



Effect of pentoxifylline on the regeneration of rat testicular germ cells after heat shock

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Abstract

High temperature is an important factor for reproduction and can induce testicular degeneration. Pentoxifylline is a methylxanthine phosphodiesterase inhibitor with anti-inflammatory and anti-apoptotic properties. Considering the protective properties of pentoxifylline and the harmful effects of heat, the present study aimed to use pentoxifylline to reduce the damage induced by heat to the testis. Adult male Wistar rats were exposed to testicular heat shock (43°C for 15 min), treated with 50 or 100 mg/kg of pentoxifylline and evaluated at 3, 7, 15, 30 and 60 days after heat shock. Pentoxifylline treatment did not change testicular weight, histomorphometrical parameters or plasma testosterone concentration. However, pentoxifylline inhibited germ cell apoptosis and reduced the severity of pathological lesions at 30 and 60 days after testicular heat shock. In conclusion, pentoxifylline treatment seemed to inhibit pro-inflammatory and apoptotic mechanisms triggered by testicular heat shock, improving spermatogenesis regeneration.

Keywords: heat shock, pentoxifylline, spermatogenesis, testicular degeneration, testis.

Introduction

Hormonal factors stimulate and modulate spermatogenesis. However, the control of testis temperature between 2 and 6°C below corporal temperature is fundamental for its normal function (Kastelic and Coulter, 1995; Setchell, 2006).

High environmental temperature is an important factor and should be taken into account for reproduction as it can result in testicular degeneration, reducing the percentage of normal and fertile sperm in the ejaculate (Jainudeen and Hafez, 1995; Turner, 2007), and negatively affecting spermatogenesis in all mammalian species (Huang *et al.*, 2000). Germ cells are vulnerable to heat stress and undergo apoptosis in temperatures above that observed in the scrotum (Kumagai *et al.*, 2002). Most studies claimed that heat effects on the testis are reversible. However, Setchell (1998) observed that testicular weight did not return to normal, even 60 days after a single exposure to heat.

According to Setchell (2006), heat directly applied to the testis has been providing new information regarding the damage triggering mechanisms on spermatogenesis and possible treatments to prevent damage to the testis by blocking apoptosis.

Pentoxifylline is a methylxanthine phosphodiesterase inhibitor with anti-inflammatory properties that increases intracellular AMPc, reduces superoxide anions and inhibits TNF- α , which is responsible for DNA fragmentation (Vadiraja and Madyastha, 2001; Chen *et al.*, 2002; Maxwell *et al.*, 2002). No previous studies on the effects of pentoxifylline on spermatogenesis or its regeneration after heat shock were found.

Considering the properties of cellular protection and apoptosis inhibition shown by pentoxifylline, the present study analyzed the effects of pentoxifylline on the recovery of spermatogenesis after testicular degeneration induced by heat shock.

Materials and Methods

Experimental design

This experiment was approved by the ethics committee (technical administrative council) of the UFRPE/DMFA (No. 31/2007). Sixty day old male Wistar rats (*Rattus norvegicus*, var. *Albinos*) from the biotery of the Animal Morphology and Physiology Department of the Federal Rural University of Pernambuco were kept in a controlled environment with a temperature of 22°C at 50% humidity and a 12-h light/dark cycle. Standard pellet food (Labina Purina) and tap water were available *ad libitum*. Seventy two rats were randomly chosen to compose the experimental groups: control rats that were exposed to heat shock (n = 18); rats that were exposed to heat shock and treated with 50 mg/kg of pentoxifylline (n = 18); rats that were exposed to heat shock and treated with 100 mg/kg of pentoxifylline (n = 18); and negative control rats that were not submitted to heat shock (n = 18).

At 120 days post-natal, the animals were intraperitoneally anesthetized with 30 mg/kg of sodium thiopental (Lue *et al.*, 1999) and the testes were immersed in water at 43°C for 15 min. After heat shock, animals were transferred to their cages (n = 6) and maintained

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at room temperature until they recovered from the anesthesia. The next day, the animals began to receive two daily intraperitoneal applications of distilled water (control), or either 50 or 100 mg/kg of pentoxifylline in agreement with their experimental group. Body weight was measured daily to calculate pentoxifylline doses. The experimental analyses were performed at 3, 7, 15, 30 and 60 days after heat shock.

Testicular perfusion

At the end of the experimental period, the animals were heparinized (125 UI/100 g; Akzo Organon Teknika), anesthetized (Thiopental 50 mg/kg; Roche) and submitted to intracardiac perfusion using a 0.9% NaCl solution plus heparin (500 UI/l) and sodium nitroprusside (100 mg/l; Sigma). After that, all rats were perfused using 4% glutaraldehyde (Vetec) in a sodium phosphate buffer (pH 7.2 and 0.01 M). After fixation, the testes were removed and weighed using a balance scale (BEL Engineering MARK 500 / BRA \pm 0.001 g).

Testicular histomorphometry and histopathology

Testicular fragments were cut at a thickness of 2 mm and re-fixed for 1 h with the same perfusion solution. Afterwards, the fragments were immersed in a phosphate buffer for 2 h and then dehydrated in a crescent series of alcohol (70 - 100%) and embedded in plastic resin composed of glycol methacrylate (LEICA). Histological sections 4 μ m in thickness were stained with 1% toluidine blue/sodium borate and analyzed morphologically and morphometrically.

Histomorphometrical and histopathological analysis of testicular components was performed according to Silva Junior *et al.* (2006) and Tenorio *et al.* (2011) using an optical microscope (Olympus BX-51, Tokyo, Japan).

Seminiferous tubule volume

The volume density of each testicular component was measured using point counting by systematic allocation through a micrometer reticle (Olympus U-OCMSQ 10 mm/100) with 441 intersection points on a histological preparation of the testis at 400X magnification. Fifteen fields were randomly counted, totaling 6615 points for each animal. The seminiferous tubule volume expressed in μ l was established from the product of volume density (%) and testicular liquid weight calculated in milligrams (mg). The value of testicular liquid weight was obtained by subtracting 6.5% (relative to the albuginea) of the testicular gross weight. Testis weight was considered equal to its volume because testicular density was approximately 1.03 to 1.04.

Tubular diameter and total length of seminiferous tubules

The diameters of 30 randomly selected round seminiferous tubules per animal were measured using a linear reticle micrometer (U-OCMSQ10/10, Olympus, Tokyo, Japan) at 100X magnification. The tubular diameter was obtained by means of two diametrically opposite measurements. The total length of seminiferous tubules (TLST) per testis, expressed in meters, was obtained by dividing the seminiferous tubule absolute volume (STAV) by r^2 (r = diameter/2) and π :

$$TLST = \frac{STAV}{\pi r^2}$$

Germ and Sertoli cell counting

To evaluate the efficiency of spermatogenesis, we estimated the corrected number of germ and Sertoli cells in stage VII of the seminiferous epithelium cycle (according to the acrosomal method). Germ cell nuclei and Sertoli cell nucleoli were counted in 10 seminiferous tubule cross sections per animal. The number of nuclei of spermatocyte I in preleptotene (SPT I PI), spermatocyte I in the pachytene stage (SPT I P), round spermatids (SPD Ar) and nucleoli of Sertoli cells was counted.

Germ or Sertoli cells counts (GSCC) were corrected for nuclear or nucleolar diameter and histological section thickness. The crude counts (CC) were corrected for section thickness (S) and the mean nuclear or nucleolar diameter (ND):

$$GSCC = CC \times \frac{S}{S + \sqrt{\left(\frac{ND^2}{2}\right) - \left(\frac{ND^2}{4}\right)}}$$

The mean nuclear or nucleolar diameters were measured using a linear reticle micrometer (U-OCMSQ10/10, Olympus, Tokyo, Japan) at 1000X magnification.

The number of Sertoli cells per testis (NSCT), also called Sertoli cell population, was determined from the corrected count of Sertoli cells per tubule cross section (CSC), section thickness (S) and the total length of seminiferous tubules (TLST):

$$NSCT = \frac{TLST \times CSC}{S}$$

Daily sperm production

Daily sperm production per testis was obtained



according to the following formula:

$$DSP = \frac{NSCT \times RSC \times RFS VII}{Stage VII Duration}$$

DSP = Daily sperm production; NSCT = Total number of Sertoli cells per testis; RSC = Round spermatid count in stage VII; RFS VII = Relative frequency of stage VII; Stage VII duration (days).

Daily sperm production per gram of testis (DSP/g) was obtained from the ratio between DSP and testicular liquid weight.

Nuclear fragmentation analysis of germ cells

Testis fragments were processed for inclusion in paraffin and histological cuts with a 5 µm thickness were stained with Nile's Blue solution to evidence the presence of nuclear fragmentation similar to apoptosis (Lee and Baehrecke, 2001; Tolosa *et al.*, 2003). To evaluate nuclear fragmentation of germ cells, five round seminiferous tubules were randomly selected per animal and germ cells positively stained with Nile's Blue solution were counted.

Plasma testosterone

Before saline perfusion, blood samples were taken from the vena cava sinus for measurement of serum testosterone. After clotting and centrifugation, the recovered serum was stored at -20°C until analysis. Testosterone was quantified by enzyme immunoassay

using a polyclonal anti-testosterone antibody (R 1567 1:7500 dilution) obtained from Coralie Munro at the University of California, Davis (Davis, CA, USA), cross reacting with testosterone 100.0%, 5α-dihydrotestosterone 57.4%, androstenedione 0.27%, and androsterone, dehydroepiandrosterone (DHEA), cholesterol, oestradiol, progesterone and pregnenolone <0.05%. Serial dilutions of pooled serum samples produced a displacement curve similar to the standard displacement curve and the assay sensitivity was 2.3 pg/well. Inter- and intra-assay coefficients of variation were less than 10%.

Statistical analyses

The Shapiro-Wilks test was used to evaluate the normality of the obtained data. Subsequently, depending on the normality of the results, we used parametric or nonparametric tests. For the data considered to be normal, we used analysis of variance (ANOVA) with Tukey-Kramer post-hoc. If the data did not follow a normal distribution, we used the Kruskal-Wallis nonparametric test with Dunn post-hoc. The data are expressed as mean ± standard deviation. All statistical analysis was outlined at P < 0.05.

Results

Testicular weight

It was observed that testicular weight did not change between animals treated or not treated with pentoxifylline at 3, 7, 15, 30 and 60 days after heat shock (Table 1).

Table 1. Testicular weight (g) of negative control rats, rats exposed to testicular heat shock (control), and rats treated with 50 or 100 mg/kg of pentoxifylline at 3, 7, 15, 30, and 60 days after testicular heat shock.

Days	Negative control	Control	50 mg/kg of pentoxifylline	100 mg/kg of pentoxifylline	P - value
3	1.7 ± 0.1	1.3 ± 0.5	1.7 ± 0.1	1.5 ± 0.1	0.19
7	1.7 ± 0.2	1.5 ± 0.2	1.5 ± 0.3	1.6 ± 0.1	0.58
15	1.7 ± 0.0	1.4 ± 0.4	1.5 ± 0.0	1.4 ± 0.1	0.14
30	1.7 ± 0.3	1.7 ± 0.2	1.5 ± 0.3	1.4 ± 0.1	0.63
60	1.7 ± 0.1	1.3 ± 0.3	1.2 ± 0.3	1.4 ± 0.1	0.07

Histopathological analysis

The testicular parenchyma of the control group 3 days after heat shock showed pathological lesions compatible with testicular degeneration. Thickening of the basal membrane, Sertoli cell vacuolation, germ cell desquamation, giant syncytial cells, spermatocyte I in pre-leptotene/leptotene with nuclear pyknosis and degenerated spermatocyte I in the pachytene stage were observed. Some seminiferous tubules contained just spermatogonia and Sertoli cells in the basal compartment (Fig. 1A, B, C, D).

Animals treated with 50 mg/kg of pentoxifylline after heat shock also showed lesions compatible with testicular degeneration. However, some tubules had preserved stages of the seminiferous epithelium cycle. Pentoxifylline inhibited the damage mechanisms induced by heat shock on the testis (Fig. 1C, D).

Three days after heat shock, the testes of animals treated with 100 mg/kg of pentoxifylline showed few areas compatible with testicular degeneration. Despite the presence of pathological lesions in the seminiferous epithelium, we observed several tubules showing stages of the seminiferous epithelium

cycle. Treatment with 100 mg/kg of pentoxifylline seemed to have an inhibitor effect of cell damage on testes exposed to heat shock (Fig. 1A, B). The animals showed more severe lesions 7 days after testicular heat shock than at 3 days after heat shock (Fig. 1A, C). During the same period, animals treated with 50 mg/kg of pentoxifylline showed some seminiferous tubule lesions consistent with degeneration. However, these animals showed most stages of the seminiferous epithelium cycle. Stage VII of the seminiferous

epithelium cycle was observed, indicating the inhibitory effect of pentoxifylline on testicular degeneration induced by heat. It is important to note that stage VII is critical in the evaluation of spermatogenesis and it was not observed in the control animals exposed to heat shock. Animals treated with 100 mg/kg of pentoxifylline also showed some seminiferous tubules with pathological lesions, but most stages of the seminiferous epithelium cycle were preserved, including stage VII (Fig. 1E, F).

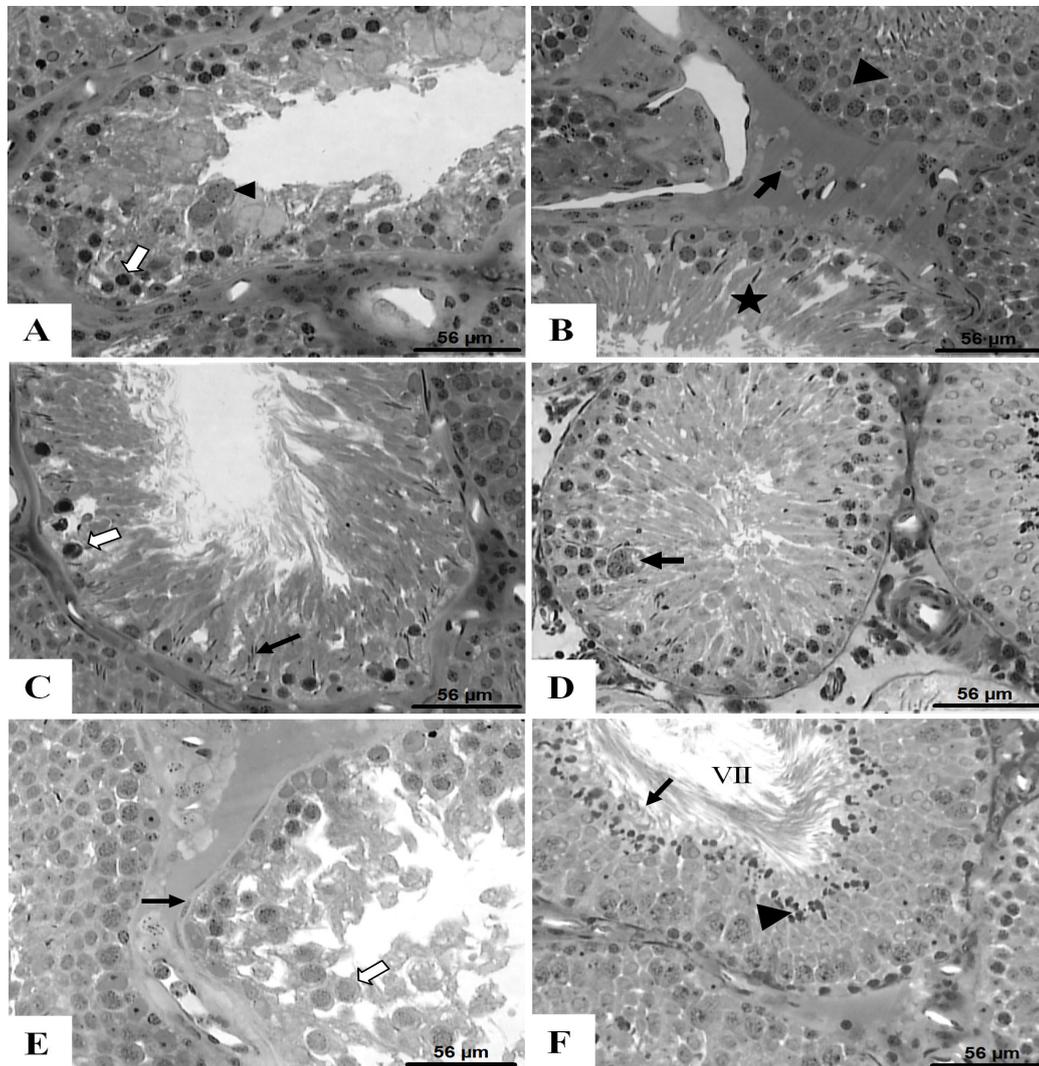


Figure 1. Photomicrographs of the testis in rats exposed to testicular heat shock (control) and treated with 50 or 100 mg/kg of pentoxifylline for 3, 7, and 15 days after testicular heat shock. Figure 1A: Control group rat at 3 days after heat shock. Note spermatocyte I in the pachytene stage with chromatin condensation (large arrow) and germ cells in desquamation (arrowhead). Figure 1B: Rats treated with 100 mg/kg of pentoxifylline at 3 days after heat shock. Note the seminiferous epithelium with incomplete cellular association without spermatocyte I in the pachytene stage (star), seminiferous epithelium with complete cellular associations (arrow head) and Leydig cells (arrow). Figure 1C: Control group rat at 7 days after heat shock. Note the degenerated elongating spermatids (arrow) and spermatocyte I with morphological changes similar to apoptosis (large arrow). Figure 1D: Rat treated with 50 mg/kg of pentoxifylline at 7 days after heat shock. Note the syncytial giant cell from round spermatids (arrow). Figure 1E: Control group rat at 15 days after heat shock. Note the thickening of the tunica propria (arrow) and seminiferous epithelium desquamation (large arrowhead). Figure 1F: Rat treated with 100 mg/kg of pentoxifylline at 15 days after heat shock. Note the tubule in stage VII of the seminiferous epithelium cycle with spermiation (arrow) and residual body release (arrow head).

The control animals exposed to heat shock also showed pathological lesions compatible with testicular degeneration 15 days after thermal injury. We did not observe the presence of stage VII of the seminiferous epithelium cycle in these animals (Fig. 1E). In animals treated with 50 and 100 mg/kg of pentoxifylline, despite the presence of some degenerated tubules, spermatogenesis was preserved because all stages of the seminiferous epithelium cycle were observed.

Thirty days after heat shock, a partial recovery of spermatogenesis in the untreated group was observed. However, degenerated seminiferous tubules were also observed. For the animals treated with either 50 or 100 mg/kg of pentoxifylline, all stages of the seminiferous epithelium cycle were shown, but tubules with incomplete germ cell associations and some pathological lesions were still observed (Fig. 2A, B).

The histopathological analysis performed on the untreated animals 60 days after heat shock showed some pathological lesions, such as seminiferous tubules with reduced amounts of germ cells in the epithelium,

Sertoli cell vacuolation, desquamated germ cells in the lumen and multinucleated syncytial cells (Fig. 2C). However, this group showed a partial regeneration of spermatogenesis at 60 days post-heat shock because tubules in stage VII of the seminiferous epithelium cycle were observed. Seminiferous tubules with typical cellular associations of normal spermatogenesis were observed in animals treated with 50 mg/kg of pentoxifylline in the same period of evaluation. Moreover, some seminiferous tubules with desquamated germ cells were observed in the lumen (Fig. 2D). Seminiferous tubules in stage VII and intertubular areas without structural changes were observed in animals treated with 100 mg/kg of pentoxifylline. There were no changes compatible with testicular degeneration in animals treated with 100 mg/kg of pentoxifylline (Fig. 2E, F). This fact indicates that pentoxifylline accelerated the regeneration of spermatogenesis, especially in the group that received the higher dose.

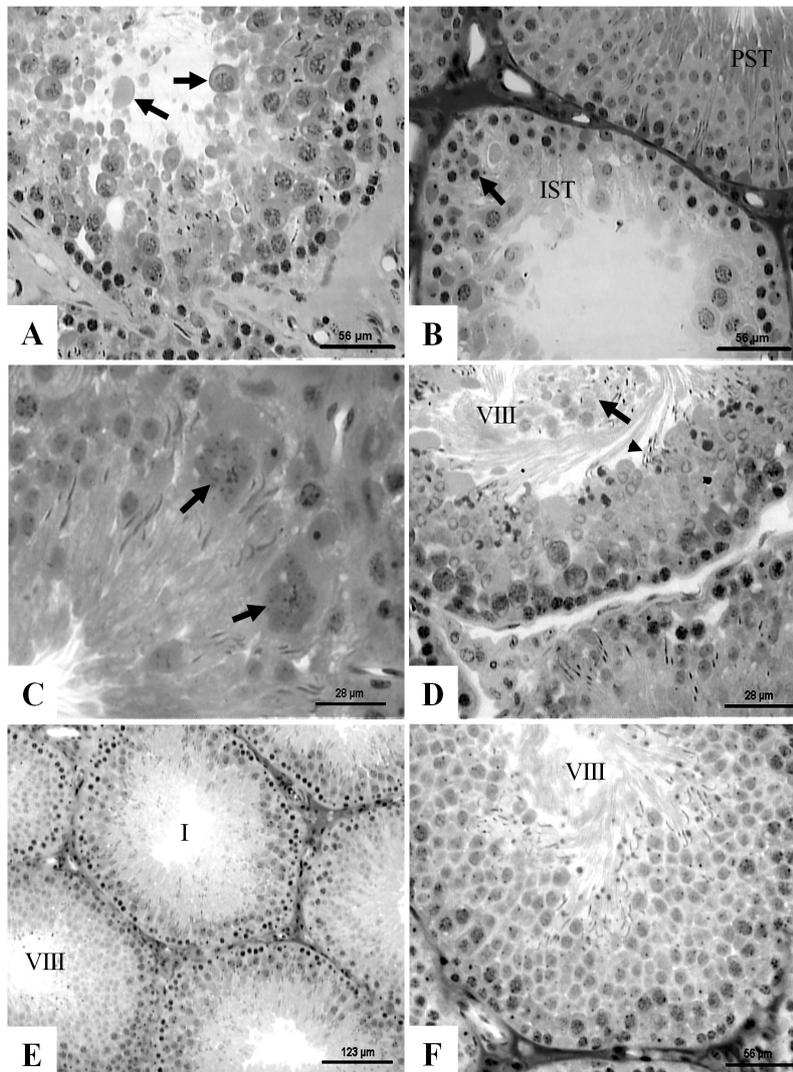


Figure 2. Photomicrographs of the testis in rats exposed to testicular heat shock (control) and treated with 50 or 100 mg/kg of pentoxifylline for 30 and 60 days after testicular heat shock. Figure 2A: Control group rat at 30 days after heat shock. Detail of a degenerated seminiferous tubule in stage VII. Note germ cells with desquamation (arrow). Figure 2B: Rat treated with 100 mg/kg of pentoxifylline at 30 days after heat shock. Note the preserved seminiferous tubules (PST) and a tubule with incomplete cellular association (IST) and some pyknotic germ cells (arrow). Figure 2C: Rat at 60 days after heat shock. Note a seminiferous tubule between stages IV-V with multinucleated syncytial cells (arrow) from round spermatids. Figure 2D: Rat treated with 50 mg/kg of pentoxifylline at 60 days after heat shock. Seminiferous tubule in stage VIII, note spermiation (arrowhead) and desquamated germ cells in the lumen (arrow). Figure 2E: Rat treated with 100 mg/kg of pentoxifylline at 60 days after heat shock. Note a seminiferous tubule in stage I and another in stage VIII. Figure 2F: Rat treated with 100 mg/kg of pentoxifylline at 60 days after heat shock. Note the seminiferous tubule in stage VIII with complete cellular association.

*Histomorphometrical analysis*

No significant changes were observed in the volume of seminiferous tubules between the experimental groups at 3, 7, 15, 30, and 60 days after heat shock (Table 2).

Sertoli cell population, daily sperm production per testis or gram of testis, Sertoli cell index and round spermatids per seminiferous tubule cross section did not change between the negative control, control or animals treated with 50 or 100 mg/kg of pentoxifylline (Table 3).

Table 2. Volume of seminiferous tubules (μ l) of negative control rats, rats exposed to testicular heat shock (control), and rats treated with 50 or 100 mg/kg of pentoxifylline at 3, 7, 15, 30, and 60 days after testicular heat shock.

Days	Negative control	Control	50 mg/kg of pentoxifylline	100 mg/kg of pentoxifylline	P - value
3	1295.5 \pm 138.9	1107.9 \pm 426.2	1426.3 \pm 89.1	1222.6 \pm 124.9	0.36
7	1320.9 \pm 253.0	1244.7 \pm 125.8	1267.5 \pm 316.4	1276.7 \pm 105.6	0.96
15	1422.6 \pm 39.6	1409.0 \pm 309.9	1126.9 \pm 147.0	1228.2 \pm 118.0	0.22
30	1360.4 \pm 263.0	1262.3 \pm 323.9	1260.4 \pm 301.7	1249.8 \pm 51.6	0.90
60	1424.4 \pm 73.2	1115.1 \pm 226.9	1050.1 \pm 274.8	1176.8 \pm 178.7	0.11

Table 3. Histomorphometrical testicular parameters of negative control rats, rats exposed to testicular heat shock (control), and rats treated with 50 or 100 mg/kg of pentoxifylline at 30, and 60 days after testicular heat shock.

	Days	Negative control	Control	50 mg/kg of pentoxifylline	100 mg/kg of pentoxifylline	P - value
Round SPT/CS	30	75.8 \pm 11.5	66.4 \pm 10.0	76.0 \pm 6.1	87.2 \pm 9.6	0.15
	60	72.9 \pm 8.2	57.4 \pm 18.0	58.6 \pm 3.2	73.9 \pm 7.3	0.18
Sertoli cell index	30	8.4 \pm 0.3	6.1 \pm 2.1	7.8 \pm 2.1	8.4 \pm 1.7	0.46
	60	8.4 \pm 1.3	6.7 \pm 3.3	7.5 \pm 0.8	6.5 \pm 0.9	0.40
Sertoli cell population $\times 10^6$	30	3.6 \pm 0.5	4.6 \pm 0.3	3.1 \pm 0.9	3.9 \pm 1.0	0.35
	60	4.3 \pm 0.5	5.2 \pm 1.4	3.7 \pm 0.1	4.8 \pm 2.3	0.25
DSP/g testis $\times 10^6$	30	14.5 \pm 4.0	12.6 \pm 7.3	12.3 \pm 2.3	17.0 \pm 8.7	0.67
	60	17.0 \pm 5.1	13.9 \pm 3.8	11.6 \pm 3.0	14.3 \pm 2.0	0.76
DSP/testis $\times 10^6$	30	22.7 \pm 5.4	18.5 \pm 10.6	17.0 \pm 0.5	23.3 \pm 12.0	0.68
	60	27.6 \pm 7.0	17.3 \pm 5.0	19.2 \pm 8.1	19.1 \pm 4.3	0.52

DSP = Daily sperm production ($\times 10^6$). CS = Cross Section. SPT = Spermatid.

Germ cell apoptosis

A significant reduction in the number of germ cells with nuclear fragmentation similar to apoptosis between control animals and animals treated with 50 or 100 mg/kg of pentoxifylline at 30 and 60 days after heat shock was observed (Fig. 3).

Plasma testosterone

There were no significant changes in plasma testosterone concentrations in animals exposed to testicular heat shock and treated or not treated with pentoxifylline (Table 4).

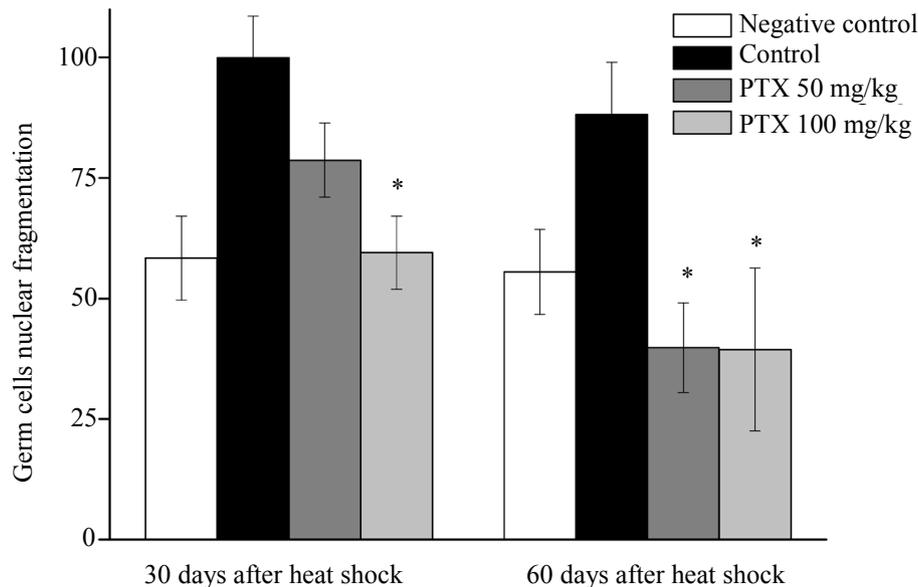


Figure 3. Number of germ cells with nuclear fragmentation similar to apoptosis per seminiferous tubule cross-section from the negative control group, animals exposed to testicular heat shock (control) and rats treated with 50 or 100 mg/kg of pentoxifylline (PTX) at 30 and 60 days after heat shock. Note the constant number of germ cells with nuclear fragmentation in animals not exposed to heat shock (negative control) and the high number of nuclear fragmentation in cells exposed to heat shock that was statistically lower in animals treated with pentoxifylline (*).

Table 4. Plasma testosterone concentration (ng/ml) of negative control rats, rats exposed to testicular heat shock (control), and rats treated with 50 or 100 mg/kg of pentoxifylline at 3, 7, 15, 30, and 60 days after testicular heat shock.

Days	Negative control	Control	50 mg/kg of pentoxifylline	100 mg/kg of pentoxifylline	P - value
3	1.2 ± 0.6	0.4 ± 0.1	0.5 ± 0.4	0.9 ± 0.1	0.10
7	0.9 ± 0.1	0.6 ± 0.2	0.9 ± 0.4	0.6 ± 0.4	0.58
15	1.3 ± 0.1	1.2 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	0.38
30	1.1 ± 0.2	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	0.91
60	1.2 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	0.43

Discussion

The reduction of testicular weight in animals exposed to heat shock was expected because acute or chronic exposure of the testes to heat led to changes in mass, scrotal circumference and sperm motility (Setchell *et al.*, 1998). Mice exposed to an environment with a temperature of 35°C for 4 days showed a decrease in testicular weight (Meistrich *et al.*, 1973). Similarly, the exposure of sheep to a hot environment for 14 days caused a reduction in testicular weight by 70% (Gomes *et al.*, 1971). Heat shock reduced testicular weight in the present study. However, treatment with pentoxifylline did not significantly change testicular weight.

Testis exposure to heat can trigger testicular degeneration, which is defined as a process that causes deterioration of testis structure, resulting in the loss of

testicular function (Turner, 2007). Embryo quality is directly linked to sperm quality; therefore, germ cells maturing into normal sperm are crucial to reproductive success (Lue *et al.*, 1999). Heat exposure can induce abnormal sperm formation, producing a gamete that can fertilize but the embryo dies (Gabaldi and Wolf, 2002).

In the present study, at 3, 7, and 15 days after heat shock, histopathological analysis showed testicular degeneration in the group that suffered a thermal injury directly to the testis but did not receive treatment. In animals treated with pentoxifylline, the pathological lesions were less severe and we observed some stages of the seminiferous epithelium cycle. A partial recovery of spermatogenesis was observed in control animals at 30 and 60 days after heat shock. Animals treated with 50 mg/kg of pentoxifylline showed less severe lesions. Animals treated with 100 mg/kg of pentoxifylline showed no testicular degeneration and all stages of the seminiferous



epithelium cycle at 60 days post-heat shock. The presence of all stages of the seminiferous epithelium cycle is indicative of completely restored spermatogenesis (Jannes *et al.*, 1998).

The testicular pathological lesions observed in the present study are consistent with those observed by Rockett *et al.* (2001). According to them, adult mice exposed to heat shock showed intratubular vacuoles, giant syncytial cells, pyknotic germ cells, and cells with apoptotic fragments. Seminiferous epithelium degeneration can occur quickly, but if the period of hyperthermia is not too long, a visible regeneration can occur 60 days after exposure to heat (McEntee, 1990). In very severe lesions where spermatogonia A are affected, azoospermia can occur (Fonseca and Chow, 1995). We observed a visible regeneration of spermatogenesis 60 days after heat shock, so spermatogonia A (testicular stem cells) were not affected. In the present study, pentoxifylline treatment seemed to inhibit the initial factors that trigger testicular degeneration, reducing the severity of pathological lesions induced by heat.

The mechanism of cell death after heat exposure appears to be apoptosis and may involve reactive oxygen species, tumor suppressor protein p53, nitric oxide synthase, translocation of the Pro-apoptotic factor Bax from the cytoplasm to a perinuclear position, the release of cytochrome C from mitochondria and several caspases (Setchell, 2006). Reactive oxygen species produced during heat shock induce germ cell death and delay testicular parenchyma regeneration (Ishii *et al.*, 2005). The anti-inflammatory properties of pentoxifylline, such as the capturing of reactive oxygen species, a reduction of lipid peroxidation associated with the plasma membrane, an increase in intracellular AMPc, a reduction of superoxide anions, and an inhibition of TNF- α (Vadiraja and Madyastha, 2001; Chen *et al.*, 2002; Maxwell *et al.*, 2002) may prevent germ cell apoptosis and reduce the severity of pathological lesions induced by heat shock.

Seminiferous tubules are composed of tunica propria (myoid cells and basement membrane), seminiferous epithelium and tubular lumen (Karl and Capel, 1998). Seminiferous tubules are the main component of the testis, occupying 89% of the testes in rats (França *et al.*, 2005). The volume of seminiferous tubules is directly correlated with testicular parameters such as sperm production and Sertoli cell populations (França and Russell, 1998) and did not change due to pentoxifylline treatment. The Sertoli cell index reflects the functional efficiency of the cell and sperm production per gram of testis reflects spermatogenesis efficiency (Russell and Peterson, 1984; Sinha-Hikino *et al.*, 1989; França *et al.*, 2005). These parameters did not change due to pentoxifylline treatment, indicating that pentoxifylline reduced the severity of pathological lesions in the testes (observed in histopathological analysis) but did not change the efficiency of spermatogenesis after testicular heat shock. Sertoli cell

population in rats is defined after birth and remains stable in adulthood (Silva Junior *et al.*, 2006). These cells are very resistant to temperature as they remain functional in cryptorchid testes (Gumińska *et al.*, 2007). Sertoli cells do not seem to be affected in animals submitted to thermal injury and treated with pentoxifylline because it supports capacity (Sertoli Cell Index) and its population was not changed due to heat.

Apoptosis is a programmed cell death that involves multiple kinases and cysteine proteases known as caspases. It is a genetically controlled suicide, characterized by cleavage of DNA and does not produce inflammation (Dorstyn *et al.*, 1998; Earnshaw *et al.*, 1999; Wolf and Green, 1999). According to Khan and Brown (2002), an increase in temperature triggers apoptosis in dividing germ cells, but does not trigger apoptosis in mature post-mitotic cells. Primary spermatocytes in the pachytene stage seem to be the germ cell type most sensitive to heat (Lue *et al.*, 1999). We also observed several primary spermatocytes in the pachytene stage with nuclear fragmentation similar to apoptosis. In the present study, we observed that pentoxifylline inhibited germ cell apoptosis at 30 and 60 days post-heat shock. This inhibition could have occurred due to the anti-inflammatory properties of pentoxifylline, which increases intracellular cAMP, reduces superoxide anions and inhibits TNF- α , which is responsible for DNA fragmentation (Vadiraja and Madyastha, 2001; Chen *et al.*, 2002; Maxwell *et al.*, 2002). Pentoxifylline can reduce degenerative effects on the testis by inhibiting germ cell apoptosis. According to Teruya *et al.* (2008), pentoxifylline can increase intracellular cAMP in erythrocytes, increase oxygen delivery to ischemic tissues and reduce free radical production. Moreover, pentoxifylline inhibits inflammatory responses such as a reduction of cell activation, phagocytosis, endothelial adhesion and tissue destruction by nitric oxide (Vadiraja and Madyastha, 2001). According to Ji *et al.* (2010), pentoxifylline suppressed TNF- α , IL-1beta, IL-6 and reduced the apoptosis level, transcriptional activating factor NF-kB and the activation of glial cells in the brains of rats exposed to burns. In rabbits submitted to ischemic spinal cord injury, pentoxifylline caused a reduction in serum TNF- α , myeloperoxidase activity, immunoreactivity of PECAM-1 and caspase-3 and the number of necrotic and apoptotic neurons (Zhu *et al.*, 2008).

In somatic cells, heat stress activates a group of genes that encode heat shock proteins. The "turn on" mechanism of these genes occurs due to an interaction between a specific transcription factor, HSF1, and a regulatory sequence called heat shock element (HSE). In higher eukaryote cells, HSF1 is present in non-heat-stressed cells as an active monomer which responds to cellular stress through trimeric aggregation to form a transcription factor for HSP70 proteins, which correct damage or induce apoptosis (Widlak *et al.*, 2003, 2007).

Germ cells exposed to heat stress can develop



repair mechanisms or can be eliminated via apoptosis (Jannes *et al.*, 1998). In the present study, we observed that treatment with 100 mg/kg of pentoxifylline can reduce germ cell apoptosis, reducing the severity of pathological lesions shown in testicular degeneration induced by heat shock. Mice exposed to heat shock showed HSP70 expression in Leydig cells (Cao *et al.*, 2009). Heat shock reduced the synthesis of progesterone and mRNA for steroidogenic regulatory protein (Star) and its activity in Leydig cell tumors of MA-10 mice. These observations provide evidence for a down-regulation in Star expression caused by heat shock and a reduction of testosterone levels (Murphy *et al.*, 2001). In the present study, there was no reduction in plasma testosterone levels. It was possible that the pentoxifylline triggered a mechanism to block the heat damage to Leydig cells and maintained normal testosterone concentrations in the blood.

In conclusion, treatment of adult rats with either 50 or 100 mg/kg of pentoxifylline did not change testicular weight, histomorphometrical parameters or plasma testosterone concentration at 3, 7, 15, 30, and 60 days after testicular heat shock. However, treatment with pentoxifylline inhibited germ cell apoptosis and reduced the severity of pathological lesions at 30 and 60 days after testicular heat shock. The present study demonstrated that pentoxifylline might be an alternative therapy to the harmful effects of heat on spermatogenesis.

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