Protection effects of pentoxifylline on cigarette smoking induced renal tissue damage in rats

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<th>Journal:</th>
<th>Toxicology and Industrial Health</th>
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<td>Manuscript ID:</td>
<td>TIH-10-0036.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>10-Jun-2010</td>
</tr>
<tr>
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<tr>
<td>Keywords:</td>
<td>cigarette, glomerular cell number, glomerular diameter, kidney, pentoxifylline, proximal tubule cell number, rat, smoking</td>
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In this study, we investigated the protective effect of pentoxifylline (PTX) on smoking-induced damage in rat kidney tissues. Twenty-seven male Wistar rats were used in the study. Animals were divided into three equal groups as follows: Group 1- control group with only saline (0.9% NaCl) injection for 8 weeks; Group 2- cigarette smoking and saline injection for 8 weeks; and Group 3- cigarette smoking and PTX injection for 8 weeks. The rats were sacrificed after 8 weeks and their kidneys were excised for histopathological analysis. Serial paraffin sections (5 µm) of the kidneys were cut and stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) method was used to assess apoptosis. Glomerular diameters, glomerular cell number and proximal tubule cell numbers were compared between the groups. Our results showed that PTX treatment prevented negative effects of smoking in rat kidneys. There was a statistically significant difference in all assessed parameters between Group 2 and other groups (p< 0.05). In conclusion, our study shows that PTX treatment is effective in preventing the negative effects of cigarette smoking on kidneys by inhibiting cell damage with its antioxidant properties.

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Abstract

In this study, we investigated the protective effect of pentoxyfilline (PTX) on smoking-induced damage in rat kidney tissues. Twenty-seven male Wistar rats were used in the study. Animals were divided into three equal groups as follows: Group 1- control group with only saline (0.9% NaCl) injection for 8 weeks; Group 2- cigarette smoking and saline injection for 8 weeks; and Group 3- cigarette smoking and PTX injection for 8 weeks. The rats were sacrificed after 8 weeks and their kidneys were excised for histopathological analysis. Serial paraffin sections (5 μm) of the kidneys were cut and stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) method was used to assess apoptosis. Glomerular diameters, glomerular cell number and proximal tubule cell numbers were compared between the groups. Our results showed that PTX treatment prevented negative effects of smoking in rat kidneys. There was a statistically significant difference in all assessed parameters between Group 2 and other groups (p< 0.05). In conclusion, our study shows that PTX treatment is effective in preventing the negative effects of cigarette smoking on kidneys by inhibiting cell damage with its antioxidant properties.

Keywords: cigarette; glomerular cell number; glomerular diameter; kidney; pentoxifylline; proximal tubule cell number; rat; smoking

INTRODUCTION
Cigarette smoking is a well known cause of morbidity and mortality from neoplastic and non-neoplastic diseases. In the early 1980’s kidneys were proved to be another target organ of smoking [1]. Epidemiologic studies suggest that smoking worsens the progression of renal injury in patients with glomerular diseases [2]. The correlation between smoking and a deterioration of renal function in patients with chronic renal and vascular diseases is presented in several clinical studies [3, 4]. Jaimes and colleagues have shown that stable compounds present in cigarette smoke may cause endothelial dysfunction by increasing the vascular production of reactive oxygen species (ROS) [5, 6].

Pentoxifylline (PTX) is a methylxanthine derivative and a nonspecific type 4 phosphodiesterase inhibitor which is used in the treatment of peripheral vascular diseases [7]. Its pharmacological mechanisms are not completely understood. However, PTX has been shown to reduce the production of collagen, the expression of interleukin-6, and the expression of transforming growth factor-beta 1 (TGFβ1) in rat hepatic stellate cells [8]. PTX also inhibits the proliferation of cultured lymphocytes, fibroblasts, and mesangial cells while reducing the production of extra cellular matrix proteins [9]. Recent studies have also confirmed the potential antioxidant effects of PTX [10-12].

Here, in this study we investigated the protective effects of PTX treatment on cigarette-induced histological changes in the kidney sections of rats.

METHODS

a) Animals
The study was approved by the Institutional Ethics Committee. Twenty-seven male Wistar rats weighing 220-260g, were housed under standard conditions (12h light/dark cycle), with free access to water and food ad libitum. Animals were divided into three equal groups as follows: Group 1- control group with only saline (0.9% NaCl) injection for 8 weeks; Group 2- cigarette smoking and saline injection for 8 weeks; and Group 3- cigarette smoking and PTX injection for 8 weeks.

PTX (25 mg/kg/day) and saline were administered intraperitoneally. Rats were exposed to cigarette smoke in an air-flow chamber for 3 h per day, 5 days per week.

The rats were sacrificed after 8 weeks and their kidneys were excised for histopathological analysis.

b) Histology and immunocytochemistry

Fixed kidneys were removed and post-fixed in the same fixative overnight. The organs were embedded in paraffin. Serial paraffin sections (5 µm) of the kidneys were cut and stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS).

Paraffin-embedded sections of 5 µm were used to calculate the glomerular cell numbers (GCN) stained with H&E. Ten different glomeruli in the peripheral cortex of each of the seven sections of each group were randomly selected and were examined under a light microscope at a magnification of x40. The nuclei of all the cells in all groups were counted. PMNL, congestion and interstitial edema were determined in the renal tissue at the magnifying area stained with H&E.
The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) method was used to assess apoptosis according to the manufacturer’s recommendations (Trevigen, Inc.TACS 2 Tdt DAB In Situ Apoptosis Detection Kit, Catalog #4810-30-K). TUNEL staining was examined by a light microscope (BX50F-3; Olympus, Tokyo, Japan). Formalin-fixed and serial paraaffin-embedded 5-µm-thick tissue sections were deparaffinized and dehydrated. Nuclear proteins were stripped from the DNA by incubating in proteinase K for 30 minutes and endogenous peroxidase was blocked with hydrogen peroxide. Sections were incubated in a buffer containing TdT and digoxigenin-labeled dUTP, followed by digoxigenin-conjugated peroxidase treatment. Diaminobenzidine was used as chromogen and the background was stained with methyl green. Positive and negative controls were included in each set of experiments. Human tonsil sections served as a positive control. Brown-labeled TUNEL-positive apoptotic cells in the glomeruli and tubulointerstitium were counted independently by two investigators in 10 fields at a microscopic magnification of x400. Apoptotic cells were identified by the presence of various types of chromatin condensation (perinuclear ring formation, patches, or apoptotic bodies), whereas cells showing diffuse cytoplasmic labeling were considered as necrotic cells and were not counted.

The diameters of each glomeruli were measured using University of Texas Health Science Center at San Antonio (UTHSCSA) image tool software. Two different (the greatest and the smallest) diameters of each glomeruli were measured and the average diameter was calculated.
The number of apoptotic cells was quantified in 0.5 mm$^2$ of the rat kidneys with a X40 objective using an ocular micrometer system (Olympus). Apoptotic cells were counted in selected sections independently of each renal region including the cortex and the medulla by each of two investigators, who were blinded to the group. Apoptotic cells were counted in 10 sections ($n=10$) for each renal region ($n=10$) in all groups.

c) Statistical Analysis

The data obtained from apoptotic cells were statistically analyzed using the SPSS statistical software package. One way ANOVA and Tukey’s HSD tests were used to determine the significant differences between group means. Data were expressed as mean ± standard error of the mean (S.E.M.). The statistical significance was determined as $p<0.05$.

RESULTS

**PTX prevents the negative effects of smoking on glomerular diameter**

Mean glomerular diameter (GD) was significantly smaller in Group 2 (Cigarette+SF) compared to the other groups (Table 1, Figure 1). Although cigarette smoke affected glomerular diameters negatively, the mean glomerular diameter did not change in Group 3 (Cigarette+PTX) compared to SF control group (Group 1) (156.92 ± 36.14 vs. 159.10 ± 25.80 µm). There was a statistically significant difference between Group 2 and Group 3 ($p<0.0001$).

**PTX prevents the negative effects of smoking on glomerular cell number**
The comparison of the mean GCN between Group 1 (67.2 ± 4.57), Group 2 (55.9 ± 1.59) and Group 3 (64.4 ± 3.97) showed that smoking led to a decrease in the number of cells but PTX treatment prevented this negative effect (Table 1). There was a statistically significant difference between Group 2 and the other groups (most importantly Group 3).

**PTX prevents the negative effects of smoking on proximal tubule cell number**

There was a significant difference between Group 1 and Group 2, in terms of the number of proximal tubule cells (PTCN) in the renal cortex ($p < 0.0001$). The comparison between Groups 2 and 3 showed a statistically significant difference ($p = 0.0001$). It was found that cigarette smoking reduces PTCN, but PTX treatment protects proximal tubule cells from the negative effects of smoking (Table 1).

**H&E, PAS and TUNEL findings**

There was more polymorphonuclear leukocyte (PMNL) accumulation, edema and congestion in Group 2 compared to Groups 1 and 3. Light microscopic histological examinations of the kidneys isolated from Group 2 showed focal glomerular mesangial proliferation, mild interstitial inflammation and tubular hydropic degeneration at the time of the sacrifice. PAS-positive material was detected in different sites of the kidney, i.e. brush border of proximal tubules, proximal tubule cells' cytoplasm, distal tubule cells' cytoplasm, glomerul, basal cell border of proximal tubules and the interstitial cells in all groups. PAS-positive reaction was due to carbohydrates and there was no difference between the three groups.
There were significantly more apoptotic cells in Group 2 than in Group 3 (Figure 2). The SF control group showed no significant apoptosis in the renal cortex.

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 various diseases [13]. The relationship between smoking and kidney pathology is still under investigation. The acute effect of chronic smoking on the kidney is mediated via sympathetic activation [14]. Endothelial cell damage appears to play a major role. Other complementary possibilities such as altered prostaglandin metabolism, diminished nitric oxide and increased endothelin production, increased interaction with and aggregation of platelets, PMNL, monocytes, as well as hypoxic damage from carbon monoxide were all published in literature [13, 15]. The negative effects of smoking on GD were previously shown by Dündar et al. They used healthy newborn rats and concluded that passive smoking might have a negative effect on renal morphometry and body growth [16].

Prevention of renal pathology in rats exposed to cigarette smoke was studied by Kurus et al. They found that some morphological alterations in rat kidney might be prevented by resveratrol [17]. PTX is a nonspecific phosphodiesterase inhibitor that has been prescribed for the treatment of peripheral vascular disease and a number of other conditions involving defective regional microcirculation due to its regulatory effects on blood flow [18]. Cytokines, especially TGFβ1 has been shown to play a pivotal role in promoting fibrosis at various sites including the kidney, heart and blood vessels [19].
the other hand, TNF-α has been implicated in the pathogenesis of many inflammatory and toxic diseases of the kidney, exerting a broad range of biological effects, including vasoconstriction, increase in oxidative stress, reduction of glomerular blood flow and glomerular filtration rate and induction of the synthesis of pro-inflammatory cytokines resulting in tubular dysfunction, cell apoptosis or death [20]. PTX may reverse renal functional alterations with its proven decreasing effects on TNF-α and TGFβ [8, 21].

The present study was conducted to evaluate the protective effects of PTX on the kidneys of rats which were exposed to cigarette smoke. Our results revealed that PTX had a significant protective effect on GD. There was a marked decrease in GD of smoking rats without PTX administration, compared to other groups. This negative effect was successfully prevented by PTX treatment. The mean GD of smoking rats with PTX treatment was similar to sham control rats.

A similar effect was observed in the evaluation of mean GCN. The rats in Group 2 had least mean GCN compared to the other groups and statistical analysis showed a significant difference between this group and the other two groups (p< 0.001 between 1 and 2; and p=0.0001 between 2 and 3). These results suggest that smoking significantly reduced glomerular cell numbers in affected kidneys while cell numbers were preserved in rats under PTX treatment.

Tubular atrophy and tubulo-interstitial fibrosis in kidneys of smoking rats were shown previously [17]. We also observed a significant decrease in number of proximal tubule cells in smoking rats which did not receive PTX. However, mean PTCN was similar between Groups 1 and 3 (p = 0.6794). These results showed that PTX treatment prevented the negative effects of smoking on PTCN.
CONCLUSION

Our study shows that PTX treatment successfully prevents the negative effects of cigarette smoking on kidney glomeruli by inhibiting cell damage with its antioxidant properties. Prevention of renal disease is the most important and effective strategy to decrease the prevalence and incidence of chronic kidney disease and end stage renal disease. Further studies, particularly clinical studies are necessary to establish successful protocols in humans.

Funding: This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

REFERENCES


Table 1: Comparison of parameters between three groups

<table>
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<tr>
<th>Parameter</th>
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<tr>
<td>Mean glomerular diameter (µm)</td>
<td>159.10 ± 25.80</td>
<td>98.63 ± 14.27*</td>
<td>156.92 ± 36.14</td>
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<td>Mean glomerular cell number</td>
<td>67.2 ± 4.57</td>
<td>55.9 ± 1.59*</td>
<td>64.4 ± 3.97</td>
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<td>Mean tubule cell number</td>
<td>7.6 ± 0.70</td>
<td>6 ± 0.66*</td>
<td>7.3 ± 0.48</td>
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* Statistically significant difference (p<0.05) compared to the other two groups
Figure 1. Photomicrographs of kidney sections of rats from 3 groups (Hematoxylin/eosin x40). (A) SF control group (B) Cigarette + SF group; note smaller glomerular diameters (C) Glomerular diameters did not change significantly in Cigarette + PTX group.

73x18mm (300 x 300 DPI)
Figure 2. Photomicrographs of TUNEL staining (X40). (A) Cigarette + SF group; black arrow indicates apoptotic cells. (B) Cigarette + PTX group; significantly less apoptotic cells.