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European Journal of Pharmacology 499 (2004) 345–354

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Melatonin treatment protects against diabetes-induced functional and biochemical changes in rat aorta and corpus cavernosum

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Received 19 March 2004; received in revised form 26 July 2004; accepted 3 August 2004

Abstract

Enhanced oxidative stress due to diabetes is accepted to lead to endothelial dysfunction, and this is known to play a key role in the pathogenesis of diabetic vascular diseases and complications. This study was designed to determine the possible protective effect of melatonin and/or insulin treatment on the functional and biochemical changes caused by hyperglycemia in aorta and corpus cavernosum of diabetic rats. Wistar albino male rats were rendered diabetic by injecting streptozotocin (60 mg/kg, intraperitoneally (i.p.)). Melatonin (10 mg/kg, i.p.) and/or insulin (6 U/kg, subcutaneously (s.c.)) were administered for 8 weeks. In the diabetic group, the contractile responses of aortic strips to phenylephrine were significantly impaired (EC_{50} 5.5×10^{-7} M in diabetic and EC_{50} 1.47×10^{-7} M in the control group, $P<0.001$). Treatment with melatonin (EC_{50} 4.6×10^{-7} M) or insulin+melatonin (EC_{50} 1.68×10^{-7} M, $P<0.001$) improved the contractile responses. Acetylcholine caused a dose-dependent relaxation response (EC_{50} 1.58×10^{-7} M) which was impaired in the diabetic group (EC_{50} 26×10^{-7} M, $P<0.001$). There was less impairment in melatonin-, insulin- and insulin+melatonin-treated groups (EC_{50} 11.61×10^{-7}, 7.3×10^{-7} and 1.41×10^{-7} M, respectively, $P<0.01$). Contractile responses to phenylephrine were also impaired in the corpus cavernosum strips (EC_{50} 2.06×10^{-5} M in diabetic and 0.94×10^{-5} M in the control group, $P<0.001$). In the melatonin- (EC_{50} 1.59×10^{-5} M) and insulin+melatonin-treated (EC_{50} 1.53×10^{-5} M, $P<0.5$) groups contractile responses were improved. In the diabetic group, the relaxation responses of corpus cavernosum strips to acetylcholine were impaired (EC_{50} 24.12×10^{-5} M, $P<0.001$), and treatment with melatonin (EC_{50} 0.68×10^{-5} M), insulin (EC_{50} 0.53×10^{-5} M) or insulin+melatonin (0.98×10^{-5} M, $P<0.001$) restored the responses to acetylcholine. In diabetic tissues, malondialdehyde levels were increased while glutathione levels were decreased, demonstrating oxidative damage. This was also prevented by treatment with melatonin or the melatonin and insulin combination. The diabetic state enhances the generation of free radicals, and both melatonin and insulin treatments reduced this oxidative stress; however, treatment with the combination was the most efficient in preventing diabetes-induced damage. Thus, our results suggested that giving diabetic patients adjuvant therapy with melatonin may have some benefit in controlling diabetic complications.

Keywords: Streptozotocin; Aorta; Corpus cavernosum; Melatonin; Insulin

1. Introduction

Vascular diseases are currently accepted to be the principal cause of morbidity and mortality in patients with diabetes mellitus, and loss of the modulatory role of the endothelium may be a critical and initiating factor in the development of diabetic vascular diseases. The endothelium controls the tone of the underlying vascular smooth muscle through the production of vasodilator mediators, and impaired endothelium-dependent vasodilatation has been demonstrated in various vascular beds of different models of diabetes (Durante et al., 1988, Gryglewski et al., 1986, Kamata et al., 1989, Langenstroer and Pieper, 1992, Pieper and Gross, 1988, Rodriguez-Manas et al., 1998). It has been suggested that the considerable increases in the levels of plasma glucose, cholesterol and free radicals that occur in

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doi:10.1016/j.ejphar.2004.08.002

diabetes may be contributing factors in the development of endothelial dysfunction (Göçmen et al., 2000: Kakkar et al., 1996; Reyes-Toso et al., 2002, Vural et al., 2001).

Diabetes mellitus has been shown to be a state of increased free radical activity, and oxygen-derived free radicals are known to be intricately involved in the regulation of endothelium-dependent relaxation (Giugliano et al., 1996; Kakkar et al., 1996; Langenstroer and Pieper, 1992; Montilla et al., 1998; Reyes-Toso et al., 2002). Pieper and Gross (1988) have reported that in chronic diabetic rat aorta, there is a selective impairment of endothelium-dependent relaxation with particular sensitivity to free radical-induced damage.

In this context, erectile dysfunction is also observed frequently in diabetic men. Pathological changes in vascular and neural structures, as well as depletion of neurotransmitters in the corpus cavernosum, have been demonstrated in human and animal studies of diabetes. It is believed that there are alterations in endothelial cell-mediated physiological mechanisms that control penile smooth muscle tone (Cartledge et al., 2000a,b, 2001; Sullivan et al., 1998). In animal models and patients, it has been claimed that defective nitric oxide (NO) release from corpus cavernosum nonadrenergic noncholinergic nerves and endothelium impairs control of penile smooth muscle relaxation and causes erectile dysfunction (Khan et al., 2001). Various animal studies suggested that in diabetes mellitus, there is an impairment of NO bioavailability (Azadzoi and De Tejeda, 1992; Yıldırım et al., 1999). One of the most important determinants of NO bioavailability is the reaction of NO with reactive oxygen species, O_2^-, which leads to an increased production of peroxynitrite ($ONOO^-$), a potent, toxic, volatile oxidant (Gryglewski et al., 1986).

In previous studies with animal models of streptozotocin-induced diabetes, chronic insulin treatment, starting from the onset of hyperglycemia, has been demonstrated to prevent the impairment of the endothelium-dependent relaxation of rat mesenteric resistance arteries or aortic rings and to prevent the attenuation of agonist-induced contraction in rat aortic rings (Kobayashi and Kamata, 1999).

There are also some reports demonstrating beneficial effects of some antioxidants, such as α-lipoic acid, vitamin E and selenium, in reversing neuronal and endothelial cell dysfunction due to oxidative stress (Baydas et al., 2002; Çınar et al., 2001; Göçmen et al., 2000; Naziroglu and Cay, 2001). In recent reports, melatonin, the main secretory product of the pineal gland, has been demonstrated to have free radical-scavenging and antioxidant properties (Reiter et al., 2000, 2001), and it has been found to protect tissues against oxidative damage induced by a variety of free radical-generating agents and processes (Guerrero et al., 1997; Hara et al., 1997; Tan et al., 1993). Melatonin reduces oxidative stress by several means: it scavenges hydrochlorous acid (Marshall et al., 1996), detoxifies highly toxic hydroxyl radical and peroxyl radical in vitro (Pieri et al., 1995) and scavenges peroxynitrite (Gilad et al., 1997). Melatonin has also been reported to increase the synthesis of glutathione (Reiter et al., 1995).

There are several studies showing the antioxidant effects of melatonin in streptozotocin-induced diabetic rats, but the relationship between oxidative stress and functional deterioration of the tissues has not been studied (Çınar et al., 2001; Montilla et al., 1998; Vural et al., 2001). Thus, the aim of this study was to investigate the effects of melatonin on diabetes-induced oxidative damage and contractile changes in the aorta and corpus cavernosum in comparison to the protective effects of insulin. We also investigated the efficacy of the combination of insulin and melatonin.

2. Materials and methods

2.1. Animals

Male Wistar albino rats (250–300 g) were housed in a room at a constant temperature of 22±2 °C, with 12-h light/dark cycles, and fed on standard pellet chow and water ad libitum. The study was approved by the Marmara University, School of Medicine, Animal Care and Use Committee.

2.2. Experimental groups

The rats were divided into three groups: diabetic rats, age-matched controls and melatonin-treated rats. The control group (n=8) received 0.1 M citrate buffer, the solvent of streptozotocin, intraperitoneally (i.p.). In the second group (n=8), melatonin (10 mg/kg/day) was administered i.p. for 8 weeks. In the third group, diabetes was induced in ether-anesthetized animals by intraperitoneal administration of a single dose of streptozotocin (60 mg/kg). About 48 h after streptozotocin treatment, hyperglycemia was confirmed by measuring blood glucose levels, using a glucometer. Rats with blood glucose levels >11.1 mM were considered diabetic. Blood samples were taken from the retro-orbital venous plexus, using a hematocrit capillary tube. About 48 h after streptozotocin injection (i.e., after the onset of diabetes), diabetic animals were divided into four subgroups. While the first group of diabetic rats was treated with melatonin (10 mg/kg/day) for 8 weeks, the second group was treated with 6 U/kg/day NPH insulin (*Lilly*, the product is accepted to be effective for 18–24 h) administered s.c. for the same period. To the third group, insulin and melatonin were administered in combination. The last group received no treatment. Each group consisted of eight rats. Melatonin (Sigma, St. Louis, MO, USA) was dissolved in absolute ethanol and further dilutions were made in saline. The final concentration of ethanol was 1%.

Animals were killed by cervical dislocation, and the corpus cavernosum and aorta were rapidly dissected. Part of the tissue was used for contractility studies, and the

remaining part was stored at −80 °C for the measurement of glutathione activity and lipid peroxidation (malondialdehyde levels).

2.3. In vitro organ bath experiments

The penis was pinned out in a dish containing chilled Krebs–Henseleit buffer aerated with 95% O_2 and 5% CO_2, and the urethra and dorsal vein were removed. After removal of the surrounding connective tissue, each corpus cavernosum was excised by separation from the thick medial septum, each corpus yielding a single strip of 2×2×15 mm, and mounted in an organ bath containing 20 ml of Krebs–Henseleit buffer (118.14 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11.1 mM glucose). The solution in the bath was aerated with 95% O_2 and 5% CO_2, and maintained at 37 °C, at pH 7.4.

Isometric contractions were recorded using a model FT03 force-displacement transducer (Grass Instruments, Quincy, Massachusetts) coupled to a model 7 polygraph (Grass Instruments). The corpus cavernosum strip was placed under a resting tension 1.0 *g*. After a 60-min period of equilibration, the strip was exposed to 124 mM KCl.

Thoracic aorta were carefully isolated, removed from open-chest animals and placed in a dish containing chilled Krebs–Henseleit buffer solution aerated with 95% O_2 and 5% CO_2. After removal of the surrounding connective tissue, aorta were cut transversely into rings approximately 4-mm wide and mounted in organ baths containing 20 ml of Krebs–Henseleit buffer aerated with 95% O_2 and 5% CO_2, maintained at 37 °C.

The rings were placed under a resting tension of 1.0 *g*. Following a 60-min period of equilibration, they were exposed to 80 mM KCl.

In both corpus cavernosum strips and aorta rings, the contractile responses to 10^{-8}–10^{-3} M phenylephrine were determined cumulatively. After a 30-min washout period, the relaxation responses of the same rings were evaluated by adding increasing cumulative concentrations of 10^{-8}–10^{-3} M acetylcholine, 10^{-8}–10^{-3} M sodium nitroprusside or 10^{-6}–10^{-4} M papaverine to rings precontracted with the submaximal dose of phenylephrine (30 μM). Contractile responses to phenylephrine are expressed as percentages of the maximal contraction induced by 124 mM KCl for the corpus cavernosum strips or 80 mM KCl for the aortic rings, and the relaxation responses to acetylcholine are expressed as percentages of the contraction caused by 30 μM phenylephrine.

2.4. Malondialdehyde and glutathione assays

Tissue samples were homogenized with ice-cold 150 mM KCl for determination of malondialdehyde and glutathione levels. The malondialdehyde levels were assayed for products of lipid peroxidation (Beuge and Aust, 1978). Results are expressed as nM malondialdehyde/g tissue. Glutathione was determined by a spectrophotometric method based on the use of Ellman's reagent (Beutler, 1975). Results are expressed in μM glutathione/g tissue.

2.5. Statistical analysis

Data are expressed as the means±S.E.M. The concentrations causing 50% of the maximal response to phenylephrine and acetylcholine were derived from the concentration–response curves using a computer-assisted probit transformation. For analyzing concentration–response curves, unpaired *t*-tests were used, and for analyzing other parameters, analysis of variance followed by Tukey–Kramer multiple-comparisons test were used. Calculations were performed by using the Instat and Prism statistical analysis packages (GraphPad Software, San Diego; California), with $P<0.05$ considered statistically significant.

3. Results

3.1. Body weight and blood glucose level

After 8 weeks, animals injected with streptozotocin had a significant weight loss compared to the age-matched controls (Table 1). Insulin treatment prevented the weight loss, but treatment with melatonin had no significant effect on weight loss in diabetic animals.

One week after injection of streptozotocin, the average blood glucose levels were significantly raised to over 19.4 mM in the diabetic group compared to the control group and remained that way during the study period of 8 weeks. In the diabetic rats, blood glucose levels were back to the control levels after 1 week of treatment with insulin, or insulin and melatonin, and remained that way during the study period (Table 1).

Table 1
Body weight and plasma glucose levels in the control group (C), diabetic group (D), melatonin-treated diabetic group (D+M), insulin-treated diabetic group (D+I), melatonin and insulin-treated diabetic group (D+I+M)

	Body weight (kg)		Plasma glucose (mM)
	Initial	At 8 weeks	At 8 weeks
C	273±6.1	289±6.7[a,b]	5.4±0.1
M	261±3.1	268±3.9	5.2±0.1
D	274±6.3	215±3.5[c,d]	21.2±0.8[c]
D+M	264±1.9	210±8.2[c,e]	20.9±0.7[c]
D+I	270±6.3	257±9.9	5.7±0.2[d]
D+M+I	261±2.6	258±8.2[e]	5.3±0.2[d]

[a] $P<0.05$ compared to C group.
[b] $P<0.05$ compared to diabetic group.
[c] $P<0.001$ compared to C group.
[d] $P<0.001$ compared to diabetic group.
[e] $P<0.01$ compared to diabetic group.

3.2. Contraction and relaxation studies of the corpus cavernosum

Tissues were contracted with 124 mM KCl and similar tensions were obtained in all groups.

In control rats, phenylephrine added cumulatively (10^{-8}–10^{-3} M) caused a concentration-dependent contraction in corpus cavernosum strips precontracted with 124 mM KCl, with a concentration of 0.94×10^{-5} M resulting in a 50% maximal response. In the diabetic group, the contraction responses of the strips to phenylephrine were significantly decreased compared to those in the control group, increasing the concentration needed to achieve the 50% maximal response to 2.06×10^{-5} M. In the melatonin-, insulin- and insulin+melatonin-treated diabetic groups, contractile responses to all doses of phenylephrine were higher than those in the diabetic group (EC_{50} values are given in Table 2). The contractile responses in the melatonin-treated control group were the same as those in the untreated control group, and because this group was included in the study to demonstrate that melatonin alone did not have a significant effect on the parameters studied, the values of this group were not included in the graphs (Fig. 2A).

Acetylcholine added cumulatively at a dose of 10^{-8}–10^{-3} M to strips precontracted with the submaximal dose (30 μM) of phenylephrine caused a dose-dependent relaxation response. In the diabetic group, the relaxation responses to acetylcholine were markedly impaired, increasing the concentration needed to achieve the 50% maximal response to 24.12×10^{-5} M. In melatonin-, insulin- and insulin+melatonin-treated diabetic groups, responses were significantly improved in comparison to those in the untreated diabetic group (EC_{50} values are given in Table 2; Fig. 2B). Sodium nitroprusside and papaverine added cumulatively at doses of 10^{-8}–10^{-3} M and 10^{-6}–10^{-4} M, respectively, to strips precontracted with the submaximal dose of phenylephrine caused concentration-dependent relaxation responses. There was no significant difference in E_{max} and pD_2 values in response to these direct smooth muscle relaxants among the groups (Fig. 2C and D). However, at certain concentrations, the relaxation induced by these agents was impaired in diabetic tissues.

3.3. Contraction and relaxation studies of the aorta

Tissues were contracted with 80 mM KCl and similar tensions were obtained in all groups.

In control rats, phenylephrine added cumulatively (10^{-8}–10^{-3} M) caused a concentration-dependent contraction in aorta rings, with a concentration of 1.47×10^{-7} M resulting in a 50% maximal response. In the diabetic group, the contraction responses of the rings to phenylephrine were significantly impaired compared to those of the control group, increasing the concentration needed to achieve the 50% maximal response to 5.5×10^{-7} M. Treatment of the diabetic rats with melatonin, insulin or insulin+melatonin improved the contractile responses to phenylephrine (EC_{50} values are given in Table 3; Fig. 1A). The contractile responses in the melatonin-treated control group were not significantly different from those of the untreated control group.

Acetylcholine added cumulatively at a dose of 10^{-8}–10^{-3} M to strips precontracted with the submaximal concentration of phenylephrine caused a dose-dependent relaxation response. In the diabetic group, the relaxation responses to acetylcholine were significantly impaired in comparison to those in the control group, whereas there was less impairment in the melatonin-, insulin- and insulin+melatonin-treated diabetic groups (EC_{50} values are given in Table 3; Fig. 1B). Sodium nitroprusside and papaverine added cumulatively at doses of 10^{-8}–10^{-3} and 10^{-6}–10^{-4} M, respectively, to rings precontracted with the submaximal dose of phenylephrine caused dose-dependent relaxation responses. Although these direct smooth muscle relaxant drugs caused similar and full relaxation of the aorta rings from all groups (Fig. 1C and D), at certain concentrations the relaxation of the diabetic tissue was significantly different from that of the control, and this was reversed following treatment with insulin and/or melatonin.

3.4. Malondialdehyde levels of tissues as a measure of lipid peroxidation

The mean levels of malondialdehyde, which is a major degradation product of lipid peroxidation, in corporal tissues and aorta were increased in the diabetic group compared

Table 2
EC_{50} values for phenylephrine (PE), acetylcholine (Ach), sodium nitroprusside (SNP) and papaverine in corpus cavernosum strips

EC_{50}(−log M)	C	M	D	D+M	D+I	D+I+M
PE (%KCl)	0.9×10^{-5}	$1.6\times10^{-5a,b}$	2.1×10^{-5a}	$1.6\times10^{-5a,b}$	1.8×10^{-5a}	$1.5.10^{-5b,c}$
Ach (%PE)	1.4×10^{-5}	1.1×10^{-5d}	24.1×10^{-5a}	0.7×10^{-5d}	0.5×10^{-5d}	0.9×10^{-5d}
SNP (%PE)	2.9×10^{-7}	3.5×10^{-7}	4×10^{-7}	4.1×10^{-7}	4.4×10^{-7}	3.5×10^{-7}
Papaverin (%PE)	1.7×10^{-5}	2.5×10^{-5}	2.1×10^{-5}	1.5×10^{-5}	1.6×10^{-5}	1.7×10^{-5}

$^{cc}P<0.01$, $^{ccc}P<0.001$ Compared to D+M group.
$^{dd}P<0.01$, $^{ddd}P<0.001$ Compared to D+I group (Also for D+M and D+I+M).
[a] $P<0.001$ compared to C group.
[b] $P<0.05$ compared to diabetic group.
[c] $P<0.01$ compared to C group.
[d] $P<0.001$ compared to diabetic group.

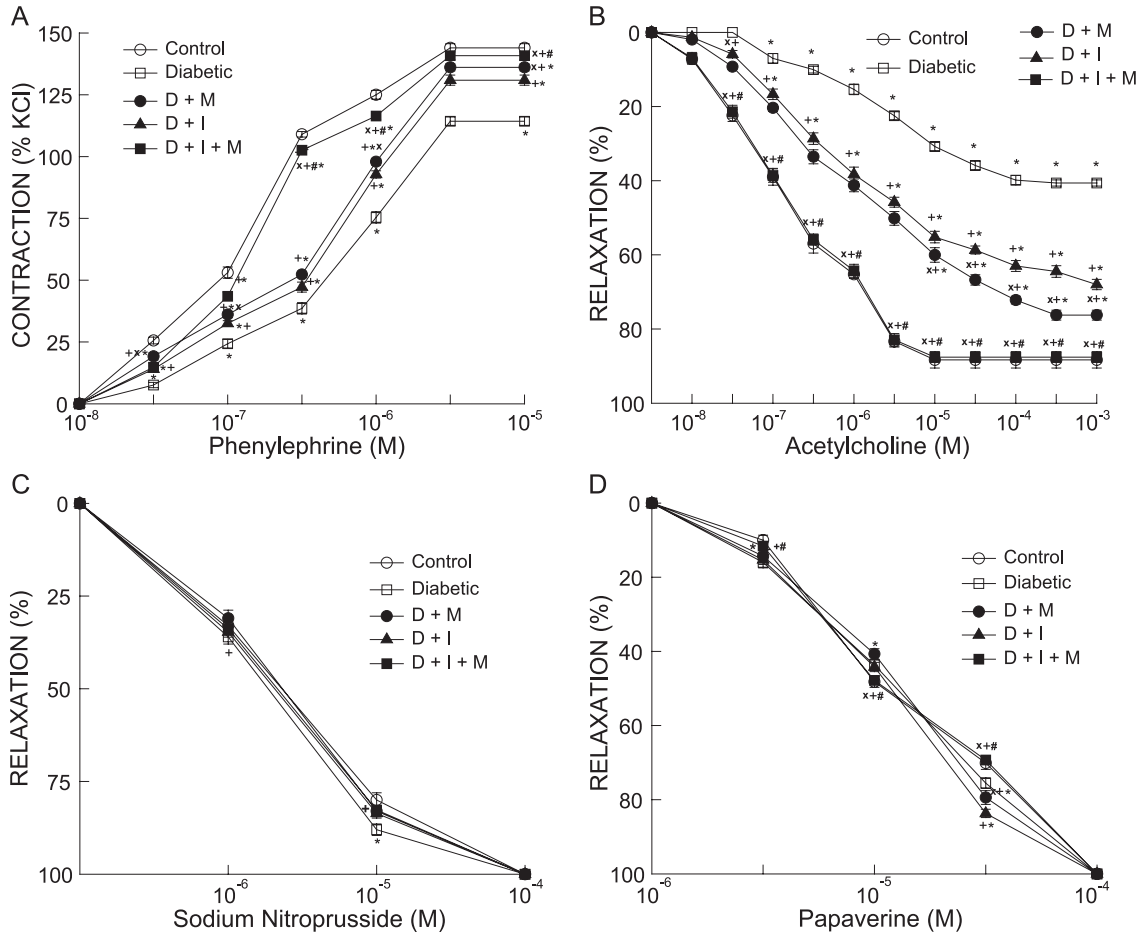

Fig. 1. (A) Concentration–response curves obtained by cumulative addition of phenylephrine (PE) to rat aortic rings. Diabetes (D) decreased contractile activity at all concentrations of PE and treatment with melatonin (M), insulin (I), or melatonin and insulin (M+I) prevented this effect. *P<0.05, compared to control (○); ^{+}P<0.05, compared to diabetic (□); $^{\#}P$<0.05, compared to D+M (●); ^{x}P<0.05, compared to D+I groups (▲). (B) Acetylcholine concentration–response curves in phenylephrine (PE)-precontracted rat aortic rings. Diabetes (D) decreased relaxation responses at all concentrations of PE, and treatment with melatonin (M), insulin (I) or melatonin and insulin (M+I) prevented this effect. *P<0.05, compared to control (○); ^{+}P<0.05, compared to diabetic (□); $^{\#}P$<0.05, compared to D+M (●); ^{x}P<0.05, compared to D+I groups (▲). (C) Sodium nitroprusside (SNP) concentration–response curves in phenylephrine (PE)-precontracted rat aortic rings. Diabetes (D) decreased relaxation responses at all concentrations of PE, and treatment with melatonin (M), insulin (I), or melatonin and insulin (M+I) prevented this effect. *P<0.05, compared to control (○); ^{+}P<0.05, compared to diabetic (□); $^{\#}P$<0.05, compared to D+M (●); ^{x}P<0.05, compared to D+I groups (▲). (D) Papaverine concentration–response curves in phenylephrine (PE)-precontracted rat aortic rings. Diabetes (D) decreased relaxation responses at all concentrations of PE, and treatment with melatonin (M), insulin (I), or melatonin and insulin (M+I) prevented this effect. *P<0.05, compared to control (○); ^{+}P<0.05, compared to diabetic (□); $^{\#}P$<0.05, compared to D+M (●); ^{x}P<0.05, compared to D+I groups (▲).

with the control group (P<0.001), indicating oxidative damage in these tissues. Melatonin alone had no effect on malondialdehyde levels. However, melatonin or melatonin and insulin treatment of the diabetic animals caused a marked decrease in malondialdehyde levels compared to those in the diabetic control animals (Fig. 3A and B).

3.5. Glutathione levels of the tissues

Glutathione levels in corpus cavernosum and aorta were decreased in the diabetic group compared with the control group. Melatonin alone had no effect on glutathione levels. However, melatonin or melatonin and insulin treatment of the diabetic group caused a marked increase in glutathione levels compared to levels in the diabetic control group (Fig. 4A and B).

4. Discussion

In the present study, following induction of diabetes by injection of streptozotocin, treatment with insulin or insulin and melatonin prevented the increase in blood glucose levels, as well as the reduction in body weight, apparently by preventing the development of metabolic changes due to diabetes. However, treatment of the diabetic rats with melatonin did not prevent hyperglycemia or the weight loss in this group. Oxidative damage was observed in the experimentally diabetic rat aorta and corpus cavernosum tissues, as assessed by the increase in malondialdehyde and the concomitant decrease in glutathione levels. In addition, the contractile responses to phenylephrine and the relaxant responses to acetylcholine were impaired in these tissues from diabetic animals. The impaired responses were partly

Table 3
EC_{50} values for phenylephrine (PE), acetylcholine (Ach), sodium nitroprusside (SNP) and papaverine in aorta rings

EC_{50}(−log M)	C	M	D	D+M	D+I	D+I+M
PE (%KCl)	1.5×10^{-7}	1.6×10^{-7}[a,b]	5.5×10^{-7}[c]	4.6×10^{-7}[a,c]	4.9×10^{-7}[c]	1.7×10^{-7}[a,b,d]
Ach (%PE)	1.6×10^{-7}	1.3×10^{-7}[a]	26×10^{-7}[c]	11.6×10^{-7}[a,c]	7.3×10^{-7}[a,e]	1.4×10^{-7}[a,f,g]
SNP (%PE)	1.5×10^{-8}	1.9×10^{-8}	1.3×10^{-8}	1.8×10^{-8}	1.5×10^{-8}	1.4×10^{-8}
Papaverin (%PE)	1.5×10^{-5}	1.3×10^{-5}	1.5×10^{-5}	1.4×10^{-5}	1.2×10^{-5}	1.5×10^{-5}

[a] $P<0.001$ compared to diabetic group.
[b] $P<0.001$ compared to D+I group (also for D+M and D+I+M group).
[c] $P<0.001$ compared to C group.
[d] $P<0.001$ compared to D+M group.
[e] $P<0.01$ compared to C group.
[f] $P<0.01$ compared to D+M group.
[g] $P<0.01$ compared to D+I group (also for D+M and D+I+M group).

restored by melatonin or insulin but were restored to the control levels following treatment with the combination of insulin and melatonin.

As a free radical-generating system, lipid peroxidation has been suggested to be closely related to diabetes-induced tissue damage and that malondialdehyde is a good indicator of the rate of lipid peroxidation. In the present study, the levels of malondialdehyde were significantly increased by diabetes. This observation is in agreement with previous studies (Arduini et al., 1989; Wali et al., 1990). Glutathione provides major protection against oxidative injury, by participating in the cellular system of defence against oxidative damage (Mandell, 1972), and it has been reported that the tissue injury induced by various stimuli is coupled to glutathione depletion (Cuzzocrea et al., 2001; Şener et al., 2000, 2002). The decrease in glutathione levels during diabetes was probably because of its consumption during oxidative stress.

Although the nature of the pathogenic link between high ambient glucose concentrations and diabetic complications remains a matter of debate, hyperglycemia is clearly recognized as the primary culprit. Hyperglycemia induces repeated acute changes in intracellular metabolism (activation of the polyol pathway, activation of diacylglycerol–protein kinase C, increased oxidative stress), as well as cumulative long-term changes in the structure and function of macromolecules, through the formation of advanced glycation end-products (De Vriese et al., 2000). Advanced glycation end products are reported to change cellular function or to generate free radicals (Yıldırım et al., 1999). The presence of advanced glycation end-products has been confirmed in both arterial walls and within the collagen of diabetic human corpus cavernosum, and it has been demonstrated that their accumulation in subendothelial collagen impairs endothelium-dependent vascular smooth muscle relaxation by quenching NO activity (Cartledge et al., 2000b).

Previous reports on the sensitivity of diabetic arteries to contractile agents are controversial. There are some studies that showed an enhanced sensitivity to contractile agents (Pieper and Gross, 1988, Taylor et al., 1992), and it was suggested that the enhanced vascular sensitivity to noradrenaline may be a result of impaired NO release from the endothelium. There are other studies reporting that responses were impaired in chemically induced diabetic rats (Heygate et al., 1995, Kamata et al., 1989). Studies done with isolated resistance arteries from insulin-dependent diabetic patients demonstrated that the contractile responses to noradrenalin were impaired (McNally et al., 1994). Thus, it was concluded that the altered reactivity found with chemically induced diabetes may depend on the vascular effect of streptozotocin per se (Heygate et al., 1995).

Conflicting results have been obtained from studies that investigated the endothelial regulation of vascular smooth muscle function in experimental diabetes: decreased, unchanged and increased responses to acetylcholine in aortic ring preparations from diabetic rats have been reported (De Vriese et al., 2000, Heygate et al., 1995, Taylor et al., 1992, Tesfamariam et al., 1993). In our study, the relaxation responses to acetylcholine were markedly reduced in the diabetic aorta and trabecular smooth muscle tissues. In support of our observation, many other investigators found a significant reduction in the relaxant response to acetylcholine in aortic rings from streptozotocin–diabetic rats (Durante et al., 1988; Kamata et al., 1989; Kobayashi and Kamata, 1999; Tesfamariam et al., 1993). However, it was reported that there are fluctuating changes in endothelium-dependent relaxation at early and intermediate stages, compared with later stages, of the disease (Öztürk et al., 1996; Pieper, 1999), and that difference in experimental conditions may also cause differences in the onset of endothelial dysfunction (Pieper, 1999). In the present study, response to acetylcholine were only partly restored following treatment with insulin or melatonin, but were completely restored by treatment with the combination of insulin and melatonin.

As NO is central to the process of relaxation in both vascular and penile smooth muscles, it will be reasonable to infer that the pathological effects that impair NO-mediated vascular smooth muscle relaxation could also be responsible for the effects seen in penile tissue. Thus, the results of the present study on aorta and corporal tissue will be discussed together.

NO is synthesized from L-arginine and diffuses to the target cell, where it exerts its activity without requiring a specific membrane receptor. The observation that diabetes

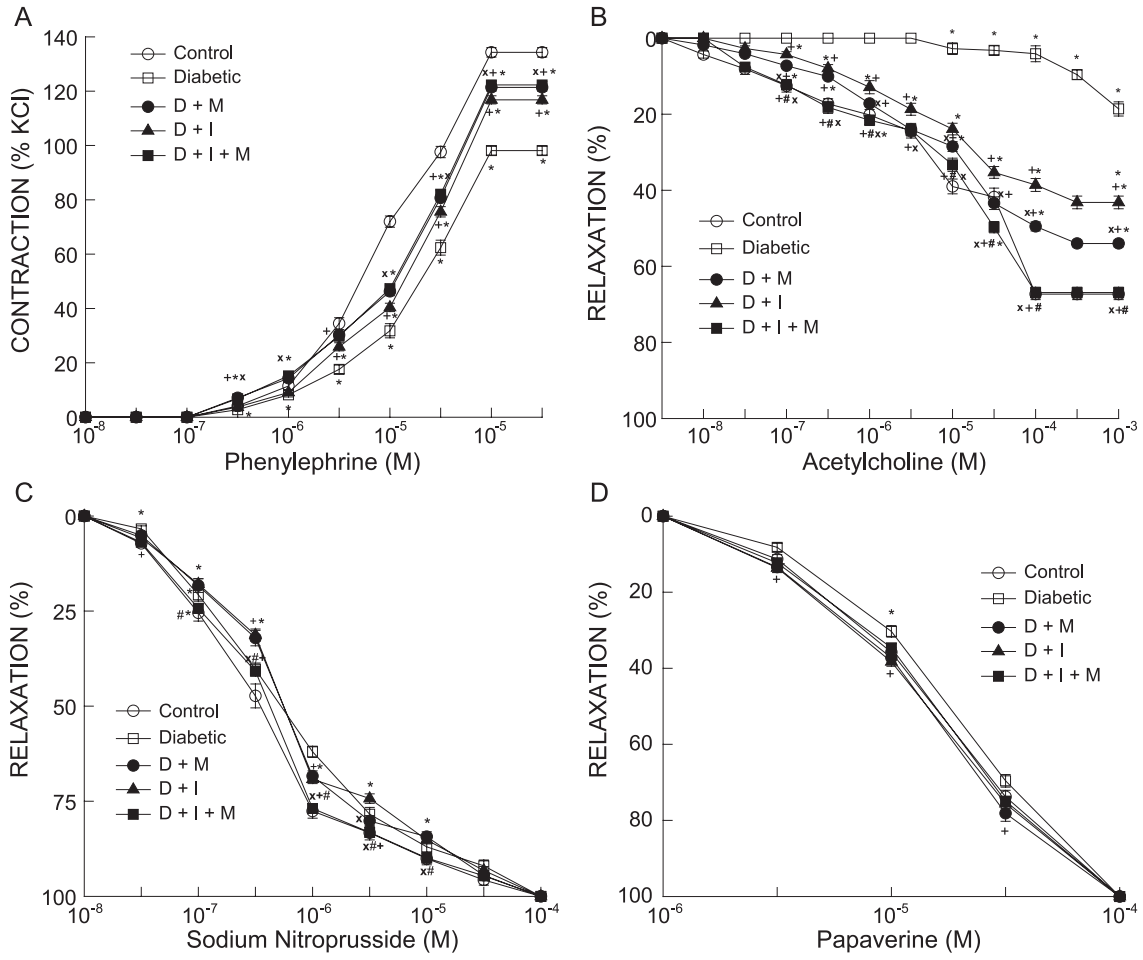

Fig. 2. (A) Concentration–response curves obtained by cumulative addition of phenylephrine (PE) to rat corporeal tissues. Diabetes (D) decreased contractile activity at all concentrations of PE and treatment with melatonin (M), insulin (I), or melatonin and insulin (M+I) prevented this effect. *P<0.05, compared to control (○); ^{+}P<0.05, compared to diabetic (□); $^{\#}P$<0.05, compared to D+M (●); ^{x}P<0.05, compared to D+I groups (▲). (B) Acetylcholine concentration–response curves in phenylephrine (PE)-precontracted rat corporeal tissues. Diabetes (D) decreased relaxation responses at all concentrations of PE, and treatment with melatonin (M), insulin (I) or melatonin and insulin (M+I) prevented this effect. *P<0.05, compared to control (○); ^{+}P<0.05, compared to diabetic (□); $^{\#}P$<0.05, compared to D+M (●); ^{x}P<0.05, compared to D+I groups (▲). (C) Sodium nitroprusside (SNP) concentration–response curves in phenylephrine (PE)-precontracted rat corporeal tissues. Diabetes (D) decreased relaxation responses at all concentrations of PE, and treatment with melatonin M), insulin (I) or melatonin and insulin (M+I) prevented this effect. *P<0.05, compared to control (○); ^{+}P<0.05, compared to diabetic (□); $^{\#}P$<0.05, compared to D+M (●); ^{x}P<0.05, compared to D+I groups (▲). (D) Papaverine concentration–response curves in phenylephrine (PE)-precontracted rat corporeal tissues. Diabetes (D) decreased relaxation responses at all concentrations of PE, and treatment with melatonin (M), insulin (I) or melatonin and insulin (M+I) prevented this effect. *P<0.05, compared to control (○); ^{+}P<0.05, compared to diabetic (□); $^{\#}P$<0.05, compared to D+M (●); ^{x}P<0.05, compared to D+I groups (▲).

impairs endothelium-mediated relaxation of the aorta or trabecular smooth muscle would suggest a possible common pathophysiologic mechanism with alteration in the NO/cGMP (nitric oxide/cyclic guanosine monophosphate) pathway. This would result in neuropathy and endothelial dysfunction.

It is also possible that diabetes impairs the relaxation of trabecular or vascular smooth muscles by diminishing their sensitivity to NO. This possibility is unlikely since corporeal strips and aorta relaxed well to the NO-producing vasodilator, sodium nitroprusside, indicating that the NO/cGMP pathway was still intact. In fact, in the present study, the relaxant response to sodium nitroprusside was increased significantly in diabetic aorta compared to the control aorta, although the EC_{50} values did not change. This finding is in support of the observation of Tesfamariam et al. (1993) that the release of endothelium-dependent NO and its stimulation of arterial guanylate cyclase are not significantly altered in diabetic animals. The response to sodium nitroprusside in the corporeal tissue was decreased, but without a change in EC_{50} values.

Corporeal strips and aorta relaxed also in response to the endothelium-independent vasodilator, papaverine, but there was again some impairment in the response to this agent, although without a change in EC_{50} values. These data, together with the observations obtained with sodium nitroprusside, suggest that, in diabetes, besides endothelial dysfunction, an abnormal morphology of vascular tissue as well as changes in collagen and elastin metabolism and nonenzymatic protein glycosylation may also contribute to

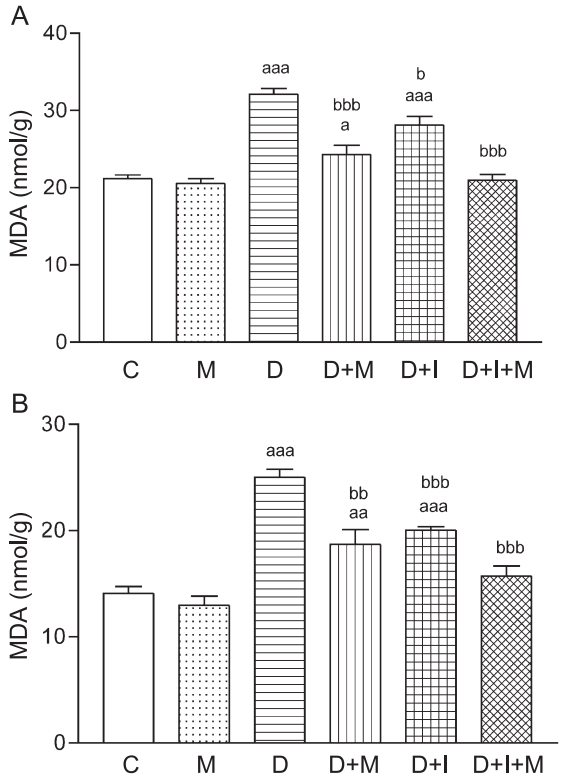

Fig. 3. Malondialdehyde (MDA) levels in (A) aorta and in (B) corpus cavernosum. C, control; M, melatonin; D, diabetic; D+M, melatonin-treated diabetic; D+I, insulin-treated diabetic group; D+I+M, insulin- and melatonin-treated diabetic groups. Each group consisted of eight rats. a, $P<0.05$; aa, $P<0.01$; aaa, $P<0.001$, compared to group C; b, $P<0.05$; bb, $P<0.01$; bbb, $P<0.001$, compared to group D.

the structural and functional changes occurring in vascular tissues, as reported previously (Heygate et al., 1995; Öztürk et al., 1996).

In the present study, treatment of diabetic rats with insulin prevented hyperglycemia and also partially restored the functional deterioration of the aortic and corporeal tissues, supporting previous reports showing that, by decreasing increased blood glucose levels, insulin treatment reverses the tissue antioxidant status (Giugliano et al., 1996; Wohaieb and Godin, 1987) and restores impaired neurogenic relaxation of the trabecular smooth muscle in streptozotocin-induced diabetic rats (Yıldırım et al., 1999). In some of the previous studies, it has been reported that insulin treatment restores the reactivity of vascular smooth muscle completely; however, higher doses of insulin were used in those studies (Kobayashi and Kamata, 1999; Rodriguez-Manas et al., 1998), and most probably, we would have observed a complete restoration of responses had we used a higher dose.

Melatonin was recently found to be a potent free radical scavenger and antioxidant (Reiter et al., 2000, 2001). It is thought to have a protective role in the initial and advanced stages of diseases, the pathogenesis of which involves damage by reactive oxygen metabolites. In the present study, melatonin had no effect on blood glucose levels. While it inhibited lipid peroxidation and increased glutathione levels significantly, endothelial function was not restored completely in melatonin-treated diabetic animals, demonstrating that in the treatment of diabetic complications, the control of glycemia is as important as the antioxidant status.

In insulin-treated diabetic rats, hyperglycemia was reduced and returned to control levels. However, malondialdehyde and glutathione levels in insulin-treated rats were still significantly different from those of the control group, demonstrating that the insulin-induced control of glycemia provided limited protection against diabetes-induced oxidative damage. Malondialdehyde and glutathione levels returned to control values when diabetic rats were treated with melatonin and insulin in combination.

In conclusion, these findings indicate that melatonin or insulin alone can provide limited protection against hyperglycemia-induced oxidative damage in diabetes. Combined treatment with insulin and melatonin can suppress hyperglycemia, prevent oxidative damage and can restore endothelial function completely, implying that treatment of diabetes mellitus with this combination would be beneficial.

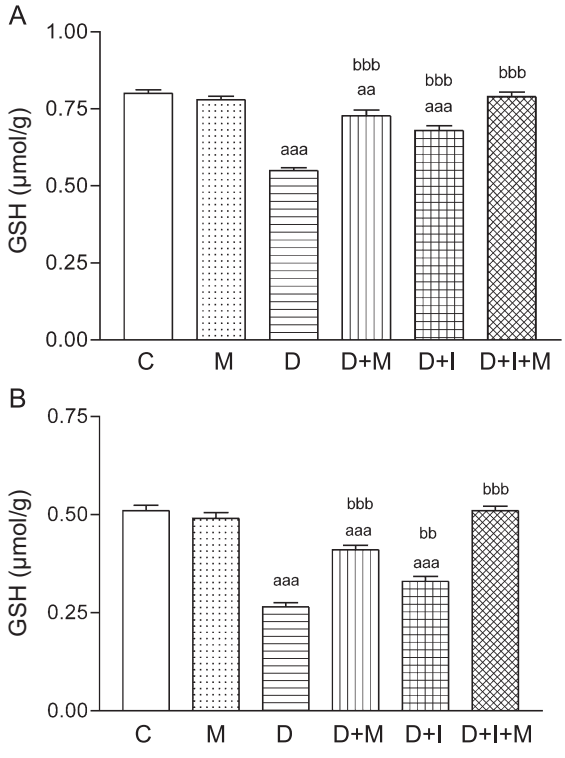

Fig. 4. Glutathione (GSH) levels in (A) aorta and in (B) corpus cavernosum. C, control; M, melatonin; D, diabetic; D+M, melatonin-treated diabetic; D+I, insulin-treated diabetic group; D+I+M, insulin- and melatonin-treated diabetic groups. Each group consisted of eight rats. a, $P<0.05$; aa, $P<0.01$; aaa, $P<0.001$, compared to group C; b, $P<0.05$; bb, $P<0.01$; bbb, $P<0.001$, compared to group D.

References

Arduini, A., Stern, A., Storto, S., Belfiglio, M., Mancinelli, G., Scurti, R., Frederici, G., 1989. Effect of oxidative stress on membrane phospholipid and protein organization in human erythrocytes. Arch. Biochem. Biophys. 273, 112–120.

Azadzoi, K.M., De Tejeda, S.I., 1992. Diabetes mellitus impairs neurogenic and endothelium-dependent relaxation of rabbit corpus cavernosum smooth muscle. J. Urol. 148, 1587–1592.

Baydas, G., Canatan, H., Türkoğlu, H., 2002. Comparative analysis of the protective effects of melatonin and vitamin E on streptozotocin-induced diabetes mellitus. J. Pineal Res. 32, 225–230.

Beuge, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302–311.

Beutler, E., 1975. Glutathione in red blood cell metabolism. A Manuel of Biochemical Methods. Grune and Strattonb, New York, pp. 112–114.

Cartledge, J.J., Eardley, I., Morrison, J.F.B., 2000. Impairment of corpus cavernosal smooth muscle relaxation by glycosylated human hemoglobin. BJU Int. 85, 735–741.

Cartledge, J.J., Eardley, I., Morrison, J.F.B., 2000. Nitric oxide-mediated corpus cavernosal smooth muscle relaxation is impaired in ageing and diabetes. BJU Int. 87, 394–401.

Cartledge, J.J., Eardley, I., Morrison, J.F.B., 2001. Advanced glycation end-products are responsible for the impairment of corpus cavernosal smooth muscle relaxation seen in diabetes. BJU Int. 87, 402–407.

Cuzzocrea, S., Riley, D.P., Capputi, A.P., Salvemini, D., 2001. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol. Rev. 53, 135–159.

Çınar, M.G., Ülker, S., Alper, G., Evinç, A., 2001. Effect of dietary vitamin E supplementation on vascular reactivity of thoracic aorta in streptozotocin–diabetic rats. Pharmacology 62, 56–64.

De Vriese, A.S., Verbeuren, T.J., Voorde, J.V., Lameire, N.H., Vanhoutte, P.M., 2000. Endothelial dysfunction in diabetes. Br. J. Pharmacol. 130, 963–974.

Durante, W., Sen, A.K., Sunahara, F.A., 1988. Impairment of endothelium dependent relaxation in aortae from spontaneously diabetic rats. Br. J. Pharmacol. 94, 463–468.

Gilad, E., Cuzzocrea, S., Zingarelli, B., 1997. Melatonin is a scavenger of peroxynitrite. Life Sci. 60, 169–174.

Giugliano, D., Cerıello, A., Paolisso, G., 1996. Oxidative stress and diabetic vascular complications. Diabetes Care 19, 257–267.

Göçmen, C., Seçilmş, A., Karabal, K.E., Ertuğ, P.U., Önder, S., Dikmen, A., 2000. Effects of vitamin E and sodium selenate on neurogenic and endothelial relaxation of corpus cavernosum in the diabetic mouse. Eur. J. Pharmacol. 398, 93–98.

Gryglewski, R.J., Palmer, R.J.M., Moncada, S., 1986. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. Nature 320, 454–456.

Guerrero, J.M., Reiter, R.J., Ortiz, G.G., 1997. Melatonin prevents increases in neural nitric oxide and cyclic GMP production after transient brain ischemia and reperfusion in the Mongolian gerbil (*Meriones unguiculatus*). J. Pineal Res. 23, 24–31.

Hara, M., Iigo, M., Ohtani-Kanelo, R., Nakamura, N., 1997. Administration of melatonin and related indoles prevents exercise-induced cellular oxidative changes in rats. Biol. Signals 6, 90–100.

Heygate, K.M., Lawrence, I.G., Bennett, M.A., Thurston, H., 1995. Impaired endothelium-dependent relaxation in isolated resistance arteries of spontaneously diabetic rats. Br. J. Pharmacol. 116, 3251–3259.

Kakkar, R., Mantha, S.V., Kalra, J., Prasad, K., 1996. Time course study of oxidative stress in aorta and heart of diabetic rat. Clin. Sci. 91, 441–448.

Kamata, K., Miyata, N., Kasuya, Y., 1989. Impairment of endothelium dependent relaxation and changes in levels of cyclic GMP in aorta from streptozotocin-induced diabetic rats. Br. J. Pharmacol. 97, 614–618.

Khan, M.A., Thompson, C.S., Jeremy, J.Y., Mumtaz, F.H., Mikhailidis, P., Morgan, R.J., 2001. The effect of superoxide dismutase on nitric oxide-mediated and electrical field-stimulated diabetic rabbit cavernosal smooth muscle relaxation. BJU Int. 87, 98–103.

Kobayashi, T., Kamata, K., 1999. Effect of insulin treatment on smooth muscle contractility and endothelium-dependent relaxation in rat aortae from established STZ-induced diabetes. Br. J. Pharmacol. 127, 835–842.

Langenstroer, P., Pieper, G.M., 1992. Regulation of spontaneous EDRF release in diabetic rat aorta by oxygen free radicals. Am. J. Physiol. 263, H257–H265.

Mandell, G.L., 1972. Functional and metabolic derangements in human neutrophils induced by a glutathione antagonist. J. Reticuloendothel. Soc. 11 (2), 129–137.

Marshall, K.A., Reiter, R.J., Poeggeler, B., 1996. Evaluation of the antioxidant activity of melatonin in vitro. Free Radic. Biol. Med. 21, 307–315.

McNally, P.G., Watt, P.A.C., Rimmen, T., Burden, A.C., Hearnshacs, R.J., Thurston, H., 1994. Impaired contraction and endothelium-dependent relaxation in isolated resistance vessels from patients with insulin dependent diabetes mellitus. Clin. Sci. 87, 31–36.

Montilla, P.L., Vargas, J.F., Tunez, I.F., Munez de Agueda, M.O., Valdelvira, M.E., Cabrera, E.S., 1998. Oxidative stress in diabetic rats induced by streptozotocin: protective effects of melatonin. J. Pineal Res. 25 (2), 94–100.

Naziroglu, M., Cay, M., 2001. Protective role of intraperitoneally administered vitamin E and selenium on the antioxidative defence mechanisms in rats with diabetes induced by streptozotocin. Biol. Trace Elem. Res. 79 (2), 149–159.

Öztürk, Y., Altan, V.M., Yildizoglu-Ari, N., 1996. Effects of experimental diabetes and insulin on smooth muscle functions. Pharmacol. Rev. 48, 69–112.

Pieper, G.M., 1999. Enhanced, unaltered and impaired nitric oxide-mediated endothelium-dependent relaxation in experimental diabetes mellitus: importance of disease duration. Diabetologia 42, 204–213.

Pieper, G.M., Gross, G.J., 1988. Oxygen free radicals abolish endothelium-dependent relaxation in diabetic rat aorta. Am. Physiol. Soc., H825–H833.

Pieri, C., Marra, M., Moroni, F., Recchioni, R., Marcheselli, F., 1995. Melatonin: a peroxyl radical scavenger more effective than vitamin E. Life Sci. 55 (15), 271–276.

Reiter, R.J., Melchiorri, D., Sewerynek, E., Poeggeler, B., Barlow-Walden, L., Chuang, J., Ortiz, G.G., Acuna-Castroviejo, D., 1995. A review of the evidence supporting melatonin's role as an antioxidant. J. Pineal Res. 18 (1), 1–11.

Reiter, R.J., Tan, D.X., Osuna, C., Gitto, E., 2000. Actions of melatonin in the reduction of oxidative stress. J. Biomed. Sci. 7, 444–458.

Reiter, R.J., Tan, D.X., Manchester, L.C., Wenbo, Q., 2001. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species. Cell Biochem. Biophys. 34, 237–256.

Reyes-Toso, C.F., Roson, M.I., Albornoz, L.E., Damiano, P.F., Linares, L.M., Cardinali, D.P., 2002. Vascular reactivity in diabetic rats: effect of melatonin. J. Pineal Res. 33 (2), 81–86.

Rodriguez-Manas, L., Angulo, J., Peiro, C., Llergo, J.L., Sanchez-Ferrer, A., Lopez-Doriga, P., Sanchez-Ferrer, C.F., 1998. Endothelial dysfunction and metabolic control in streptozotocin-induced diabetic rats. Br. J. Pharmacol. 123, 1495–1502.

Sullivan, M., Thompson, C.S., Mikhailidis, D.P., Morgan, R.J., Angelini, G.D., Jeremy, J.Y., 1998. Differential alterations of prostacyclin, cyclic AMP and cyclic GMP formation in the corpus cavernosum of the diabetic rabbit. BJU 82, 578–584.

Şener, G., Şatıroğlu, H., Kabasakal, L., Arbak, S., Oner, S., Ercan, F., Keyer-Uysal, M., 2000. The protective effect of melatonin on cisplatin nephrotoxicity. Fundam. Clin. Pharmacol. 14 (6), 553–560.

Şener, G., Paskaloğlu, K., Şehirli, A.Ö., Ayanoğlu-Dülger, G., Alican, I., 2002. The effects of melatonin on ischemia/reperfusion induced changes in rat corpus cavernosum. J. Urol. 167 (6), 2624–2627.

Tan, D.X., Chen, L.D., Poeggeler, B., 1993. Melatonin: a potent, endogenous hydroxyl radical scavenger. Endocr. J. 1, 57–60.

Taylor, P.D., McCarthy, A.L., Thomas, C.R., Poston, L., 1992. Endothelium-dependent relaxation and noradrenalin sensitivity in mesenteric resistance arteries of steptozotocin-induced diabetic rats. Br. J. Pharmacol. 107, 393–399.

Tesfamariam, B., Palacino, J.J., Weisbrod, R.M., Cohen, R.A., 1993. Aldose reductase inhibition restores endothelial cell function in diabetic rabbit aorta. J. Cardiovasc. Pharmacol. 21, 205–211.

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.

Wali, R.K., Dudeja, P.K., Bolt, M.J., Strin, M.D., Brasitus, T.A., 1990. Correction of abnormal small intestinal cytoplasmic protein kinase C activity in STZ induced diabetes by insulin therapy. Biochem. J. 272, 653–658.

Wohaieb, S.A., Godin, D.V., 1987. Alterations in free radical tissue–defence mechanisms in streptozotocin-induced diabetes in rat. Diabetes 36, 1014–1018.

Yıldırım, S., Ayan, S., Sarıoğlu, Y., Gültekin, Y., Bütünet, C., 1999. The effects of long term oral administration of L-arginine on the erectile response of rabbits with alloxan induced diabetes. BJU Int. 83, 679–685.