balance in various disorders [25,30-33].

Experimental evidence shows that oxidative damage permanently occurs to lipids of cellular membranes, proteins and DNA. In nuclear and mitochondrial DNA, 8-OHdG or 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is one of the predominant forms of free radical-induced oxidative lesions, and has therefore been widely used as a biomarker for oxidative stress and disease [34]. This may be explained by the high level

of H₂O₂ generated from free radicals produced under oxidative stress conditions. Current evidence suggests that 8-OHdG lesions present in DNA during cellular replication results in somatic mutation, which may contribute to smooth muscle proliferation in the pathogenesis of atherosclerotic plaques (35). No study has yet been undertaken on the effects of melatonin on plasma level 8-OHdG in STZ induced diabetes mellitus and associated hyperglycemia. In the current study, the effect of melatonin, as a supplemental antioxidant nutrient on the oxidative DNA damage in diabetes, were investigated. Melatonin, as an antioxidant, lowered the 8-OHdG levels in plasma. Thus, the use of melatonin as supplemental nutrient may ameliorate the DNA damage seen in diabetic patients.

Melatonin has some beneficial effects on diabetes-induced oxidative stress: decreasing the protein oxidation, lipid peroxidation and oxidative DNA damage. Our results suggest that oxidative mechanisms play an important role in STZ-induced

tissue damage and that melatonin, by balancing the oxidant-antioxidant status, ameliorates the oxidative organ injury that arises as a result of hyperglycemia. Thus, oral hypoglycemic agents, or insulin or a combination of insulin and melatonin may be beneficial in preventing the progression of diabetes-induced complications.

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