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ORIGINAL PAPER

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The effects of melatonin and the antioxidant defence system on apoptosis regulator proteins (Bax and Bcl-2) in experimentally induced varicocele

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Abstract Our aim was to examine the effects of melatonin on the testicular tissue of adult rats with experimentally-induced left varicocele, and to determine the relationship between melatonin and apoptosis regular proteins in the anti-oxidant defence system. Forty adult male Wistar rats were divided equally into four groups. A sham operation was performed on the rats in group I, and experimental left varicocele was created in groups II, III and IV. Melatonin was administered intraperitoneally at doses of 5 mg/kg and 10 mg/kg to rats in groups III and IV, respectively. An immunohistochemical analysis of the left testicular tissue was performed to evaluate the expression of Bax and Bcl-2, while tissue malondialdehyde (MDA) and antioxidant enzyme activities were assessed in homogenates to determine the role of the oxygen defence system. The immunohistochemical analysis revealed an increased ratio of pro-apoptotic protein Bax in groups II and III, whereas no significant activity was observed in the sham operated rats ($P < 0.05$). Similarly, the tissue MDA level increased and a significantly decreased level of antioxidant enzymes was observed in these groups ($P < 0.05$). Although rats in group IV showed a slightly increased ratio of the pro-apoptotic marker Bax, there was no significant difference between groups I and IV. Similarly, group IV showed decreased levels of MDA and increased levels of anti-oxidant enzyme activity with decreased Bax expression. The close relationship between pro-apoptotic/anti-apoptotic markers, reactive oxygen species and antioxidant agents provided a useful in vivo model for studying the pathophysiology of varicocele and evaluating the role of antioxidants in the prevention testicular damage.

Keywords Varicocele · Apoptosis · Bax · Bcl-2 · Melatonin · Reactive oxygen species

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Introduction

Although varicocele is the most common 'correctable' cause of male infertility, its pathophysiology has not yet been clearly defined [7]. Recently, testicular germ cell death observed in patients with varicocele was reported to be associated with apoptosis [24]. There is a growing body of literature suggesting an association between apoptosis and varicocele related to hormone deficiency and increased temperature [7].

Apoptosis is often a genetically encoded process of self-destruction. However, for the initiation of this destructive pathway, several physiological and non-physiological stimuli are required. Elevated temperature of the testis due to various diseases, such as varicocele, was reported to be one of the causes of increased programmed cellular death [23]. It is generally accepted that the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family proteins is the critical determinant in the fate of the cell, with an excess of Bcl-2 resulting in cell survival and an excess of Bax resulting in cell death [9]. Thus, apoptotic regulator proteins seem to play a major role in the regulation of cell death. In another study, the addition of exogenous reactive oxygen species (ROS) was also found to trigger the apoptotic cascade [6]. Recently, a role for oxidative stress in the induction of apoptosis has been provided by several studies, and it was observed that various antioxidants, such as N-acetylcysteine, can inhibit cell death [14, 18].

Melatonin, an endogenous hormone secreted by the pineal gland in a circadian rhythm, can enhance the antioxidant defence system and acts as a free oxygen radical scavenger [12]. In this study, we evaluated the

effects of melatonin on the testicular tissue of adult rats with experimentally induced left varicocele, and determined the role of oxidative stress as well as the expression of the apoptosis regulator proteins Bax and Bcl-2 in varicocele testes.

Materials and methods

Animals, experimental design and the creation of varicocele

Approval from the local ethics committee was obtained for all experimental procedures. The study was performed on 40 adult male Wistar rats (aged 12–14 weeks). All rats were kept in individual cages and were exposed to 12 h of daylight at a temperature of 20–25°C. They were fed with standard rat food and tap water.

The rats were divided equally into four groups. In group I, they underwent a sham operation. Partial left renal vein ligation was performed on the rats in group II. Similarly, rats in groups III and IV underwent partial ligation and subsequently received 5 mg/kg and 10 mg/kg melatonin, respectively. After a midline incision, the left renal vein was exposed, and with fine dissection, a 4-0 silk suture was tied around the vein. A metal probe with various diameters, ranging from 0.5 to 0.85 mm and selected according to vein size, was placed into the left renal vein at the point medial to the insertion of the adrenal and spermatic vein into the renal vein. A ligature was tied around the vein to contain the metal probe and, following this, the probe was gently removed and the vein allowed to expand within the boundary of ligature. Sham operated animals underwent all of these procedures except for the actual ligation of the vessel. The midline incisions were closed with interrupted 3-0 silk sutures in all animals.

Immunohistochemistry for pro-apoptotic and anti-apoptotic markers

The animals were killed 30 days after the experimental varicocele was constructed and the left testes were removed. Half of each testis was preserved at −20°C for histopathological examination, and the other was used for biochemical assessment. Immunohistochemical staining was performed on formalin fixed, paraffin embedded specimens after deparaffinization and rehydration. This technique involved the sequential incubation of the specimen with an unconjugated primary antibody specific to the target antigen, a biotinylated secondary antibody which reacts with the primary antibody, enzyme-labeled streptavidin, and substrate-chromogen. To reduce unspecific background staining due to endogenous peroxidase, the slides were incubated in an hydrogen peroxide block and then washed several times in buffer. Reactive immunoglobulins were blocked and primary antibody (monoclonal Bax or monoclonal Bcl-2, Dako, Carpinteria, Calif.) was applied and incubated according to the manufacturer's protocol. Specimens were stained separately for each antibody. Immunoreactivity was detected using biotinylated goat anti-mouse antibody followed by streptavidin-peroxidase incubation. The specimens were then rinsed in buffer and incubated with peroxidase compatible chromogen according to the manufacturer's recommendations. Bcl-2 and Bax positivity were assessed as described previously [11]. More than 10% or diffuse staining for Bax and Bcl-2 was considered positive (++), whereas <10% staining, which is denoted as (+), or no staining were both classified as negative. An excess of Bax over Bcl-2 staining was considered to be an indirect determinant of cell fate via apoptosis [9].

Determination of tissue malondialdehyde and antioxidant enzyme activities

Malondialdehyde (MDA), which is the end product of lipid peroxidation, was measured in the homogenates using the method described by Satoh [21] and Yagi [25]. MDA reacts with TBA to produce a chromogenic product which is measured spectrophotometrically. Homogenization was carried out with a Teflon end homogenizator (Elvenjem Potter, Du Pont Instruments, Newton, Conn.) and the homogenates were centrifuged at 1,500 rpm for 10 min at 4°C. The plasma was divided and stored at –20°C for a few days before the analyses were performed.

The superoxide dismutase (SOD) level was measured spectrophotometrically at 560 nm as described previously [8].

Glutathione peroxidase (GSH-Px) is an enzyme that catalyzes, with high specificity, the in vitro detoxification of hydrogen peroxide as a substrate by the oxidation of reduced glutathione [19]. GSH-Px activity is measured by following the decrease in absorbance of the reaction mixture at 340 nm, as NADPH is converted to NADP. The decomposition of hydrogen peroxide, which is a measure of catalase activity, can be followed by measuring the decrease in absorbance at 240 nm. The decrease in absorbance was recorded every 15 s, and, for measurement, absorbance differences with 1 min intervals were calculated [2].

Administration of melatonin

Under sterile conditions and avoiding direct exposure to light, melatonin (Sigma, St Louis, Mo.) was dissolved in pure ethanol and mixed with 0.9% NaCl, amounting to a final concentration of 1:10. Throughout the experiment, 5 mg/kg and 10 mg/kg of melatonin were administered daily to rats in groups III and IV, respectively. The drug was administered intraperitoneally at 4 or 5 pm in the afternoon. At this time, melatonin is considered to have its lowest natural concentration in the blood [12]. Sham operated rats received injections with an equivalent amount of saline intraperitoneally, everyday.

Statistical analysis

The data are expressed as mean ± SD. Statistical analysis was performed using the Student's t-test and the χ^2-test.

Results

Immunohistochemical staining for Bax in the sham operated rat testes showed no staining in any of the germ cell types (Fig. 1). Strong Bcl-2 staining in the sperma-

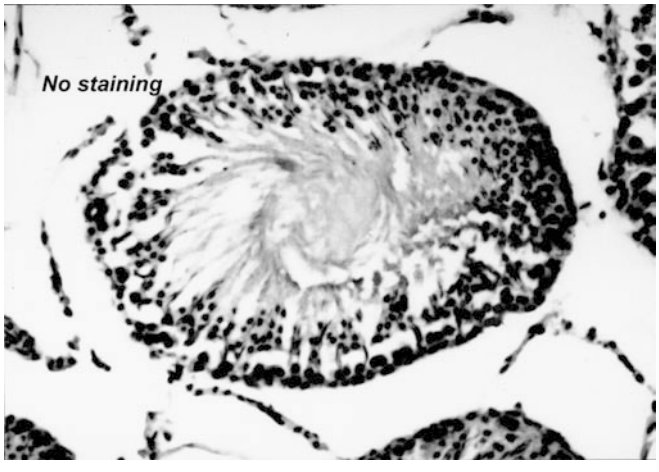

Fig. 1 Negative immunohistochemical staining for Bax in the sham operated rat testes demonstrating no apoptotic stimuli (×200)

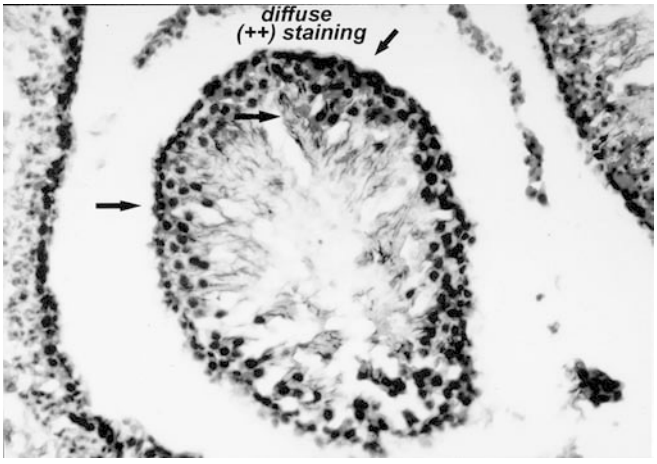

Fig. 2 An increased apoptotic signal demonstrated by diffuse (++) Bax staining in spermatogonia, spermatocytes, and spermatids in the testes in the varicocele induced group II and varicocele plus 5 mg/kg melatonin receiving group III (×200)

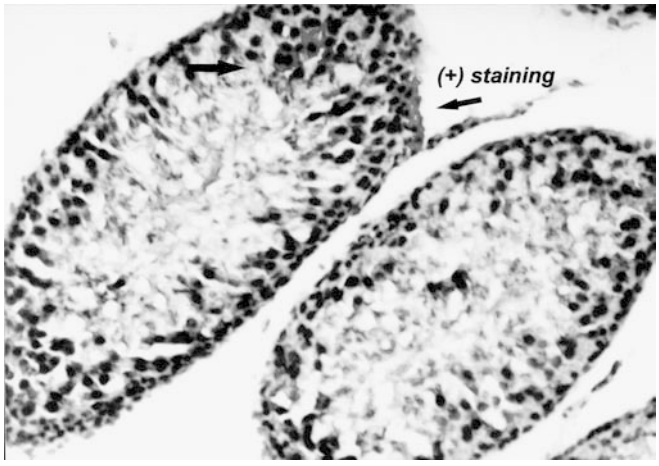

Fig. 3 Significantly decreased Bax staining (< 10%) in the testes of rats receiving 10 mg/kg melatonin in group IV indicating decreased apoptotic activity (×200)

togonia, spermatocytes, and spermatids of these rats suggested no stimuli for the induction of apoptosis. In groups II and III, there was a significant increase in the positive staining for Bax which indicated an increased ratio of Bax to Bcl-2 and concomitantly appearing apoptotic stimuli when compared to group I (between groups I and II, $P < 0.001$, between groups I and III, $P < 0.001$) (Fig. 2). In group IV, there was significantly decreased Bax protein staining (+ staining) (Fig. 3). There was no significant difference between the sham operated rats and group IV rat testes with respect to increased cellular death ($P = 0.606$). However, a significantly increased ratio of pro-apoptotic Bax protein with low or no staining of Bcl-2 was determined in groups II and III.

The tissue levels of MDA and the activities of antioxidant enzymes are shown for all groups in Table 1. In groups II and III, the level of MDA increased significantly, whereas there was a significant decrease in the level of GSH-Px and catalase in groups II and III when compared to sham operated rats and rats in group IV($P < 0.05$). Increased levels of tissue MDA and decreased antioxidant enzyme levels in these groups correlated with the increased expression of Bax protein. In group III, the level of SOD was higher than in group II, however, it did not help to decrease the level of apoptotic stimuli in this group. Group IV showed no significant difference among the level of tissue MDA and antioxidant enzyme activities when compared to sham operated rat testes. Although there were still pro-apoptotic stimuli in group IV, these were less than 10% and were not as prominent as in groups II and III.

Discussion

The Bcl-2 family of proteins, which contains both pro-apoptotic (such as Bax) and anti-apoptotic (such as Bcl-2) members, plays an important role in the determination of a cell's fate via apoptosis [1]. Bcl-2 and Bax have also been implicated as modulators of germ cell apoptosis and to be critical determinants of apoptosis, such that elevated levels of Bcl-2 favors extended survival of cells and increasing levels of Bax accelerate cell death [13]. Simsek et al. [24] proposed that apoptosis should also be expected in patients with varicocele, and determined the mean number of apoptotic cells in varicocele patients to be seven times higher than in a control group. Similarly, Lin et al. [15] reported increased apoptosis during hypospermatogenesis in comparison with normal spermatogenesis. Lue et al. [16], demonstrated that a single, transient testicular hyperthermia

Table 1 Tissue levels of malondialdehyde (MDA) and activities of the antioxidant enzymes glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (mean ± SD). * Mean level was significantly lower than that in the sham operated group and group IV ($P < 0.05$)

	Group I	Group II	Group III	Group IV
MDA (nmol/g protein)	6.67 ± 1.01	13.43 ± 2.25*	9.5 ± 1.76*	6.81 ± 0.77
	(6.0–9.30)	(11.2–17.3)	(8.0–13.6)	(6.10–8.50)
GSH-Px (U/g protein)	61.4 ± 4.52	28.7 ± 5.33*	38.2 ± 3.85*	59.8 ± 6.26
(55–67)	(21–38)	(31–44)	(56–75)	
SOD (U/mg protein)	133.65 ± 30.69	43.27 ± 6.57*	79.25 ± 6.82*	127.23 ± 15.01
	(110–210)	(33–53)	(69–51)	(92–115)
Catalase (catalase/g protein)	13.44 ± 1.76	7.21 ± 2.34*	10.85 ± 2.98*	12.57 ± 3.52
(7–21)	(4–27)	(5–31)	(7–42)	

(43°C for 15 min) induces the activation of germ cell apoptosis. In the present study, we assessed the Bax/Bcl-2 ratio in order to evaluate the effects of experimental varicocele, and showed a significantly increased expression of pro-apoptic Bax protein in experimental group II.

Although pathophysiology of the varicocele has not yet been completely determined, elevated testicular temperature or exposure to increased blood flow in the testes was shown to increase reactive oxygen species (ROS) and to suppress the activity of antioxidant enzyme activities [6, 10]. Ikeda et al. [10] investigated the role of ROS in testicular germ cell apoptosis induced by heat stress, and administered the radical oxygen molecules exogenously to culture media containing testicular cells isolated from immature rats. Treatment with catalase, an antioxidant defense enzyme, significantly attenuated heat or stress-induced apoptosis in their study [10]. Similarly, a role for oxidative stress in the induction of apoptosis was also shown in studies in which the addition of low levels of ROS induced apoptosis, and the observation that various antioxidants such as N-acetylcysteine can inhibit cell death [14, 18]. In a previous study, we also showed the relationship between experimental varicocele and degenerative changes in the germinal epithelium as well as atrophy of the seminiferous tubules in rats, and revealed the role of reactive nitrogen species and ROS in the disease status as well as the protective effects of melatonin as an antioxidant [22]. Further assessment of the protective effects of other antioxidants on varicocelized rats revealed that exogenously supplied taurine, catalase or SOD led to significant protection of the testicular functions and epididymal sperm maturation [23]. These results support the potential role of ROS and antioxidants in the pathophysiology of heat induced apoptosis. In the present study, we used an in vivo model rather than culture media and performed experimental left varicocele, subsequently treating the rats with 5 or 10 mg/kg melatonin. Following the administration of 10 mg/kg melatonin, a dose-dependent increase in the level of GSH-Px, SOD and catalase prevented the expression of pro-apoptotic Bax. Although, in a limited human study [17], the administration of 3 mg melatonin/day to healthy men over a 6 month period led to a decline in sperm density in 3/8 subjects, this finding was not confirmed in other studies [3]. Bornman et al. [3] determined the melatonin levels in both the plasma and seminal plasma of patients with good or impaired sperm motility and forward progression. There was no correlation between seminal plasma melatonin and impaired motility or any other semen parameter. High seminal plasma melatonin concentrations were not necessarily associated with impaired sperm motility. From this, it can be concluded that seminal plasma melatonin does not play an important role in sperm motility [3]. In our study, we used high doses of melatonin to induce adequate levels of antioxidant enzyme activity to prevent the expression of pro-apoptotic proteins.

Although dilation of the left internal spermatic vein was observed immediately after partial ligation of the renal vein, data in the literature suggest that experimentally induced varicocele is associated with a detrimental effect after 30 days. We assessed the protective action of melatonin after this period [5]. Melatonin is both hydrophilic and lipophilic, and can easily pass the blood-testis barrier. It may reach sufficient levels to induce direct antioxidant activity and increase the levels of antioxidant enzymes [4]. It has also been accepted as a more potent antioxidant than the well known vitamin E [20].

In the present study, experimentally induced left varicocele revealed an increased ratio of pro-apoptotic Bax protein in testicular germ cells, whereas this effect was antagonized by using the potent endogenous hormone melatonin at a dose of 10 mg/kg/day. The reversal of this effect was attributed to the prevention of damage induced by oxidative stress. We believe that our study presents an indirect but useful model for the study of the pathophysiology of varicocele-induced apoptosis, and may further help to elucidate the role of antioxidants in the prevention of varicocele induced testicular damage.

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