JPP Journal of Pharmacy and Pharmacology

JPP 2004, 56: 359–366

Received August 27, 2003
Accepted November 19, 2003
DOI 10.1211/0022357022818
ISSN 0022-3573

Melatonin reverses urinary system and aorta damage in the rat due to chronic nicotine administration

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Abstract

We have evaluated the changes in contractile activity and oxidant damage of corpus cavernosum, urinary bladder, kidney and aorta after chronic nicotine administration in rats. The effects of melatonin on these parameters were investigated also. Male Wistar albino rats were injected intraperitoneally with nicotine hydrogen bitartrate (0.6 mg kg^{-1} daily for 21 days) or saline. Melatonin (10 mg kg^{-1}, i.p.) was administered either alone or with nicotine injections. Corpus cavernosum, bladder and aorta were used for contractility studies, or stored with kidneys for the measurement of malondialdehyde and glutathione levels. Corpus cavernosum, bladder, and aorta samples were examined histologically and the extent of microscopic tissue damage was scored. In the nicotine-treated group, the contraction of corpus cavernosum, bladder and aorta samples and the relaxation of corporeal and aorta tissues decreased significantly compared with controls. However, melatonin treatment restored these responses. In the nicotine-treated group, there was a significant increase in the malondialdehyde levels of the corporeal tissue, bladder, kidney and aorta, with marked reductions in glutathione levels compared with controls. Melatonin treatment reversed these effects also. Melatonin administration to nicotine-treated animals caused a marked reduction in the microscopic damage of the tissues compared with those of the untreated group. In this study, nicotine-induced dysfunction of the corpus cavernosum, bladder and aorta of rats was reversed by melatonin treatment. Moreover, melatonin, as an antioxidant, abolished elevation in lipid peroxidation products, and reduction in the endogenous antioxidant glutathione, and protected the tissues from severe damage due to nicotine exposure.

Introduction

Nicotine, a major toxic component of cigarette smoking, has long been recognized to result in oxidative stress by inducing the generation of reactive oxygen species in the periphery and central nervous system. Experiments have shown that chronic administration of nicotine caused increased lipid peroxidation products in serum and tissues of rats (Latha et al 1993; Ashakumary & Vijayammal 1996). The increased concentration of lipid peroxidation products observed in nicotine-treated rats was found to be associated with decreased activity of scavenging enzymes such as catalase and superoxide dismutase (Ashakumary & Vijayammal 1996). A decrease in the activities of these enzymes can lead to the excessive availability of superoxides and peroxyl radicals, which in turn generate hydroxyl radicals, resulting in the initiation and propagation of lipid peroxidation. In addition to its direct effect on contractile apparatus, the superoxide anion inactivates nitric oxide which is essential for smooth muscle contractile activity (Xie et al 1997; Mayhan & Sharpe 1999). On the other hand, lipid peroxidation is known to cause cellular injury by inactivation of membrane enzymes and receptors, depolymerization of polysaccharide, as well as protein cross-linking and fragmentation. Since membrane lipids are vital for the maintenance and integrity of cell function, the breakdown of membrane phospholipids and lipid peroxidation are expected to change membrane structure, fluidity, transport and antigenic properties, all of which play an important role in the pathogenesis of organ disorders.

Melatonin, which is the chief secretory product of the pineal gland, was found to be a potent free radical scavenger and antioxidant (Allegra et al 2003). In-vitro and

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in-vivo studies have found melatonin to protect tissues against oxidant damage induced by various free radical generating agents and processes (i.e. lipopolysaccharide, kainic acid, Fenton reagents, potassium cyanide, L-cystein, excessive exercise, ischaemia-reperfusion and radiation) (Tan et al 1993b; Melchiorri et al 1995a; Sewerynek et al 1995; Guerrero et al 1997; Hara et al 1997). It has been demonstrated that melatonin reduces lipid peroxidation, scavenges the hydroxyl radical, which is a potent initiator of lipid peroxidation, and the peroxyl radical, which propagates the process of lipid peroxidation (Tan et al 1993a; Pieri et al 1994). It scavenges hypochlorous acid at a rate sufficient to protect catalase against inactivation by this molecule (Marshall et al 1996). Moreover, peroxynitrite has been shown to be directly scavenged by melatonin (Gilad et al 1997). Another effect of melatonin is to stimulate the activity of the endogenous antioxidant enzyme glutathione peroxidase, which may be due to the effect of the hormone in removing hydrogen peroxide (Barlow-Walden et al 1995).

Based on these findings, we have investigated the effects of melatonin on contractile changes and oxidant-induced tissue damage by measuring malondialdehyde (an end-product of lipid peroxidation) and glutathione levels in corpus cavernosum, urinary bladder, kidney and aorta of rats following chronic nicotine administration.

Materials and Methods

Animals

Male Wistar albino rats (200–250 g) were housed in a room at a constant temperature of $22 \pm 2\,^{\circ}C$ (mean ± s.e.m.) with a 12-h light–dark cycle. The animals had free access to standard pellet chow and water. The study was approved by the Marmara University School of Medicine Animal Care and Use Committee.

Experimental groups

Rats were divided into groups of eight. One group of rats was injected intraperitoneally with nicotine hydrogen bitartrate at a dose of $0.6\,mg\,kg^{-1}$ daily for 21 days (Helen et al 2000). The control group received saline injections. Two other groups of animals were injected intraperitoneally with $10\,mg\,kg^{-1}$ melatonin either alone or with nicotine injections for 21 days. Nicotine hydrogen bitartrate (Sigma Chemical Co., St Louis, MO) was dissolved in saline. Melatonin (Sigma Chemical Co.) was dissolved in absolute ethanol and diluted in saline. The final concentration of ethanol was 1%. After the 21 days, all animals were killed by decapitation and penis, urinary bladder, kidneys and thoracic aorta were carefully dissected. Corporeal tissues, bladder and aorta were immediately placed in organ baths for contractility studies, or stored at $-70\,^{\circ}C$ for the measurement of malondialdehyde and glutathione levels. Samples were taken of bladder, corpus cavernosum and aorta for histological evaluation and scoring. Trunk blood was collected, and the plasma samples and all other tissues were stored for the measurement of malondialdehyde and glutathione levels. For the evaluation of kidney functions, plasma blood urea nitrogen (BUN) and creatinine levels were assessed (Bauer et al 1968).

In-vitro organ bath experiments

Corpus cavernosum excised from the penis of the rats was dissected free of the tunica albuginea, and cut into $2 \times 2 \times 15$ mm strips. The bladder dome was immediately removed and separated from the bladder base at the level of ureteral orifices. Longitudinal strips of the posterior of the bladder dome (1.5×5 mm) were prepared. The aortic segments were cut into 4-mm rings. All tissues were mounted in 20-mL organ baths. Corporeal strips and aortic rings were bathed in Krebs-bicarbonate buffer (containing in mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.2 $KHPO_4$ and 11.1 glucose), whereas bladder strips were bathed in Tyrode solution (containing in mM: 124.9 NaCl, 2.6 KCl, 23.8 $NaHCO_3$, 0.5 $MgCl_2$, 0.4 NaH_2PO_4, 1.8 $CaCl_2$ and 5.5 glucose). The solutions were aerated with 95% O_2–5% CO_2 (pH 7.4) at 37 °C. The tissues were equilibrated for 60 min under a resting tension of 1 g. Isometric contractions were recorded using a Model FT03 force displacement transducer (Grass Instruments, Quincy, MA) coupled to a Model 7 polygraph (Grass Instruments). After equilibration, the tissues were exposed to 124 mM KCl. The contractile responses of the corporeal tissues and aortic rings to 10^{-8}–10^{-3} M phenylephrine were determined cumulatively. The contractile responses of the bladder strips to 10^{-9}–10^{-5} M carbachol were obtained cumulatively. The contraction responses were expressed as a percentage of the maximal contraction induced by KCl.

After a 30-min wash-out, the relaxation responses in corporeal tissues and aorta were evaluated by adding increasing cumulative concentrations of 10^{-7}–10^{-3} M and 3×10^{-9}–10^{-4}M acetylcholine (ACh), respectively, to strips precontracted with the submaximal dose of 30 μM phenylephrine. The relaxation responses to ACh were expressed as a percentage of the contraction caused by 30 μM phenylephrine.

Malondialdehyde (MDA) and glutathione (GSH) assays

Tissue samples were homogenized with ice-cold 150 mM KCl for determination of MDA and GSH levels. The MDA levels were assayed for products of lipid peroxidation (Buege & Aust 1978). Results were expressed as nmol MDA $(g\,tissue)^{-1}$. Glutathione was determined by the spectrophotometric method, which was based on the use of Ellman's reagent (Beutler 1975). Results were expressed in μmol GSH $(g\,tissue)^{-1}$.

Histological scoring

Bladder, corpus cavernosum and aorta tissue samples were investigated at light microscopic level by an experienced histologist who was unaware of the treatment

conditions. Tissue samples were prepared for routine light microscopic examination. Paraffin sections were stained with haematoxylin and eosin, and examined with an Olympus BH-2 photomicroscope. At least five areas were examined to score the specimen.

Corporal tissues were evaluated by scoring the extent of endothelial desquamation, endothelial degeneration, precapillary and interstitial oedema and pericapillary leucocyte infiltration as 0–3 (0 = none; 1 = mild; 2 = moderate; 3 = severe). Bladder samples were evaluated by scoring the degree of epithelial degeneration, mucosal oedema, eosinophilic infiltration and vasocongestion as 0–3 (0 = none; 1 = mild; 2 = moderate; 3 = severe). Aorta samples were evaluated by scoring endothelial degeneration, subendothelial oedema and disorganized elastic fibres as 0–3 (0 = none; 1 = mild; 2 = moderate; 3 = severe). To express the total score, the score of each criterion was summed for all tissues.

Statistical analysis

Data were expressed as the mean ± s.e.m. The concentration causing 50% of the maximal response (EC50) of phenylephrine, carbachol or ACh was derived from the concentration–response curves using a computer assisted probit transformation. The concentration–response curves were analysed by unpaired *t*-test, the biochemical parameters were analysed by analysis of variance followed by the Tukey–Kramer multiple tests and the histological scores were compared by Mann–Whitney U non-parametric test. Calculations were performed using the Instat and Prism statistical analysis packages (GraphPad Software, San Diego, CA) with $P < 0.05$ considered statistically significant.

Results

Organ bath experiments

Adding 10^{-8}–10^{-3} M phenylephrine cumulatively to precontracted (with 124 mM KCl) corpus cavernosum strips and aortic rings from control rats resulted in a concentration-dependent contraction, with an EC50 of 6.37×10^{-6} and 6.05×10^{-8} M, respectively. Similarly, carbachol added cumulatively to bladder strips precontracted with KCl caused a concentration-dependent contraction, with an EC50 of 1.46×10^{-7} M. In the nicotine-treated group, the contraction responses of all tissues were significantly decreased compared with the control group ($P < 0.05$–0.001; ED50 = 4.60×10^{-5}, 3.29×10^{-7} and 3.17×10^{-7} M for corpus cavernosum, aorta, and bladder, respectively). In the nicotine plus melatonin group, the contractile responses of the tissues were higher than in the untreated nicotine group ($P < 0.05$–0.001), resulting in EC50 values of 9.40×10^{-6}, 6.22×10^{-8} and 2.28×10^{-7} M for corpus cavernosum, aorta, and bladder, respectively. The contractile responses of the melatonin-treated group were not different from those of the untreated control group (Figure 1).

ACh added cumulatively at doses of 10^{-7}–10^{-3} M to corporeal tissues and at doses of 3×10^{-9}–10^{-4} M to aortic

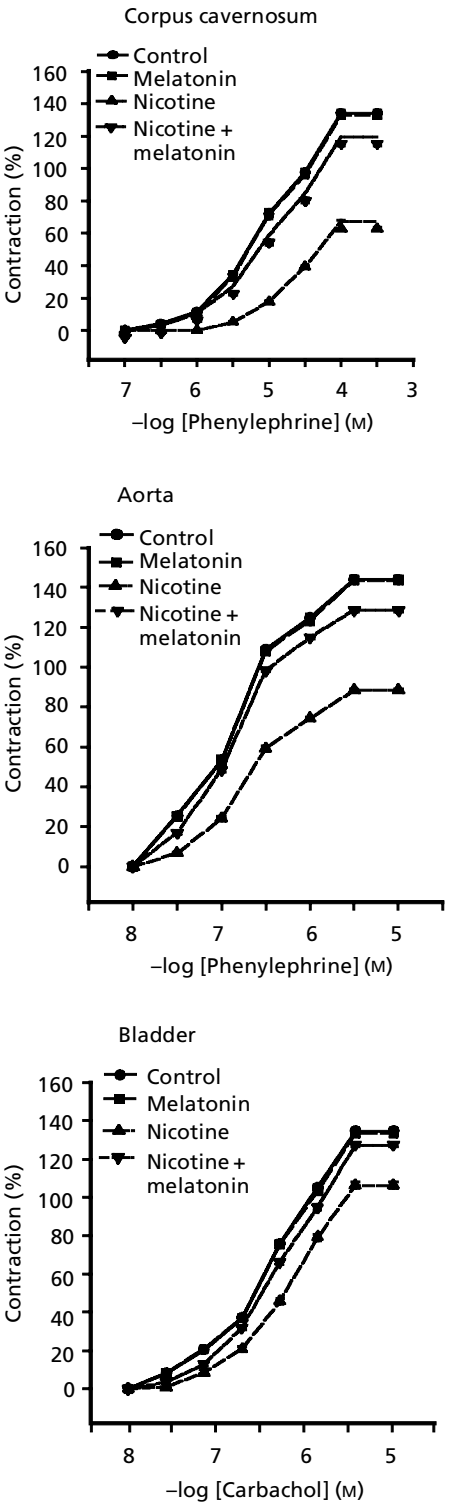

Figure 1 Concentration–response curves obtained by cumulative addition of phenylephrine to rat corpus cavernosum and thoracic aortic rings, and carbachol to bladder strips. Chronic nicotine administration decreased contractile activity at all concentrations of phenylephrine and carbachol ($P < 0.001$). Melatonin treatment in the nicotine group prevented this effect ($P < 0.001$). Points indicate percent of contraction induced by 124 mM KCl. Values are shown as mean ± s.e.m. of eight experiments.

rings, which had been precontracted with the submaximal dose of 30 μM phenylephrine, caused a dose-dependent relaxation response (ED50 = 7.67×10^{-6} and 8.87×10^{-7} M for corpus cavernosum and aorta, respectively). In the nicotine-treated group relaxation responses of corpus cavernosum strips and aortic rings to ACh were markedly lower compared with the control group ($P < 0.001$), with ED50 values of 3.49×10^{-4} and 7.43×10^{-5} M, respectively. In the melatonin-treated nicotine group, the relaxation responses of both tissues were significantly higher compared with the untreated-nicotine group ($P < 0.001$) with an ED50 of 1.83×10^{-5} and 1.25×10^{-6} M, respectively. Similar to the contractile responses, melatonin alone had no significant effect on the relaxation response (Figure 2).

Malondialdehyde (MDA) levels

The mean level of MDA, which is a major degradation product of lipid peroxidation, was increased in corporeal tissue, bladder, kidney, and aorta after nicotine treatment compared with the control group ($P < 0.001$, Figure 3). Melatonin treatment to the nicotine group caused a marked decrease in mean MDA compared with nicotine alone ($P < 0.001$), whereas melatonin alone had no effect (Figure 3). Moreover, the serum MDA level was higher in the nicotine group compared with the control ($P < 0.001$), and melatonin treatment reversed this effect significantly ($P < 0.001$, Table 1).

Glutathione (GSH) levels

The mean GSH levels of corpus cavernosum, bladder, kidney and aorta in the nicotine-treated group were significantly lower than in the control group ($P < 0.01$–0.001, Figure 4). Melatonin treatment to the nicotine group reversed the decrease in GSH level in all tissues ($P < 0.05$–0.001), whereas melatonin treatment alone had no significant effect (Figure 4). Similarly, the decrease in serum GSH levels in nicotine-treated animals ($P < 0.01$) was significantly reversed by melatonin treatment ($P < 0.01$, Table 1).

Histological scoring

As shown in Table 2, the microscopic examination of tissue samples revealed severe damage characterized by cellular degeneration and desquamation, oedema and inflammatory cell infiltration in nicotine-treated rats. Melatonin administration to nicotine-treated animals caused marked reductions in the microscopic score values of the tissues ($P < 0.05$).

Plasma BUN and creatinine levels

As shown in Table 1, plasma BUN and creatinine levels were significantly higher in nicotine-treated animals compared with controls ($P < 0.05$–0.01). Melatonin administration in the nicotine group abolished the rise in these values ($P < 0.05$–0.01).

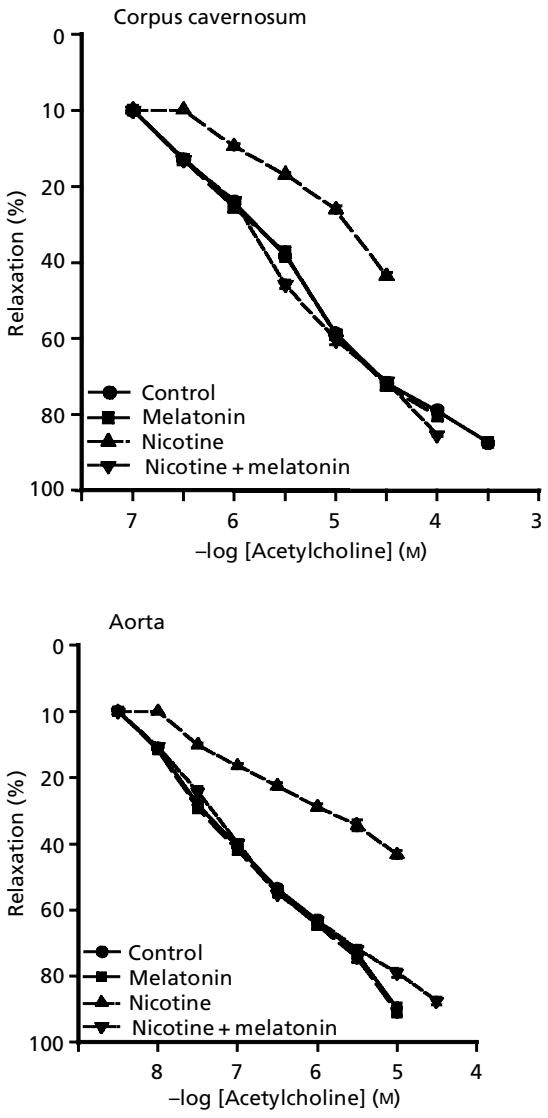

Figure 2 Concentration–response curves obtained by cumulative addition of acetylcholine to rat corpus cavernosum strips and thoracic aortic rings precontracted with 30 μM phenylephrine. Chronic nicotine administration decreased relaxation responses at all concentrations of acetylcholine ($P < 0.001$), and melatonin treatment in the nicotine group prevented this effect ($P < 0.001$). Values are shown as mean ± s.e.m. of eight experiments.

Discussion

We observed that rat corporeal strips exposed to nicotine treatment had lower responses to both contractile and relaxant agents compared with controls. Previous in-vitro data and clinical observations suggested that smoking impaired erectile function. Juenemann et al (1987) showed significant decreases in the intracorporeal pressure and flow through the internal pudendal artery of healthy adult mongrel dogs after cigarette smoking and nicotine injections. Although it has been found that nitrite release

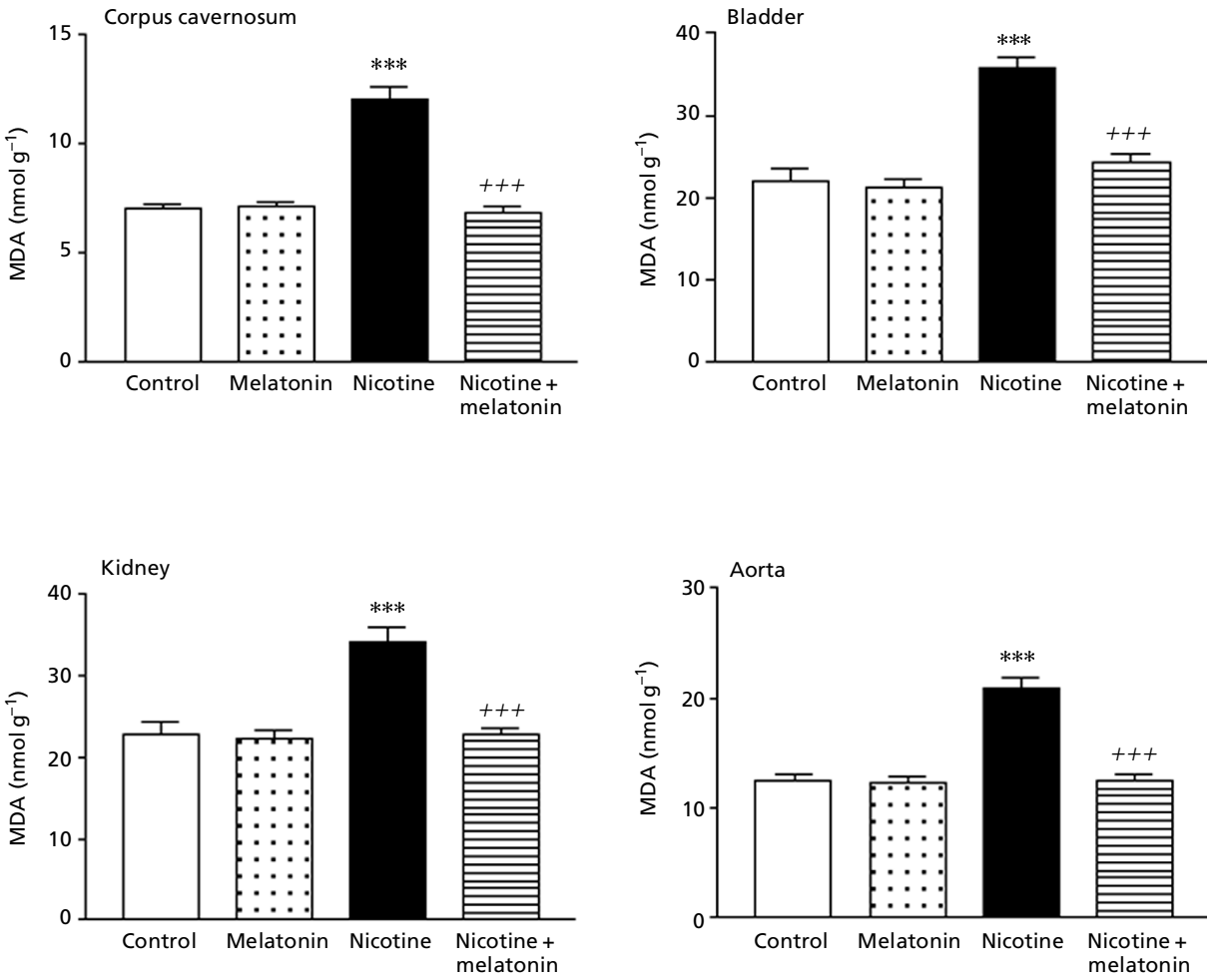

Figure 3 Malondialdehyde (MDA) levels in corpus cavernosum, bladder, kidney, and thoracic aorta of control, untreated and melatonin-treated nicotine groups, and the effect of melatonin alone on control rats. ***$P < 0.001$ compared with control. $^{+++}P < 0.001$ compared with the untreated nicotine group.

Table 1 The plasma malondialdehyde (MDA), glutathione (GSH), blood urea nitrogen (BUN) and creatinine levels in control, melatonin, nicotine and nicotine + melatonin groups (n = 8 per group).

	Control	Melatonin	Nicotine	Nicotine + melatonin
MDA (nmol mL^{-1})	2.25 ± 0.1	2.2 ± 0.1	4.7 ± 0.1***	2.66 ± 0.2^{+++}
GSH (μmol mL^{-1})	27.8 ± 2.2	31.0 ± 1.4	17.0 ± 0.7**	26.1 ± 2.1^{++}
BUN (mg dL^{-1})	17.5 ± 0.9	17.3 ± 0.7	23.9 ± 1.6**	16.7 ± 1.1^{++}
Creatinine (mg dL^{-1})	0.94 ± 0.1	0.87 ± 1.1	1.65 ± 0.2*	0.96 ± 0.1^{+}

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control. $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ compared with nicotine group.

by rat cavernosa slices, in-vitro, is not inhibited by nicotine or its metabolite cotinine, the alterations in the release of agents other than nitric oxide that control smooth muscle contractility may be involved in this effect (Xie et al 1997).

In this study, we observed marked reductions in contraction and relaxation responses of the aortic rings from rats exposed to nicotine. Nicotine is a toxic compound that produces diffuse damage to endothelial cells (Hladovec 1978; Booyse et al 1981). It has been known

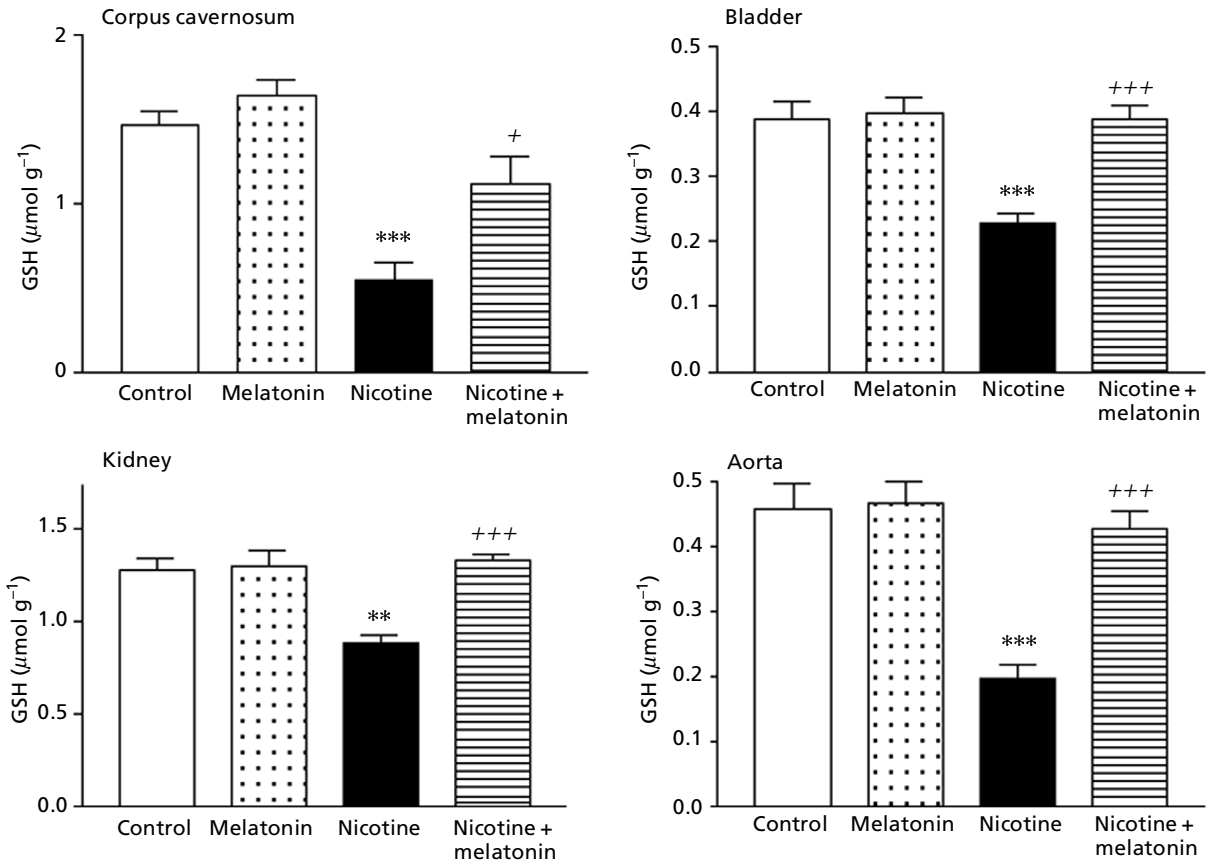

Figure 4 Glutathione (GSH) levels in corpus cavernosum, bladder, kidney, and thoracic aorta of control, untreated and melatonin-treated nicotine groups, and the effect of melatonin alone on control rats. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control. $^{+}P < 0.05$, $^{+++}P < 0.001$ compared with the untreated nicotine group.

Table 2 The histological scores of the bladder, corpus cavernosum and thoracic aorta samples from nicotine and nicotine + melatonin groups (n = 8 per group).

Tissue	Control	Melatonin	Nicotine	Nicotine+melatonin
Corpus cavernosum	0.07 ± 0.03	0.03 ± 0.02	11.00 ± 0.70*	3.25 ± 0.20^{+}
Bladder	0.04 ± 0.02	0.03 ± 0.02	11.50 ± 0.30**	3.00 ± 0.40^{+}
Aorta	0.07 ± 0.02	0.05 ± 0.03	10.50 ± 0.05*	3.25 ± 0.25^{+}

$^{*}P < 0.05$, $^{**}P < 0.01$ compared with control; $^{+}P < 0.05$ compared with nicotine group.

to impair endothelium-dependent arteriolar dilatation. In-vitro and in-vivo studies on cheek pouch arterioles demonstrated that chronic exposure to nicotine produced selective impairment of endothelium-dependent arteriolar dilatation via a mechanism related to the synthesis/release of oxygen-derived free radicals (Mayhan & Sharpe 1998, 1999). Although the precise mechanism of impaired contractile function due to oxidants has not been clarified, oxidants may alter vascular reactivity through the impairment of a signal transduction system, as demonstrated in the coronary endothelial cells (Mayhan & Patel 1997). A study showing the impairment of nitric oxide synthase-mediated dilatation of resistance arterioles after infusion of nicotine in hamsters (Lefer et al 1992) support the data. An in-vitro study by Zhang et al (2001) showed that nicotine exposure of human coronary artery endothelial cells resulted in increased mRNA levels of endothelial nitric oxide synthase, angiotensin-I converting enzyme, tissue-type plasminogen activator, plasminogen activator inhibitor-I, von Willebrand factor, and vascular cell adhesion molecule-I. These findings suggested that nicotine altered the expression of a number of endothelial genes whose

products play important roles in regulating the vascular tone. In addition, nicotine has been shown to reduce vascular synthesis of prostacyclin, an endothelial cell-derived local vasodilator (Chahine et al 1990). Reduced prostacyclin synthesis may alter vascular tone, and aggravate tissue ischaemia.

Cytotoxic reactive oxygen metabolites (ROMs) have been implicated in the pathogenesis of a variety of cardiovascular, pulmonary, and neoplastic disorders for which cigarette smoking is a prominent risk factor. ROMs, such as superoxide radical, hydroxyl radical, and hydrogen peroxide induce tissue damage through peroxidation of lipids, oxidation of protein sulfuryl groups, and disruption of DNA strands. On the other hand, the endogenous antioxidant glutathione reacts directly with ROMs, protects essential thiol groups from oxidation, and serves as a substrate for several enzymes, including glutathione peroxidase. Thus, a decrease in glutathione not only impairs cell defense against toxic compounds, but results in enhanced oxidative stress and tissue damage.

We observed that chronic nicotine administration resulted in a significant increase in MDA levels in all tissue samples including plasma, compared with control animals. It also caused significant reductions in the GSH content of the tissues and plasma, indicating the utilization of the endogenous antioxidants for protection against oxidant injury. Previous studies have shown that neutrophils exposed to nicotine in-vitro exhibited enhanced superoxide anion generation and chemotactic responses (Jay et al 1986). Accordingly, acute or subchronic nicotine injections have potentiated phorbol ester-induced superoxide anion generation, and f-met-leu-phe-induced chemotaxis (Gillespie 1987). Therefore, nicotine-induced exacerbation of neutrophil derived ROM production may be involved in the risk of multiple organ failures in individuals who smoke.

The pineal secretory product melatonin was shown recently to have free radical scavenging ability (Reiter et al 1994; Melchiorri et al 1995b). It has been demonstrated that melatonin scavenges the hydroxyl radical, which is very potent in initiating lipid peroxidation and peroxyl radical which propagates the chain reaction of lipid peroxidation (Tan et al 1993a; Pieri et al 1994). Melatonin has the ability to scavenge peroxynitrite directly (Gilad et al 1997). In accordance with previous findings, in this study melatonin administration reduced tissue MDA contents of the animals and restored the decrease in tissue GSH levels in rats after nicotine exposure. These beneficial effects were parallel to the changes in the morphological appearance of the tissues. The disruption of cellular integrity in corpus cavernosum, bladder and aorta after nicotine exposure was significantly prevented by melatonin treatment. In addition to this, we observed that melatonin treatment caused a significant reduction in inflammatory cell infiltration to these tissues as assessed by microscopic examination. This observation was in accordance with previous findings showing that the hormone was effective in scavenging hypochlorous acid (derived from activated neutrophils). Thus, it is conceivable that the antioxidant effect of melatonin was one of the mechanisms of its anti-inflammatory effect. Melatonin was effective also in preserving renal functions – assessed by plasma BUN and creatinine levels – after nicotine exposure. Therefore, these data indicated that the maintenance of the balance between oxidant and antioxidant systems in the tissues was one of the major mechanisms of the beneficial effects of melatonin on nicotine-induced tissue damage.

In a study investigating the possible interaction between nicotine and melatonin in the body, Gaddnas et al (2002) showed that chronic nicotine did not alter the daily rhythm of pineal melatonin in mice maintained on a light–dark cycle, but it slightly suppressed melatonin production. In rat pinealocyte cultures nicotine or acetylcholine resulted in decreased melatonin synthesis via inhibiting norepinephrine (noradrenaline)-dependent serotonin N-acetyltransferase activity (Yamada et al 1998). In contrast, Tarquini et al (1994) showed that in man there were higher circulating melatonin levels in smokers than in non-smokers. Since melatonin is a potential inhibitor of the development and growth of tumours, higher melatonin levels in smokers could be considered as an attempt to counterbalance the carcinogenesis induced by smoking (Tarquini et al 1994).

In view of our data and previous observations, melatonin, the potent free radical scavenger and antioxidant agent, seems to be a highly promising agent for protecting tissues from oxidative damage and preventing organ dysfunction due to nicotine.

References

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**: 1–13

Ashakumary, L., Vijayammal, P. L. (1996) Additive effect of alcohol and nicotine on LPO and antioxidant defence mechanism in rats. *J. Appl. Toxicol.* **16**: 305–308

Barlow-Walden, L. R., Reiter, R. J., Abe, M., Pablos, M., Menendez-Pelaez, A., Chen, L. D., Poeggeler, B. (1995) Melatonin stimulates brain glutathione peroxidase activity. *Neurochem. Int.* **26**: 497–502

Bauer, J. D., Ackermann, P. G., Toro, G. (1968) *Bray's clinical methods*. CV Mosby, St Louis, MO, pp 308–315

Beutler, E. (1975) Glutathione in red blood cell metabolism. In: *A manual of biochemical methods*. Grune & Stratton, New York, pp 112–114

Booyse, F. M., Osikowicz, G., Quarfoot, A. J. (1981) Effects of chronic oral consumption of nicotine on rabbit aortic endothelium. *Am. J. Pathol.* **102**: 229–238

Buege, J. A., Aust, S. D. (1978) Microsomal lipid peroxidation. *Methods Enzymol.* **53**: 302–311

Chahine, R., Calderone, A., Navarro-Delmalsure, C. (1990) The in vitro effects of nicotine and cotinine on prostacyclin and thromboxane biosynthesis. *Prostaglandins Leukot. Essent. Fatty Acids* **40**: 261–266

Gaddnas, H., Pietila, K., Alila-Johannson, A., Ahtee, L. (2002) Pineal melatonin and brain transmitter monoamines in CBA mice during chronic oral nicotine administration. *Brain Res.* **957**: 76–83

Gilad, E., Cuzzocrea, S., Zingarelli, B., Salzman, A. L., Szabo, C. (1997) Melatonin is a scavenger of peroxynitrite. *Life Sci.* **60**: 169–174

Gillespie, M. N., Owasoyo, J. O., Kojima, S., Jay, M. (1987) Enhanced chemotaxis and superoxide anion production by polymorphonuclear leukocytes from nicotine-treated and smoke-exposed rats. *Toxicology* **45**: 45–52

Guerrero, J. M., Reiter, R. J., Ortiz, G. G., Pablos, M. I., Sewerynek, E., Chuang, J. I. (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., Suziki, T., Reiter, R. J., Hirata, K. (1997) Administration of melatonin and related indoles prevents exercise-induced cellular oxidative changes in rats. *Biol. Signals.* **6**: 90–100

Helen, A., Krishnakumar, K., Vijayammal, P. L., Augusti, K. T. (2000) Antioxidant effect of onion oil (*Allium cepa* Linn.) on the damages induced by nicotine in rats as compared to alpha-tocopherol. *Toxicol. Lett.* **116**: 61–68

Hladovec, J. (1978) Endothelial injury by nicotine and its prevention. *Experienta* **34**: 1585–1586

Jay, M., Kojima, S., Gillespie, M. N. (1986) Nicotine potentiates superoxide anion generation by human neutrophils. *Toxicol. Appl. Pharmacol.* **86**: 484–487

Juenemann, K. P., Lue, T. F., Luo, J. A., Benowitz, N. L., Abozeid, M., Tanagho, E. A. (1987) The effect of cigarette smoking on penile erection. *J. Urol.* **138**: 438–441

Latha, M. S., Vijayammal, P. L., Kurup, P. A. (1993) Effect of nicotine administration on lipid metabolism in rats. *Indian J. Med. Res.* **98**: 44–49

Lefer, D. J., Nakaniski, K., Vinten-Johansen, J., Ma, X. L., Lefer, A. M. (1992) Cardiac venous endothelial dysfunction after myocardial ischemia and reperfusion in dogs. *Am. J. Physiol.* **263**: H850–H856

Marshall, K. A., Reiter, R. J., Poeggeler, B., Aruoma, O. I., Halliwell, B. (1996) Evaluation of the antioxidant activity of melatonin in vitro. *Free Radic. Biol. Med.* **21**: 307–315

Mayhan, W. G., Patel, K. P. (1997) Effect of nicotine on endothelium-dependent arteriolar dilatation in vivo. *Am. J. Physiol.* **272**: H2337–H2342

Mayhan, W. G., Sharpe, G. M. (1998) Superoxide dismutase restores endothelium-dependent arteriolar dilatation during acute infusion of nicotine. *J. Appl. Physiol.* **85**: 1292–1298

Mayhan, W. G., Sharpe, G. M. (1999) Chronic exposure to nicotine alters endothelium-dependent arteriolar dilatation: effect of superoxide dismutase. *J. Appl. Physiol.* **86**: 1126–1134

Melchiorri, D., Reiter, R. J., Atia, A., Hara, M., Burgos, A., Nistico, G. (1995a) Potent protective effect of melatonin on in vivo paraquat induced toxicity. *Life Sci.* **56**: 83–89

Melchiorri, D., Reiter, R. J., Sewerynek, E., Chen, L. D., Nistico, G. (1995b) Melatonin reduces kainate-induced lipid peroxidation in homogenates of different brain regions. *FASEB J.* **9**: 1205–1210

Pieri, C., Marra, M., Moroni, F., Recchioni, R., Marcheselli, F. (1994) Melatonin: a peroxyl radical scavenger more effective than vitamin E. *Life Sci.* **55**: PL271–PL276

Reiter, R. J., Tan, D. X., Poeggeler, B., Menendez-Pelaez, A., Chen, L. D., Saarela, S. (1994) Melatonin as a free radical scavenger: implications for aging and age-related diseases. *Ann. N. Y. Acad. Sci.* **719**: 1–12

Sewerynek, E., Melchiori, D., Reiter, R. J., Ortiz, G. G., Lewinski, A. (1995) Lipopolisaccharide-induced hepatotoxicity is inhibited by the antioxidant melatonin. *Eur. J. Pharmacol.* **293**: 327–334

Tan, D. X., Chen, L. D., Poeggeler, B., Manchester, L. C., Reiter, R. J. (1993a) Melatonin: a potent, endogenous hydroxyl radical scavenger. *Endocr. J.* **1**: 57–60

Tan, D. X., Poeggeler, B., Reiter, R. J., Chen, L. D., Chen, S., Manchester, L. C., Barlow-Walden, L. R. (1993b) The pineal hormone melatonin inhibits DNA adduct formation induced by the chemical carcinogen safrole in vivo. *Cancer Lett.* **70**: 65–71

Tarquini, B., Perfetto, F., Poli, R., Tarquini, R. (1994) Daytime circulating melatonin levels in smokers. *Tumori* **80**: 229–232

Xie, Y., Garban, H., Ng, C., Rajfer, J., Gonzalez-Cadavid, N. F. (1997) Effect of long-term passive smoking on erectile function and penile nitric oxide synthase in the rat. *J. Urol.* **157**: 1121–1126

Yamada, H., Ogura, A., Koizumi, S., Yamaguchi, A., Moriyama, Y. (1998) Acetylcholine triggers L-glutamate exocytosis via nicotinic receptors and inhibits melatonin synthesis in rat pinealocytes. *J. Neurosci.* **18**: 4946–4952

Zhang, S., Day, I., Ye, S. (2001) Nicotine induced changes in gene expression by human coronary artery endothelial cells. *Atherosclerosis* **154**: 277–283