Protective effects of pentoxifylline on the seminiferous tubules morphology in smoking rats

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Abstract

In this study, we investigated the protective effects of pentoxifylline on the testes of male rats exposed to cigarette smoke. Twenty-seven male Wistar rats were divided into 3 equal groups: Group 1 – control group with only saline (0.9% NaCl) injection for 8 weeks, Group 2 – cigarette smoke + saline for 8 weeks, and Group 3 – cigarette smoke + pentoxifylline for 8 weeks. The rats were killed after 8 weeks; the testes were fixed in paraformaldehyde 4% solution and processed for histological study. Paraffin sections (5 μm) were stained with hematoxylin and eosin. TUNEL method was used to identify apoptotic cells. Immunoreactivity was examined by light microscopy. For statistical analysis, each mean was compared using the Mann-Whitney U-Wilcoxon rank sum W-test and level of significance was taken as p<0.05. Our results showed that the mean number of atrophic seminiferous tubules and apoptotic cells was significantly increased in Group 2, compared to other groups (p<0.05). Degeneration and apoptosis of germ cells were the most prominent findings in Group 2. Focal loss of germinal epithelium was seen locally in group 3, but very mild compared to Group 2. This study showed that cigarette smoke significantly increased tubular epithelial atrophy and apoptosis, which was successfully prevented by pentoxifylline administration. Further studies are needed to clarify clinical usefulness of this agent in smoking individuals.

Key words: Apoptosis; pentoxifylline; seminiferous tubules; smoking, testis.

INTRODUCTION

Several reports have suggested decreasing sperm counts and increase in reproductive disorders in some areas during the past 50 years (Bonde, 2010). Cigarette smoking is a well-known cause of morbidity and mortality from neoplastic and non-neoplastic diseases. The incidence of infertility has been gradually but increasingly related to environmental exposure such as cigarette smoking, etc. (Dorfman, 2008; Foster et al., 2008). Cigarette smoking causes atrophy of normal testicular components and reduction in the number of spermatogenic cells. Chronic exposure of male rats to cigarette smoke is reported to be either directly or indirectly toxic to spermatogenesis (Guven et al., 1999).

Pentoxifylline (PTX) is a methylxanthine derivate that has been used for its regulatory effects on the blood flow. It increases the flexibility of red and white blood cells, reduces the blood viscosity by decreasing plasma fibrinogen concentrations, and decreases the platelet aggregation and thrombus formation (Ward and Clissold, 1987). On the other hand, PTX has been shown to reduce production of collagen, and expression of interleukin-6 and transforming growth factor-beta 1.
(TGFβ1) in rat hepatic stellate cells (Hernández et al., 2002). PTX also inhibits the proliferation of cultured lymphocytes, fibroblasts, and mesangial cells while reducing the production of extracellular matrix proteins (Hewitson et al., 2000). Recent studies have also confirmed the potential antioxidant effects of PTX (Bhat and Madyastha, 2001; Horvath et al., 2002; Lin et al., 2002). It was shown that PTX enhances sperm motility in initiating motility in testicular spermatozoa (Tasdemir et al., 1993).

Pozor et al. (2011) have investigated the effects of PTX on testicular perfusion and sperm production in stallions in miniature horse stallions. They stated that PTX delayed the seasonal decrease of testicular perfusion in stallions, sperm quality and quantity were not significantly affected; perhaps they would have been enhanced by prolonged treatment. So we aimed to evaluate the protective effects of PTX on testis histology of male rats exposed to cigarette smoke.

MATERIALS AND METHODS

Animals

The study was approved by the Institutional Ethics Committee. Twenty-seven male Wistar rats weighing 220 - 260 g were housed under standard conditions (12 h light-dark cycle), with water and standard rat Chow available ad libitum. Animals were divided into 3 equal groups: Group 1; Control group with only intraperitoneal saline (0.9% NaCl) injection for 8 weeks, Group 2; Cigarette smoke + intraperitoneal saline for 8 weeks, and Group 3; cigarette smoke + intraperitoneal PTX (25 mg/kg/day) for 8 weeks. Rats were exposed to cigarette smoke in an air-flow chamber for 3 h daily. The animals were killed after 8 weeks and both testses were excised for histopathological analysis.

Laboratory studies

All the testes were removed and fixed in paraformaldehyde 4% solution and processed for histological study. Paraffin sections (5 μm) were stained with hematoxylin & eosin (H&E) and TACS™ 2 TdT-DAB in situ Apoptosis Detection Kits (Catalog# 4810-30-K, Trevigen) were used to identify apoptotic cells by detecting DNA fragmentation through a combination of enzymology and immunohistochemistry techniques. The enzyme reaction generates an insoluble colored precipitate where DNA fragmentation has occurred. To discriminate apoptotic cells from necrotic cells, the samples are counterstained to aid in the morphological verification of apoptosis. DAB-stained samples are examined using a light microscope.

Immunocytochemistry

Following removal of both testes, tissues were fixed in paraformaldehyde 4% for 16-18 h at 4°C and then postfixed overnight in the same fixative. Testes were embedded in paraffin. Tissue sections (5 μm) were deparaffinized, rehydrated, and incubated with cytoxin (Trevigen, Gaithersburg, MD) for 30 min at 37°C in a humidity chamber, hydrogen peroxide 3% for 5 min at room temperature, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) mixture (TdT and Label solution) for 60 min at 37°C finally counterstained with methylene green. Control sections were stained using the same procedure, but DNase was used instead of TUNEL mixture. Immunoreactivity was examined by light microscopy (BX50F-3; Olympus, Tokyo, Japan).

Quantitative (Histoplanimetrical) and statistic analysis

In each immunostained section, quantification of apoptotic cells was performed in 2.5 mm² fields of seminiferous epithelium with a ×40 objective using an ocular micrometer system (Olympus). Apoptotic germ cells were counted in 100 seminiferous tubules per section. Atrophic tubules were counted in 100 seminiferous tubules for each section. Germinal epithelium of seminiferous tubules were randomly examined per section in group 2 and group 3 compared with the control group. Atrophic tubules were evaluated in 100 seminiferous tubules from each section same as Johnsen’s criteria (Johnsen, 1970).

Data are presented as means with standard deviations (SD). For statistical analysis, each mean was compared using the Mann-Whitney U-Wilcoxon rank sum W-test (SPSS Inc., Illinois, USA) and level of significance was taken as p<0.05.

RESULTS

Control group with only intraperitoneal saline injection for 8 weeks (Group 1)

The seminiferous tubules were composed of Sertoli cells and germ cells in different spermatogenic stages (Figures 1 and 2). The mean numbers of atrophic tubules and apoptotic germ cells were 5.6±3.8 and 1.4 ±0.7, respectively (Table 1).

<table>
<thead>
<tr>
<th>Groups and Seminiferous tubules</th>
<th>Group 1 (Control)</th>
<th>Group 2 (Smoking + saline)</th>
<th>Group 3 (Smoking + PTX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The number of atrophic tubules (mean±SD)</td>
<td>5.6±3.8</td>
<td>24.8±4.9*</td>
<td>17.6±5.4</td>
</tr>
<tr>
<td>The number of apoptotic cells (mean±SD)</td>
<td>1.4±0.7</td>
<td>4.5±1.01*</td>
<td>2.6±1.1</td>
</tr>
</tbody>
</table>

* Statistically significant difference compared to group 1 and group 3; p<0.05. There was no significant difference between group 1 and group 3 in both parameters. PTX: Pentoxifylline.
Cigarette smoke + intraperitoneal saline for 8 weeks (Group 2)

It was observed that while some of seminiferous tubular epithelial cells were normal, most of them were degenerated in the group. Degeneration and apoptosis of germ cells were determined as the most prominent degenerative findings. Within the tubules, there was partial atrophy of germinal epithelium adjacent to the lumen and the degenerated cytoplasm was darkly stained. Separation, desquamation, vacuolization, depletion and apoptosis of germ cells in the seminiferous epithelium were found locally at microscopic examination (Figures 3 and 4). The mean numbers of atrophic tubules and apoptotic germ cells were 24.8±4.9 and 4.5 ±1.01, respectively (Table 1). A significantly increased number of atrophic tubules and apoptotic cells were found in this group compared to Groups 1 and 3 (p<0.05).

Cigarette smoke + intraperitoneal PTX for 8 weeks (Group 3)

Light microscopic evaluation revealed partly degenerative changes of germ cells, such as separation, desquamation, vacuolization and apoptosis of germ cells in seminiferous epithelium. Focal loss of germinal epithelium was seen locally in Group 3, but the loss of germinal epithelium was less than that in Group 2. The mean numbers of atrophic tubules and apoptotic cells were 17.6±5.4 and 2.6±1.1, which were significantly decreased compared to Group 2 (p<0.05) (Figures 5 and 6). Results are summarized in Table 1.

DISCUSSION

Smoking is associated with significant morbidity and mortality. Smoking still remains one of the leading causes...
of preventable death. The health risks associated with cigarette smoking play a part in many diseases (Orth et al., 2000).

Sharpe (2010) has reviewed potential causes involving adverse effects on testis development in perinatal life (primarily effects on Sertoli cell number), which are probably irreversible, or effects on the process of spermatogenesis in adulthood, which are probably mainly reversible. Several lifestyle-related (obesity, smoking) and environmental (exposure to traffic exhaust fumes, dioxins, combustion products) factors have been appeared to negatively affect both the perinatal and adult testes, emphasizing the importance of environmental/lifestyle impacts throughout the life course.

Experimental animal studies suggested that exposure to nicotine, cigarette smoke, and/or polycyclic aromatic hydrocarbons may produce testicular atrophy, block spermatogenesis, and alter sperm morphology (Stillman et al., 1986). Cigarette smoking was found to be associated with impaired spermatogenesis process. The mean diameter of the seminiferous tubules, and the index and number of the Sertoli cells were shown to be reduced, which could indirectly impair spermatogenesis in smoking rats (Ahmadnia et al., 2007). In other studies, the effects of smoking on morphometric changes of the testicular tissue were evaluated, which showed apoptosis and reduction in the number of germ cells, and decrease in the height of germinal epithelium and diameter of the tubules. In our study, the number of atrophic tubules and apoptotic cells increased in Group 2 (smoking and saline) compared to Groups 1 (control) and 3 (smoking + PTX). Ahmadnia et al. (2007) have determined a concurrent reduction in the number of germ cells expose to the rats to the smoke. Their findings were compatible and they stated that cigarette smoke has a rather obvious effect on spermatogenesis in rats which may be due to toxic substances in the cigarette or the histologic reactions due to hypoxemia induced by smoke. They have emitted that although further documentation, especially in humans is required, the potential impact of smoking on fertility in men should be considered in public health education (Rajpurkar et al., 2000; Rajpurkar et al., 2002). Zhang et al. (2009) have also determined that smoking damages spermatogenic epithelia, Leydig cells and Sertoli cells, reduces the testosterone and LH levels, and block the proliferation of spermatogenic cells.

Antioxidant preparations has been examined and compounds identified that are capable of crossing the blood testes barrier and protecting the germinal epithelium and Leydig cells from oxidative stress in experimental animal models. It has been stated that superficial phenomenology that characterizes the clinical investigations in this area in an attempt to (i) gain insights into the underlying causes of oxidative stress in the male reproductive system and (ii) develop optimized antioxidants to treat pathologies arising from an imbalance status of these tissues. The process will be long and difficult but more prospective than the empirical approach that characterizes the current approach to treating the infertile male (Aitken and Roman, 2008).

Spermatozoa must be processed and identified from aspirates, seminiferous tubules or pieces of testicular tissue in andrology laboratory. A motility stimulant such as PTX is commonly used to calculate the number of functionally competent spermatozoa. After recovery, spermatozoa may be used immediately for IVF-ICSI, incubated overnight prior to IVF-ICSI, or cryopreserved for future use (Muller and Pagel, 2013). Aliabadi et al. (2013) have stated that in vitro administration of L-carnitin and PTX to extracted testicular sperm samples led to increased sperm motility and lactate dehydrogenase C4 enzyme activity. They have concluded that application of non-toxic antioxidant, L-carnitin is more suitable for ART protocol than PTX (Aliabadi et al., 2013).
Tesarik et al. (2000) stated that the differentiation-promoting effect of FSH was connected to protection against germ cell apoptosis and that both effects could be mimicked by the intracellular cyclic AMP (cAMP)-elevating drug pentoxifylline. Their data showed that the in-vitro effects of supraphysiological concentrations of FSH on human spermatogenesis were mediated by the classical FSH signal transduction pathway involving cAMP as a second messenger. PTX may thus be useful as an alternative means for intracellular cAMP elevation in men with high circulating FSH concentrations leading to desensitization of the FSH receptor.

According to Rajpurkar et al. (2000) chronic cigarette smoke inhalation is associated with an increase in the level of oxidants and a simultaneous decrease in the level of antioxidants in the rat testis. This abnormality of the oxidant-antioxidant balance may be one of the mechanisms leading to testicular tissue damage and abnormal spermatogenesis in the rat testis following chronic inhalation of cigarette smoke. On the other hand, chronic cigarette smoke induces apoptosis in the rat testis. Apoptosis may be one of the pathogenic mechanisms responsible for defective spermatogenesis in the rat following chronic cigarette smoking (Rajpurkar et al., 2002). The study showed that cigarette smoke clearly induced apoptosis compared to control group in rats. Tubular epithelial atrophy was significantly higher in smoking rats without PTX treatment (Group 2). Taken together, the available information suggests that apoptosis is a result of chronic cigarette smoking in rats.

The mechanism of apoptosis in testis is still under investigation. Nitric oxide is an important mediator of cell death via either apoptosis or necrosis, depending on the intensity and duration of injury. NO is synthesized by three isoforms of NO synthase (NOS), namely, the neuronal, endothelial, and inducible isoforms. NOS isoforms have been shown to regulate a number of functions, e.g., sperm motility and maturation and germ cell apoptosis in the testes. Endothelial NOS (eNOS) and inducible NOS (iNOS) are known to be involved in germ cell apoptosis. Whether iNOS and eNOS use the same pathway to regulate germ cell number is not known, but clearly both are positive regulators of germ cell apoptosis (Zini et al., 1996; Zini et al., 1998; Lee and Cheng, 2004). There could be two reasons for the elevation of tissue NO levels after exposure to cigarette smoke: increased synthesis due to the injury of the vascular endothelium, or the activation of neutrophils in damaged testicular tissue, leading to synthesis of NO (Koltukusz et al., 2000).

Phosphodiesterase inhibitors are shown to be among the most potent iNOS modulator agents. There are at least 11 PDE types, each being fairly selective for either cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP), or catalyzing the degradation of both in a nonspecific fashion (Markovic et al., 2003). Pentoxifylline is a phosphodiesterase inhibitor of the methylxanthine group, which suppresses NO production from interferon γ (IFN γ) - and lipopolysaccharide (LPS) costimulated macrophages. Inhibition of NO production is associated with elevated total cellular cAMP (Beshay et al., 2001). Evidence suggests that cAMP profoundly contributes to the regulation of iNOS gene expression and subsequent NO production. Although in vitro studies were cell specific, both stimulatory and inhibitory effects of cAMP-elevating PDE inhibitors on iNOS expression, the latter seem to prevail in vivo (Markovic et al., 2003). This mechanism may explain the protective effect of PTX during the apoptotic process exposed to cigarette smoke in the study.

Conclusions

The present study showed that cigarette significantly increased tubular epithelial atrophy and apoptosis in rats which may be due to toxic substances or histological changes due to hypoxemia induced by smoke. Nevertheless, PTX administration significantly prevented these negative effects on testicular histological architecture of rats when compared with the cigarette smoke + saline group. PTX is already in clinical use for the treatment of vascular diseases and may find a new application in this context. Further studies are needed to clarify clinical usefulness of this agent in smoking individuals.

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REFERENCES


Bonferroni test for multiple comparisons was used to determine statistical significance. The level of significance was set at p < 0.05.

Results

Pentoxifylline significantly improved sperm motility and fertilization rates in normal and FSH-induced spermatogenic dysfunction. Furthermore, pentoxifylline treatment improved testicular NO levels and reduced testicular injury.

Discussion

Pentoxifylline is a non-selective phosphodiesterase inhibitor that has been shown to have anti-inflammatory and anti-oxidant properties. These properties may be beneficial in the treatment of spermatogenic dysfunction.

Conclusion

Pentoxifylline treatment significantly improves sperm motility and fertilization rates in normal and FSH-induced spermatogenic dysfunction. These findings suggest that pentoxifylline may be a promising treatment option for spermatogenic dysfunction.

References