

Melatonin attenuates metabolic disorders due to streptozotocin-induced diabetes in rats

Elena Ju. Sudnikovich^a, Yuri Z. Maksimchik^a, Svetlana V. Zabrodskaya^a, Valeri L. Kubyshev^a,
Elena A. Lapshina^a, Maria Bryszewska^c, Russel J. Reiter^d, Ilya B. Zavodnik^{a,b,*}

^a State Research and Innovation Center “Institute for Pharmacology and Biochemistry of the National Academy of Sciences of Belarus”,
BLK-50, 230017 Grodno, Belarus

^b Department of Biochemistry, Yanka Kupala State University of Grodno, 230023 Grodno, Belarus

^c Department of General Biophysics, University of Łódź, 90-237 Łódź, Poland

^d Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, TX, USA

Received 14 March 2007; received in revised form 10 May 2007; accepted 15 May 2007

Available online 5 June 2007

Abstract

Enhanced oxidative stress and impairments in nitric oxide synthesis and bioavailability are of considerable importance in the pathogenesis of diabetic vascular diseases. The aim of the present work was to evaluate the metabolic effects of pharmacological doses of the melatonin, a known antioxidant, on streptozotocin-induced diabetic damage in rats. We investigated the indolamine's influence on the cellular redox-balance, nitric oxide (NO) level, and the activities of antioxidative defence enzymes, as well as the activities of enzymes involved in phase II detoxication and NADPH-generating pentose phosphate pathway. Blood glucose, glycated hemoglobin, bilirubin, as well as plasma alanine aminotransferase activities increased and body weight was reduced in rats with streptozotocin-induced (60 mg/kg, i.p.) diabetes (25 days). The NO level was markedly increased in diabetic plasma (by 50%) and aortic tissue (by 30%). The hyperglycemia resulted in reduced activities of glutathione peroxidase (by 25%), catalase (by 20%), glucose-6-phosphate dehydrogenase (by 55%) and transketolase (by 40%) in liver tissue of diabetic animals. Melatonin treatment (10 mg/kg, 18 days) did not influence the level of hyperglycemia or glycated hemoglobin and it had little effect on the activities of antioxidative enzymes. However, melatonin markedly reversed the activities of glucose-6-phosphate dehydrogenase and transketolase in liver tissue of diabetic rats. The most pronounced effect of the melatonin administration was the prevention of an increase in nitric oxide levels in blood plasma and aortic tissue during diabetes. In *in vitro* experiments, nitrosomelatonin formation in the presence of nitrosodonor was observed. This implies that melatonin might operate as an NO scavenger and carrier. Thus, melatonin treatment may have some beneficial effects in controlling diabetic vascular complications.

© 2007 Published by Elsevier B.V.

Keywords: Diabetes; Melatonin; Nitric oxide; Nitrosomelatonin

1. Introduction

Vascular diseases are major long-term complications in patients with diabetes mellitus. The mechanisms by which hyperglycemia injures vascular cells and leads to functional changes are multifunctional and include oxidative stress,

increased metabolism of glucose *via* the sorbitol pathway, enhanced cholesterol levels, non-enzymatic glycation of proteins and changes in the production of vasoactive substances such as endothelin, prostanoids and nitric oxide (Calles-Escandon and Cipolla, 2001). It was suggested that NO production may be increased in diabetes, but NO-availability was reduced due to NO inactivation by reactive oxygen species (Bojunga et al., 2004). Vascular production of excessive reactive oxygen and nitrogen species may contribute to endothelial dysfunction during diabetes, as well as oxidative modification of low density lipoproteins induced by high glucose concentrations (Kojda and Harrison, 1999). Stadler et al. (2003) suggested that oxidative

* Corresponding author. State Research and Innovation Center “Institute for Pharmacology and Biochemistry of the National Academy of Sciences of Belarus”, BLK-50, 230017 Grodno, Belarus. Tel.: +375 152 437935; fax: +375 152 434121.

E-mail address: zavodnik_il@mail.ru (I.B. Zavodnik).

stress increased at a very early stage of diabetes and that high levels of NO and peroxynitrite could play a decisive role in the development of numerous complications in vasculature and kidney of diabetics. The accumulation of products of non-enzymatic chemical protein modification by reducing sugars (advanced glycation end products) is also thought to contribute to the development of diabetic complications (Metz et al., 2003).

Targeted dysfunction of the inducible nitric oxide synthase (iNOS) gene partially protected against streptozotocin-induced diabetes in the mouse (Flodstrom et al., 1999). On the other hand, NO participates in the development of insulin signaling (Wittmann et al., 2001).

Mechanisms that contribute to the formation of free radicals in diabetes may include metabolic stress resulting from changes in energy metabolism, levels of inflammatory mediators, non-enzymatic glycosylation and glucose autooxidation (Baynes, 1991; Evans et al., 2003; Wolff, 1993). Recently, we demonstrated an impairment of the antioxidative defence system during streptozotocin-induced diabetes (Lapshina et al., 2006). In addition to their ability to inflict direct damage on macromolecules, reactive oxygen and nitrogen species activate a number of cellular stress-sensitive pathways, including nuclear factor κ B and p38 mitogen-activated protein kinase, which play a key role in the development of the late complications in type 1 and type 2 diabetes and the insulin resistance (Evans et al., 2003).

It was suggested that antioxidant treatment might be an important therapeutic option for preventing vascular complications in diabetes mellitus (Bojunga et al., 2004). Melatonin, *N*-acetyl-5-methoxy-tryptamine, the main secretory product of the pineal gland, has a number of membrane receptors and regulates mammalian circadian and seasonal rhythms, reproduction in seasonal breeders and retinal function (Becker-Andre et al., 1994; Brzezinski, 1997). Antioxidant protection afforded by melatonin at the level of the cell membrane, mitochondria, and nucleus has been shown both *in vivo* and *in vitro* (Reiter, 2000). Melatonin, as well as its metabolites (Tan et al., 2007), possesses redox properties because of the presence of an electron rich system which allows these molecules to act as an electron donors (Allegra et al., 2003).

Blood glucose levels exhibit a circadian rhythm. The evaluation of the relationships between diabetes, glucose metabolism and the effects of melatonin is the topic of great interest (Derlacz et al., 2005). It was shown earlier that melatonin could effectively normalize the impaired antioxidative status in rats with streptozotocin-induced diabetes (Anwar and Meki, 2003). Similarly, long-term melatonin administration reduced hyperlipidemia and hyperinsulinemia, and restored altered ratios of polyunsaturated fatty acids in serum and tissues of diabetic rats (Nishida, 2005). The potential existence of a signaling pathway cross-talk between melatonin and insulin was suggested (Nishida, 2005). The relaxation and contractile responses in the aorta and trabecular smooth muscle tissues of streptozotocin-diabetic rats were markedly reduced (Paskaloglu et al., 2004). Melatonin exerts a protective effect on the functional and biochemical changes in the aorta and corpus cavernosum of diabetic rats. It was demonstrated that combined treatment with insulin and melatonin suppresses hyperglycemia, prevents oxidative damage and

restores completely endothelial function in the aorta and corpus cavernosum of diabetic rats (Paskaloglu et al., 2004).

The aim of the present work was to investigate the metabolic effects of pharmacological doses of melatonin under conditions of experimental diabetes in rats. We measured the activities of the antioxidative enzymes, glutathione peroxidase and catalase, that of glutathione *S*-transferase (which catalyzes the reaction of drug detoxication), as well as those of the pentose phosphate pathway enzymes, transketolase and glucose-6-phosphate dehydrogenase. Since nitric oxide levels may correlate with vascular complications during diabetes, a possible melatonin effect on the nitric oxide levels in diabetic blood plasma, liver and aorta was examined. We also studied the interaction of melatonin with nitric oxide *in vitro*.

2. Materials and methods

2.1. Chemicals

Melatonin, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), glutathione reduced (GSH), thiobarbituric acid, trichloroacetic acid, NADP, *tert*-butyl hydroperoxide and glucose-6-phosphate disodium salt hydrate were from Sigma-Aldrich, St. Louis, USA. 1-chloro-2,4-dinitrobenzene (CDNB) and streptozotocin (Streptozocin) were from Fluka Chemie AG, Buchs, Switzerland. All other reagents were of analytical grade and were purchased from Reakhim, Moscow, Russia. All the solutions were made with water purified in the Milli-Q system.

2.2. Animal model

The investigations were performed using 40 albino male Wistar rats (150–180 g). A standard balanced diet and tap water were provided *ad libitum*. The animals were adapted to intermittent 12-h light and dark phase cycle for 1 week and were divided into two groups. Lights were on daily from 08.00 to 20.00 h. The procedures of animal treatment were approved by the Institute for Pharmacology and Biochemistry of the National Academy of Sciences of Belarus, Ethics Committee.

The first group (10 animals) received physiological saline containing 5% ethanol intraperitoneally (i.p.) and was kept as control; the animals in this group were injected daily with physiological saline solution containing 5% ethanol (i.p.). The second group (30 animals) was injected with a single dose of streptozotocin (60 mg/kg, i.p.), dissolved in 0.01 M citrate buffer, pH 4.5, immediately before use. Three days later, blood glucose levels were determined in whole blood samples. The rats injected with streptozotocin were considered diabetic if their fasting blood glucose was >200 mg/dl (Blood Glucose Sensor Electrodes, MediSense, Abbot Laboratories, Bedford, UK). Animals diagnosed as diabetic were further divided into two subgroups: the first subgroup was injected daily with physiological saline solution containing 5% ethanol (the diabetes group), the second subgroup received daily 10 mg melatonin/kg b.w. (i.p.) (diabetes + melatonin). Melatonin was prepared as a 0.3% solution in the physiological saline, containing 5% ethanol and injected at 08.00 h. Melatonin treatment was initiated 7 days

after the streptozotocin administration and rats were sacrificed after 18 days of melatonin (or saline) treatment. Blood samples were drawn by an abdominal aorta into tubes containing hirudin (50 µg/ml). The liver and aorta were excised immediately, dried on filter paper, weighed, and used immediately.

After removing of plasma by centrifugation, the erythrocytes were washed three times with cold phosphate buffered saline (140 mM NaCl, 1.9 mM NaH₂PO₄, 8.1 mM Na₂HPO₄), pH 7.4, and hemolyzed. Livers were homogenized in cold 1.15% KCl solution (1:3, w/v), and homogenates were centrifuged at 12,000 *g* for 30 min to isolate post-mitochondrial supernatant for biochemical measurements. Aortic tissue was homogenized in cold 1.15% KCl solution (1:10, w/v) and the homogenates were centrifuged at 3000 *g* for 5 min. To determine glutathione *S*-transferase activity in the isolated cytosolic fraction, the post-mitochondrial supernatant was further centrifuged at 105,000 *g* for 90 min.

2.3. Biochemical measurements

The post-mitochondrial hepatocyte fraction was used for analysis of membrane lipid peroxidation and reduced glutathione (GSH) content. GSH was assayed with Ellman's reagent, using the molar absorption coefficient $\epsilon_{412} = 1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Ellman, 1959). The accumulated products of membrane lipid peroxidation (thiobarbituric acid-reactive substances) were monitored assuming that the molar absorption coefficient was $\epsilon_{532} = 1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (Stocks and Dormandy, 1971). A stable form of glycated hemoglobin, containing 1-deoxy-1(*N*-valyl)fructose, the activities of the serum marker enzymes, alanine aminotransferase and aspartate aminotransferase, indicating the degree of hepatolysis, as well as conjugated, free and total plasma bilirubin was assayed using reagent sets (Pliva-Lachema a.s., Brno, Czech Republic). Nitrite plus nitrate levels as an indicator of NO generation in blood plasma, liver and aorta tissues were measured using the Griess reagent (*N*-(1-naphthyl) ethylenediamine dihydrochloride, sulphanilamide) and cadmium as a reductant (Green et al., 1982). Protein content was measured by the method of Lowry et al. (1951).

The activity of cytosolic glutathione *S*-transferase was measured employing the method of Habig et al. (1974). The reactions were carried out in the presence of 10 to 30 µg cytosolic protein, 1 mM CDNB (as substrate), 1 mM GSH and 100 mM sodium-phosphate buffer, pH 7.5, at 30 °C in the final volume of 3 ml. A complete assay mixture without the enzyme was used as a control. The conjugation of CDNB with GSH was monitored at 340 nm, using the molar absorption coefficient of $9600 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as µmoles of GSH-CDNB conjugate/min/mg sample protein (Habig et al., 1974).

The activity of glutathione peroxidase in the post-mitochondrial fraction was determined according to the method of Martinez et al. (1979). The reaction mixture contained 0.1 M Tris-HCl, pH 8.0, 1 mM EDTA, 12 mM sodium azide, 2 mM *tert*-butyl hydroperoxide and 4.8 mM GSH (as cosubstrates of glutathione peroxidase). The reaction was started by addition of the sample and was stopped by 0.2 ml 25% trichloroacetic acid after 10-min incubation at 37 °C. The reaction plot was linear at

this time interval. The protein concentration in the reaction mixture was 40 µg/ml, the reaction volume was 1 ml. The activity was measured as the amount of GSH oxidized in the glutathione peroxidase reaction, using Ellman's reagent.

The method of Aebi (1984) was used to measure catalase activity. The reaction was started by addition of 0.3 ml of 30 mM hydrogen peroxide (H₂O₂) to 0.65 ml of 50 mM potassium phosphate buffer and 50 µl (15 µg protein) of the sample. The H₂O₂ decomposition was monitored at 240 nm, 37 °C for 3 min. The catalase activity was expressed as micromoles of H₂O₂ consumed per minute per milligram of sample protein using the molar absorption coefficient of $36 \text{ M}^{-1} \text{ cm}^{-1}$.

Glucose-6-phosphate dehydrogenase activity was measured as described by Costa Rosa et al. (1995). The assay mixture contained 100 mM Tris/HCl, 5 mM MgCl₂, 0.5 mM NADP⁺, 1 mM glucose-6-phosphate at pH 7.6. Enzyme activity was assayed by following the rate of NADPH production at 340 nm and 30 °C. The protein concentration was 50 µg/ml.

Transketolase activity was determined in a reaction mixture (1.7 ml) containing 15 mM ribose 5-phosphate, 250 µM NADH, 0.1 mM Tris-HCl, pH 7.8, 200 units/ml glycerol-3-phosphate dehydrogenase, and triosephosphate isomerase at 18±1 °C (Hammes et al., 2003). The enzyme reaction was started by the addition of 100 µl of the sample (500 µg protein), and the NADH oxidation at 340 nm was measured.

2.4. Statistical analysis

Data for 8–10 rats in each group are presented as a mean±S.E.M. for the normally distributed parameters or as a median and interquartile range for data showing departures from normality. The nested design of hierarchical ANOVA was used to test for the simultaneous effects of group (control vs. diabetic) and pharmacological treatment (melatonin). We used the standard Student *t* test for the comparison of raw and transformed data showing no departures from normality (according to Shapiro–Wilk's test), and the non-parametric Mann–Whitney *U* test for the remaining variables. *P*<0.05 was taken to indicate statistical significance.

3. Results

3.1. Effects of diabetes and melatonin treatment on biochemical parameters

As was expected, streptozotocin-induced diabetes in rats (25 days) resulted in significant hyperglycemia, increased levels of hemoglobin glycosylation and retarded growth of the animals (Table 1). Melatonin administration (18 days) did not affect these parameters.

An elevation of the activity of the marker hepatocyte enzyme, alanine aminotransferase (1.2-fold) (Fig. 1A) and a decrease of aspartate aminotransferase activity (1.2-fold) (Fig. 1B) in blood plasma of diabetic animals were observed. Blood plasma levels of total and conjugated bilirubin increased during diabetes (Fig. 2). Melatonin treatment reduced the elevated level of bilirubin in diabetic blood plasma.

Table 1
Blood glucose, glycated hemoglobin and body weight in control and streptozotocin-diabetic rats under melatonin treatment (18 days)

	Control	Diabetes	Diabetes+ melatonin
Blood glucose, mM	5.5±0.4	29.3±2.9 ^a	30.2±1.5 ^a
Glycated hemoglobin, μmol fructose/g hemoglobin	1.76±0.48	4.36±0.55 ^a	4.3±0.38 ^a
Body weight in grams (change from initial body weight)	+61.5±4.5	-14.5±4.5 ^a	-6.5±6.0 ^a

Data, presented as a mean±S.E.M., represent values at the termination of the experiment (25 days of experimental diabetes).

^a $P < 0.05$ vs control non-treated animals.

Diabetes was accompanied by an enhancement of blood plasma NO levels (1.5-fold) (Table 2). In a similar manner, the nitric oxide titers markedly increased in diabetic aortic tissue (1.3-fold) but they did not increase in the liver (Table 3). It should be emphasized that the nitric oxide level was significantly higher in the aorta compared to that in the liver. Melatonin administration reversed the elevated nitric oxide level in blood plasma and aorta tissue (Tables 2 and 3). Diabetes did not cause any appreciable changes in the levels of GSH or lipid peroxidation products (thiobarbituric acid-reactive substances) in erythrocytes (Table 2) but reduced thiobarbituric acid-reactive substances levels in liver tissue (Table 3). Melatonin administration to diabetic animals did not change significantly the GSH or thiobarbituric acid-reactive substances levels in rat erythro-

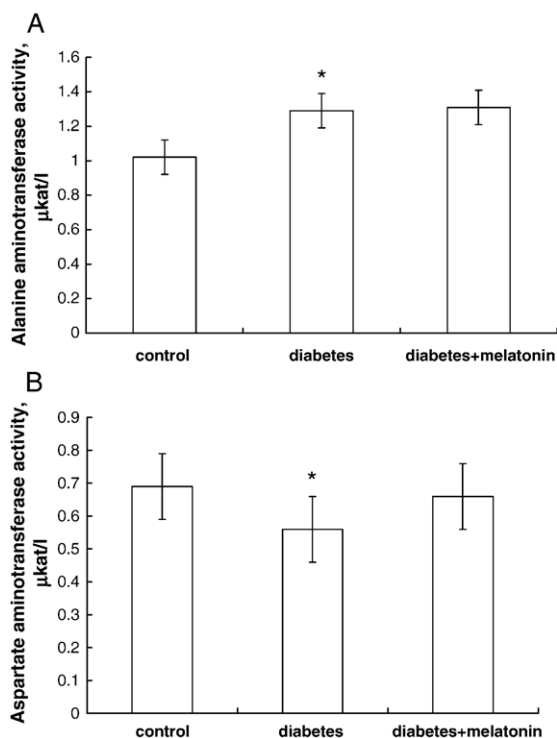


Fig. 1. Blood plasma alanine aminotransferase (A) and aspartate aminotransferase (B) activities in control, diabetic and melatonin-treated rats (18 days). * $P < 0.05$ in comparison with control values.

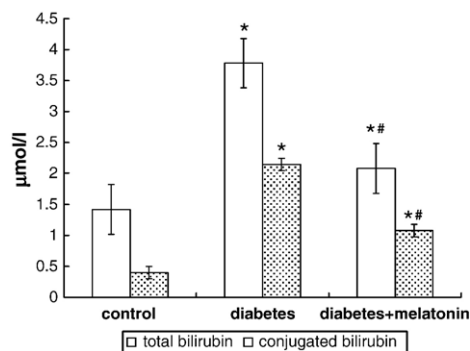


Fig. 2. Blood plasma total and conjugated bilirubin levels in control, diabetic and melatonin-treated rats (18 days). * $P < 0.05$ in comparison with control values, [#] $P < 0.05$ in comparison with diabetic values.

cytes, but decreased thiobarbituric acid-reactive substances levels in the liver.

3.2. Effects of diabetes and melatonin treatment on the activities of antioxidative, drug-metabolizing and pentose phosphate pathway enzymes

A marked decrease in glucose-6-phosphate dehydrogenase activity (2.2-fold) in the post-mitochondrial fraction of diabetic liver tissue was observed (Fig. 3A). The activity of transketolase also decreased substantially (1.7-fold) in the liver of diabetic animals in comparison to control rats (Fig. 3B). Melatonin treatment of diabetic rats partially prevented the diabetes-induced inactivation of these enzymes, increasing liver glucose-6-phosphate dehydrogenase (1.3-fold) and transketolase (1.2-fold) activities (Fig. 3). A similar reduction in the activities of the antioxidative enzymes glutathione peroxidase (1.3-fold) (Fig. 3C) and catalase (1.2-fold) (Fig. 3D) in the diabetic liver were noted.

Table 2

Effects of diabetes and melatonin treatment (18 days) on the activities of glutathione peroxidase, catalase and glucose-6-phosphate dehydrogenase, GSH and membrane lipid peroxidation product levels in erythrocytes and nitrite/nitrate level in rat blood plasma

	Control	Diabetes	Diabetes+ melatonin
Glutathione peroxidase, μmol GSH/min/ml packed cells	448±31	476±32	502±17
Catalase, mmol H ₂ O ₂ /min/ml packed cells	12.9±0.8	13.3±0.7	11.8±0.6
Glucose-6-phosphate dehydrogenase, μmol NADPH/min/ml packed cells	2.69±0.18	3.17±0.08 ^a	2.96±0.08
GSH, mM	1.34±0.16	1.25±0.14	1.21±0.06
Thiobarbituric acid-reactive substances, nmol/ml packed cells	3.9±2.1	5.0±0.7	4.6±1.3
Nitrite/nitrate, μM	22.1±1.4	32.3±3.5 ^a	22.0±3.3 ^b

Data, presented as a mean±S.E.M., represent values at the termination of the experiment (25 days of experimental diabetes).

^a $P < 0.05$ vs control non-treated animals.

^b $P < 0.05$ vs diabetic group.

Table 3
Effects of diabetes and melatonin treatment (18 days) on GSH, membrane lipid peroxidation products and nitric oxide levels in rat liver tissue and nitric oxide level in rat aorta tissue

	Control	Diabetes	Diabetes + melatonin
GSH, nmol/mg protein	36.6±3.3	31.4±2.9	26.8±2.8
Thiobarbituric acid-reactive substances, nmol/mg protein	0.027±0.003	0.022±0.002 ^a	0.018±0.004 ^{a,b}
Nitrite/nitrate (liver), nmol/mg protein	0.59±0.12	0.56±0.07	0.46±0.10 ^b
Nitrite/nitrate (aorta), nmol/mg protein	3.92±0.7	5.19±1.49	2.75±0.25 ^b

Data, presented as a mean±S.E.M., represent values at the termination of the experiment (25 days of experimental diabetes).

^a $P < 0.05$ vs control non-treated animals.

^b $P < 0.05$ vs diabetic group.

The activity of glutathione *S*-transferase, a drug-metabolizing enzyme, did not change in the post-mitochondrial fraction of liver tissue after 25-day diabetes as opposed to those in non-diabetic rats (Fig. 3E). Melatonin administration to the diabetic rats partially reversed liver catalase activity but did not affect glutathione peroxidase activity (Fig. 3). In diabetic rat erythrocytes, the activities of glutathione peroxidase and catalase remained unchanged (Table 2). The activity of the pentose phosphate pathway enzyme, glucose-6-phosphate dehydrogenase, increased in diabetic erythrocytes in comparison with the controls (Table 2). Melatonin did not influence these enzyme activities in erythrocytes.

3.3. Direct scavenging of nitric oxide by melatonin

Melatonin administration to diabetic rats drastically decreased the elevated nitric oxide concentration. We assessed, *in vitro*, the

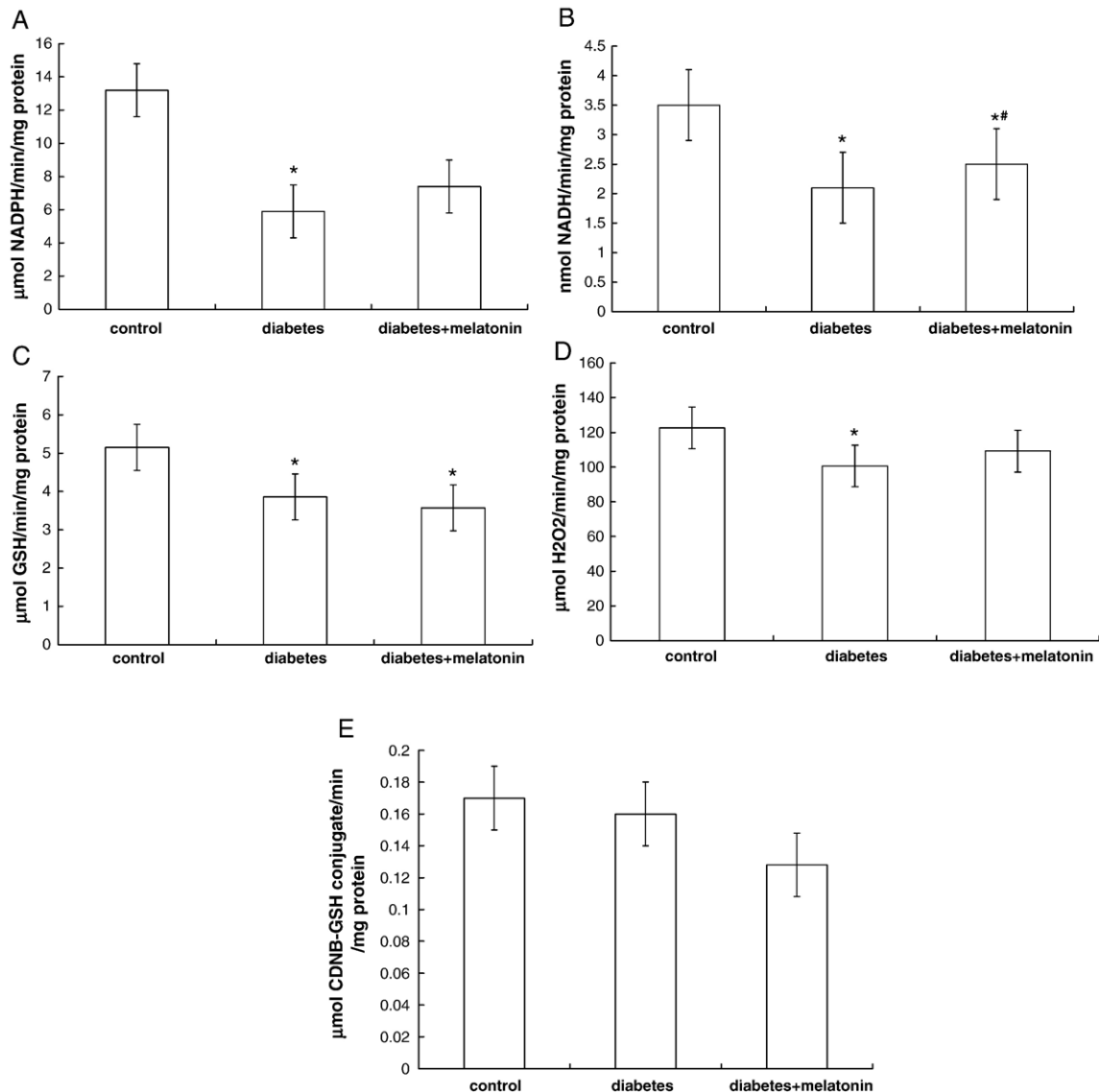


Fig. 3. Effect of diabetes and melatonin treatment on the activities of glucose-6-phosphate dehydrogenase (A), transketolase (B), glutathione peroxidase (C), catalase (D) and glutathione *S*-transferase (E) in the rat liver. * $P < 0.05$ in comparison with control values, # $P < 0.05$ in comparison with diabetic values.

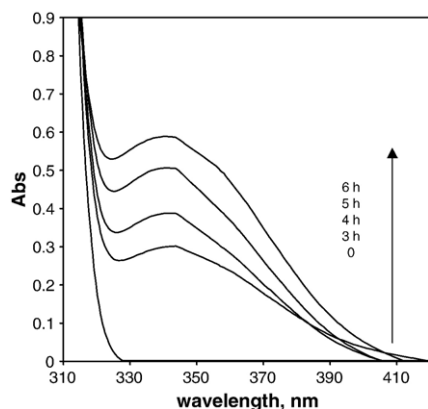


Fig. 4. Nitrosomelatonin formation during incubation of nitrosoglutathione (1 mM) with melatonin (1 mM) for 0, 3, 4, 5 and 6 h measured as the differential absorption spectra of nitrosoglutathione in the presence of melatonin versus nitrosoglutathione. Phosphate buffered saline, pH 7.4, 18 °C.

possibility of a direct melatonin interaction with nitric oxide liberated by NO donor, nitrosoglutathione (Gabor et al., 1997). The nitrosoglutathione decomposition in the presence of melatonin resulted in nitrosomelatonin formation that was demonstrated in terms of increasing characteristic absorbance at 346 nm (Fig. 4). We extracted nitrosomelatonin by ethylacetate from the reaction mixture (nitrosoglutathione and melatonin) and characterized as a specific absorption band (Blanchard-Fillion et al., 2001).

4. Discussion

Melatonin causes a broad spectrum of metabolic and physiological effects, binding to the target proteins (receptors) and directly neutralizing a number of reactive oxygen, nitrogen and chlorine species (Reiter, 2000). A large number of studies have shown melatonin and its metabolites to be highly effective free radical scavengers and ubiquitously acting antioxidants, which play an essential role in reducing oxidative stress under a variety of experimental settings (Reiter, 2000; Tan et al., 2007). Melatonin scavenges singlet oxygen (the rate constant was $2.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$), hydrogen peroxide (the rate constant during the initial reaction phase was $2.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$), forming *N*¹-acetyl-*N*²-formyl-5-methoxykynuramine, a hydroxyl radical (the rate constant was in the range of 1.25×10^{10} – $4.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$), forming cyclic 3-hydroxymelatonin, and hypochlorous acid (the rate constant was $7.0 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) (Reiter et al., 2001). At the same time it was reported that melatonin has little or no ability to scavenge the superoxide anion radical (Reiter et al., 2001).

Earlier we found that melatonin effectively interacted with alkoxy and peroxy radicals generated in oxidant-treated cells and cell-free systems (Zavodnik et al., 2006). From the thermodynamic standpoint, *N*¹-acetyl-*N*²-formyl-5-methoxykynuramine was the most stable end oxidative product of melatonin (Zavodnik et al., 2006).

Melatonin has been documented to exert beneficial effects under diabetic conditions. Vural et al. (2001) observed that melatonin (10 mg/kg per day, i.p., 6 weeks) prevented reductions in the thiol antioxidant (GSH) level in red blood cells of the

diabetic rats and the activities of glutathione peroxidase and superoxide dismutase and inhibited lipid peroxidation, stimulating the antioxidant status of diabetic rats. It was shown that 100 nM melatonin stimulated acceleration of flux through fructose-1,6-biphosphotase and increased both glucose and lactate synthesis in rabbit kidney-cortical tubules (Derlacz et al., 2005). Treatment of diabetic animals with melatonin (200 µg/kg) for 15 days significantly raised plasma levels of total thiols, increased ceruloplasmin activities and decreased plasma lipid peroxides, uric acid, blood glucose, triglycerides, cholesterol and nitric oxide levels (Anwar and Meki, 2003).

In the present study, we observed that experimental (25 days) streptozotocin-induced diabetes affected the activities of the liver antioxidative enzymes, glutathione peroxidase and catalase, the key enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase, which plays an essential role in regulation of the cellular redox level, generating NADPH, and transketolase; the latter is the non-oxidative part of the pentose phosphate pathway. We demonstrated that the activity of glutathione *S*-transferase, catalyzing detoxification of electrophilic/oxidizing xenobiotics, did not change.

The literature contains a confusing array of data showing marked effects of diabetes on the activities of antioxidative enzymes, those of the pentose phosphate shunt and drug-metabolizing enzymes in various tissues. Streptozotocin-induced diabetic rats showed a significant decrease in the activities of hepatic hexokinase, phosphofructokinase and glucose-6-phosphate dehydrogenase, as well as an increase in glucokinase activity (Ugochukwu and Babady, 2003). Uluşu et al. (2003) reported that glucose-6-phosphate dehydrogenase activity in the brain of rats with streptozotocin-induced diabetes was markedly elevated in comparison to controls, the aorta glucose-6-phosphate dehydrogenase activity of diabetic rats amounting to 52% of the control value. Brain, heart and kidney glutathione peroxidase activities were increased in streptozotocin-induced diabetes (Uluşu et al., 2003).

It was shown previously that glutathione *S*-transferase activity in erythrocytes, as well as liver and kidney tissues, dropped slightly during diabetes and significantly increased in melatonin-treated diabetic rats (Anwar and Meki, 2003).

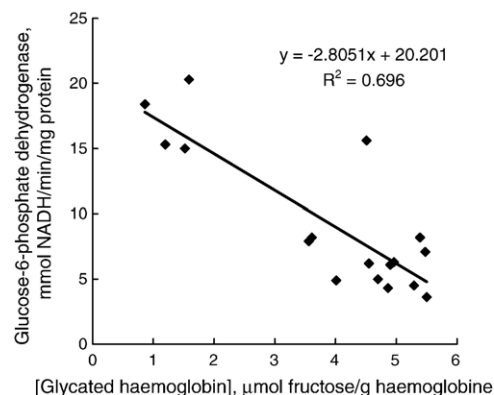


Fig. 5. Relationships between blood glycated hemoglobin level and liver glucose-6-phosphate dehydrogenase activity for all groups of rats.

According to [Uluslu et al. \(2003\)](#), glutathione *S*-transferase activity did not significantly change in the brain or aorta of diabetic rats, whereas catalase was activated in the diabetic heart and depressed in the diabetic kidney. By comparison, [Sheweita et al. \(2002\)](#) observed that alloxan-induced diabetes in rats increased the hepatic activities of cytochrome P450, NADPH-cytochrome reductase, and some other enzymes of phase I and phase II drug-metabolism. Alterations in the activities of phase I and phase II drug-metabolizing enzymes should be considered during drug administration to diabetic patients ([Sheweita et al., 2002](#)). The changes in enzyme activities during diabetes could be due to either an altered enzyme gene expression ([Bojunga et al., 2004](#)) or a post-translational enzyme modification (glycation), or a combination thereof. We observed a correlation between the level of protein glycosylation and glucose-6-phosphate dehydrogenase activity ([Fig. 5](#)).

Diabetes significantly reduced the level of antioxidative defence and the redox state of the diabetic tissues in comparison with the controls. These data are in line with our previous findings obtained for long-term streptozotocin-induced diabetes ([Lapshina et al., 2006](#)). At the same time, we did not observe any changes in the GSH level and additional thiobarbituric acid-reactive substances accumulation in diabetic red blood cells or liver tissue. A possible reason for the reduction of lipid peroxidation products accumulation in the liver of diabetic rats might be a disruption of lipid content in cell membranes during diabetes ([Watala and Winocour, 1992](#)). [Raza et al. \(2004\)](#) showed that mitochondrial GSH level was moderately increased (20–35%) in different tissues of streptozotocin-diabetic animals, whereas GSH in the cytosolic fraction was increased only in the liver (~10%) and reduced by ~20% in the kidney.

Hyperglycemia (25 days) resulted in an expected high glycosylated hemoglobin level, growth retardation, hepatolysis reflected by increased blood plasma alanine aminotransferase activity and bilirubin levels. The decrease of blood plasma aspartate aminotransferase activity despite of the increased alanine aminotransferase activity might be explained by hepatic aspartate aminotransferase modification (glycation) during diabetes ([Okada et al., 1997](#)). In our experiment, neither hypoglycemic effect of melatonin nor decreased level of glycosylated hemoglobin was observed after melatonin administration to diabetic animals. We did note a reduction in plasma bilirubin levels after melatonin administration to diabetic animals; this demonstrates hepatoprotective properties of melatonin. Melatonin administration to diabetic rats partially reversed both the activities of the pentose phosphate pathway enzymes, glucose-6-phosphate dehydrogenase and transketolase, and catalase activity in the diabetic liver. Earlier a stimulatory effect of melatonin (125–500 mg/kg) on the activity of hepatic and cerebral glucose-6-phosphate dehydrogenase in mice and a protective effect of the indole on the development of alloxan-induced diabetes were shown ([Pierrefiche and Laborit, 1995](#)).

In the present study, the levels of nitrite/nitrate, as the end products of nitric oxide conversion, were increased in blood plasma and aortic tissue from diabetic rats in comparison with non-diabetic animals. The literature contains variable data

related to the influence of diabetes on the activity of NO synthases as well as on NO levels. [Xia et al. \(2006\)](#) showed that streptozotocin-induced diabetes in rats was accompanied by a significant reduction of plasma stable metabolites of NO; the protective effect of *N*-acetylcysteine under diabetes was attributed to restoration of NO bioavailability in the circulation. A similar attenuation of nitric oxide levels was observed in other studies ([Sailaja Devi and Suresh, 2000](#); [Yoshioka et al., 2005](#)). On the contrary, it was demonstrated that iNOS-mRNA was significantly up-regulated and NO production increased in diabetes and the reduced reactivity of the vascular smooth muscle to NO was a possible reason for vascular damage ([Anwar and Meki, 2003](#); [Bojunga et al., 2004](#); [Ho et al., 1999](#); [Welsh et al., 1994](#)). The changes in nitric oxide levels due to melatonin administration might be a result of either NOS gene expression down-regulation or direct NO scavenging by pharmacological concentrations of the indolamine. It has been reported that melatonin reduces the expression of inducible isoform of NO synthase *via* inhibition of the transcription factor NF- κ B ([Gilad et al., 1998](#)) and effectively inhibits iNOS activity ([Poza et al., 1997](#)). We have shown that melatonin influences the rate of decomposition of NO donors (as in the case of GSH-NO in our experiment *in vitro*) or regulation of NO bioavailability, acting as an NO scavenger in the reactions of transnitrosylation ([Blanchard-Fillion et al., 2001](#)). On the other hand, the reduction of reactive nitrogen species by melatonin has been reported ([Blanchard-Fillion et al., 2001](#)). It was recently demonstrated that melatonin is *N*-nitrosated by nitrite at acidic pH and by NO in the presence of oxygen under neutral conditions. 1-Nitrosomelatonin behaves as an NO-donor regenerating melatonin ([Blanchard-Fillion et al., 2001](#)).

Melatonin might be considered as a factor regulating glucose metabolism by affecting glucose-metabolizing enzyme activities, restoring tissue redox-balance and nitric oxide bioavailability. The decrease in nitric oxide levels may be related to the anti-inflammatory effect of melatonin ([Carrillo-Vico et al., 2005](#)). Melatonin treatment has beneficial effects in controlling diabetic vascular complications and hepatotoxicity.

References

- Aebi, H., 1984. Catalase in vitro. *Meth. Enzymol.* 105, 121–126.
- Allegra, M., Reiter, R.J., Tan, D.X., Gentile, C., Tesoriere, L., Livrea, M.A., 2003. The chemistry of melatonin's interaction with reactive species. *J. Pineal. Res.* 34, 1–10.
- Anwar, M.M., Meki, A.R.M., 2003. Oxidative stress in streptozotocin-induced diabetic rats: effects of garlic oil and melatonin. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 135, 539–547.
- Baynes, J.W., 1991. Role of oxidative stress in development of complications in diabetes. *Diabetes.* 40, 405–412.
- Becker-Andre, M., Wiesenberg, I., Schaeren-Wiemers, N., Andre, E., Missbach, M., Saurat, J.H., Carlberg, C., 1994. Pineal gland hormone melatonin binds and activates an orphan of nuclear receptor super family. *J. Biol. Chem.* 269, 28531–28534.
- Blanchard-Fillion, B., Servy, C., Ducrocq, C., 2001. 1-Nitrosomelatonin is a spontaneous NO-releasing compound. *Free Radic. Res.* 35, 857–866.
- Bojunga, J., Dresar-Mayert, B., Usadel, K.H., Kusterer, K., Zeuzem, S., 2004. Antioxidative treatment reverses imbalances of nitric oxide synthase isoform expression and attenuates tissue-cGMP activation in diabetic rats. *Biochem. Biophys. Res. Commun.* 316, 771–780.

- Brzezinski, A., 1997. Melatonin in humans. *N. Engl. J. Med.* 336, 186–195.
- Calles-Escandon, J., Cipolla, M., 2001. Diabetes and endothelial dysfunction: a clinical perspective. *Endocr. Rev.* 22, 36–52.
- Carrillo-Vico, A., Guerrero, J.M., Lardone, P.J., Reiter, R.J., 2005. A review of the multiple actions of melatonin on the immune system. *Endocrine* 27, 189–200.
- Costa Rosa, L.F., Curi, R., Murphy, C., Newsholme, P., 1995. Effect of adrenaline and phorbol myristate acetate or bacterial lipopolysaccharide on stimulation of pathways of macrophage glucose, glutamine and O₂ metabolism. Evidence for cyclic AMP-dependent protein kinase mediated inhibition of glucose-6-phosphate dehydrogenase and activation of NADP⁺-dependent 'malic' enzyme. *Biochem. J.* 310, 709–714.
- Derlacz, R.A., Poplawski, P., Napierala, M., Jagielski, A.K., Bryla, J., 2005. Melatonin-induced modulation of glucose metabolism in primary cultures of rabbit kidney-cortex tubules. *J. Pineal. Res.* 38, 164–169.
- Ellman, J., 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82, 70–77.
- Evans, J.L., Goldfine, I.D., Maddux, B.A., Grodsky, G.M., 2003. Are oxidative stress-related signaling pathways mediators of insulin resistance and β -cell dysfunction? *Diabetes* 52, 1–8.
- Flodstrom, M., Tyrberg, B., Eizirir, D.L., Sandler, S., 1999. Reduced sensitivity of inducible nitric oxide synthase-deficient mice to multiple low-dose streptozotocine-induced diabetes. *Diabetes* 48, 706–713.
- Gabor, G., Allon, N., Weetall, H.H., 1997. Are thiols the carrier of nitric oxide in biological systems? A kinetic model. *Microchem. J.* 56, 177–187.
- Gilad, E., Wong, H.R., Zingarelli, B., Virag, L., O'Connor, M., Salzman, A.L., Szabo, C., 1998. Melatonin inhibits expression of the inducible isoform of nitric oxide synthase in murine macrophages: role of inhibition of NF κ B activation. *FASEB J.* 12, 685–693.
- Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [¹⁵N]nitrate in biological fluids. *Anal. Biochem.* 126, 131–138.
- Habig, W.H., Pabst, M.J., Jacoby, W.B., 1974. Glutathione-S-transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130–7139.
- Hammes, H.P., Du, X., Edelstein, D., Taguchi, T., Matsumura, T., Ju, Q., Lin, J., Bierhaus, A., Nawroth, P., Hannak, D., Neumaier, M., Bergfeld, R., Giardino, I., Brownlee, M., 2003. Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy. *Nat. Med.* 9, 294–299.
- Ho, E., Chen, G., Bray, T.M., 1999. Supplementation of N-acetylcysteine inhibits NF κ B activation and protects against alloxan-induced diabetes in CD-1 mice. *FASEB J.* 13, 1845–1854.
- Kojda, G., Harrison, D., 1999. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc. Res.* 43, 562–571.
- Lapshina, E.A., Sudnikovich, E.Ju., Maksimchik, Ju.Z., Zbrodskaya, S.V., Zavodnik, L.B., Kubyshin, V.L., Nocun, M., Kazmierczak, P., Dobaczewski, M., Watala, C., Zavodnik, I.B., 2006. Antioxidative enzymes and glutathione S-transferase activities in diabetic rats exposed to long-term ASA treatment. *Life Sci.* 79, 1804–1811.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Martinez, J.I.R., Launay, J.M., Dreux, C., 1979. A sensitive fluorimetric microassay for the determination of glutathione peroxidase activity. Application to human blood platelets. *Anal. Biochem.* 98, 154–159.
- Metz, T.O., Alderson, N.L., Chachich, M.E., Thorpe, S.R., Baynes, J.W., 2003. Pyridoxamine traps intermediates in lipid peroxidation reactions in vivo. Evidence on the role of lipids in chemical modification of protein and development of diabetic complications. *J. Biol. Chem.* 278, 42012–42019.
- Nishida, S., 2005. Metabolic effects of melatonin on oxidative stress and diabetes mellitus. *Endocrine* 27, 131–136.
- Okada, M., Murakami, Y., Miyamoto, E., 1997. Glycation and inactivation of aspartate aminotransferase in diabetic rat tissues. *J. Nutr. Sci. Vitaminol. (Tokyo)* 43, 463–469.
- Paskaloglu, K., Sener, G., Ayangolu-Dulger, G., 2004. Melatonin treatment protects against diabetes-induced functional and biochemical changes in the rat aorta and corpus cavernosum. *Eur. J. Pharmacol.* 499, 345–354.
- Pierrefiche, G., Laborit, H., 1995. Oxygen free radicals, melatonin, and aging. *Exp. Gerontol.* 30, 213–227.
- Pozo, D., Reiter, R.J., Calvo, J.R., Guerrero, J.M., 1997. Inhibition of cerebellar nitric oxide synthase and cyclic GMP production by melatonin via complex formation with calmodulin. *J. Cell. Biochem.* 65, 430–442.
- Raza, H., Prabu, S.K., Robin, M.A., Avadhani, N.G., 2004. Elevated mitochondrial cytochrome P450 2E1 and glutathione S-transferase A4-4 in streptozotocin-induced diabetes rats: tissue specific variations and roles in oxidative stress. *Diabetes* 53, 185–194.
- Reiter, R.J., 2000. Melatonin: lowering the high price of free radicals. *News Physiol. Sci.* 15, 246–250.
- Reiter, R.J., Tan, D.X., Manchester, L.C., Qi, W., 2001. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence. *Cell Biochem. Biophys.* 34, 237–256.
- Sailaja Devi, M.M., Suresh, Y., Das, 2000. Preservation of the antioxidant status in chemically-induced diabetes mellitus by melatonin. *J. Pineal. Res.* 29, 108–115.
- Sheweita, S.A., Newairy, A.A., Mansour, H.A., Yousef, M.I., 2002. Effect of some hypoglycemic herbs on the activity of phase I and II drug-metabolizing enzymes in alloxan-induced diabetic rats. *Toxicology* 174, 131–139.
- Stadler, K., Jenei, V., von Bolcschazy, G., Somogyi, A., Jakus, J., 2003. Increased nitric oxide levels as an early sign of premature aging in diabetes. *Free Radic. Biol. Med.* 35, 1240–1251.
- Stocks, J., Dormandy, T.L., 1971. The autooxidation of human red cells lipids induced by hydrogen peroxide. *Br. J. Haematol.* 20, 95–111.
- Tan, D.X., Manchester, L.C., Terron, M.P., Flores, L.J., Reiter, R.J., 2007. One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species? *J. Pineal. Res.* 42, 28–42.
- Ugochukwu, N.H., Babady, N.E., 2003. Antihyperglycemic effect of aqueous and ethanolic extracts of *Gongronema latifolium* leaves on glucose and glycogen metabolism in livers of normal and streptozotocin-induced diabetic rats. *Life Sci.* 73, 1925–1938.
- Ulusu, N.N., Sahilli, M., Avci, A., Canbolat, O., Ozansov, G., Ari, N., Bali, M., Stefek, M., Stolic, S., Gaidosik, A., Karasu, C., 2003. Pentose phosphate pathway, glutathione-dependent enzymes and antioxidant defense during oxidative stress in diabetic rodent brain and peripheral organs: effects of stobadine and vitamin E. *Neurochem. Res.* 28, 815–823.
- Vural, H., Sabuncu, T., Arslan, S.O., Aksoy, N., 2001. Melatonin inhibits lipid peroxidation and stimulates the antioxidant status of diabetic rats. *J. Pineal. Res.* 31, 193–198.
- Watala, C., Winocour, P.D., 1992. The relation of chemical modification of membrane proteins and lipoproteins to reduced membrane fluidity of erythrocytes from diabetic subjects. *Eur. J. Clin. Chem. Clin. Biochem.* 30, 513–519.
- Welsh, N., Eizirik, D.L., Sandler, S., 1994. Nitric oxide and pancreatic beta-cell destruction in insulin dependent diabetes mellitus: don't take no for an answer. *Autoimmunity* 18, 285–290.
- Wittmann, I., Koszegi, T., Wagner, L., Wagner, Z., Mazak, I., Nagy, J., 2001. Insulin-induced peroxynitrite production in human platelet-rich plasma. *Redox Rep.* 6, 251–255.
- Wolff, S.P., 1993. Diabetes mellitus and free radicals. Free radicals, transition metals and oxidative stress in the aetiology of diabetes mellitus and complications. *Br. Med. Bull.* 49, 642–652.
- Xia, Z., Nagareddy, P.R., Guo, Z., Zhang, W., McNeill, J.H., 2006. Antioxidant N-acetylcysteine restores systemic nitric oxide availability and corrects depressions in arterial blood pressure and heart rate in diabetic rats. *Free Radic. Res.* 40, 175–184.
- Yoshioka, N., Adachi, J., Ueno, Y., Yoshida, K., 2005. Oxysterols increase in diabetic rats. *Free Radic. Res.* 39, 299–304.
- Zavodnik, I.B., Domanski, A.V., Lapshina, E.A., Bryszewska, M., Reiter, R.J., 2006. Melatonin directly scavenges free radicals generated in red blood cells and a cell free system: chemiluminescence measurements and theoretical calculations. *Life Sci.* 79, 391–400.