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Histomorphometric and Hormonal Studies on the Protective Role of Testosterone in Nicotine-Induced Testicular Toxicity

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ABSTRACT
The consumption of nicotine through smoking is a serious public health issue. Nicotine is associated with infertility and the alteration of spermatogenesis and metabolism of male reproductive hormones. Testosterone propionate is a testosterone ester with great potency relative to testosterone. This study was initiated to investigate the protective role of testosterone in nicotine-induced testicular toxicity. Eighteen (18) adult Wistar were randomly divided into three (3) groups as follows: Group A: Nicotine only (N); Group B: Nicotine + Testosterone (NT); and Group C: Normal Control (NC). 0.8 mg/kg body weight of nicotine and 2.5 mg/kg of testosterone were administered respectively for a period of 30 days after which the rats were sacrificed and the testes fixed for histological and histomorphometrical analysis. The reproductive hormones, FSH, LH and testosterone were also assayed.
Histological sections of the testes of N rats revealed desquamated cells, atrophic germinal epithelium, thickened basement membrane, congested blood vessels, depleted lumina and reduced tubular diameter as well as increased luminal diameter compared to the normal control. The reproductive hormones were also significantly reduced compared to the NT and control groups. These deleterious effects were however mitigated in the treated rats. These findings indicate that testosterone ameliorates the severity of toxicity induced by nicotine in rat testes.

Keywords: *Nicotine, Testosterone, Histomorphometry, Germinal epithelium, Infertility*

INTRODUCTION

Nicotine is regarded as the primary chemical in tobacco that is responsible for endangering tobacco use and dependence[1]. It is a pharmacologically active and addictive alkaloid component of the cigarette smoke[2]. It is a widely used licit drug with high toxicity and is absorbed rapidly through the gastrointestinal tract, respiratory tract, mouth mucosa and skin. About 80-90% of it is metabolized in the liver with the kidney and lungs taking care of the rest[3]. Cotinine is the main metabolite with a long half-life. Both nicotine and the cotinine affects spermatogenesis adversely often due to the oxidative stress produced by free oxygen radicals[4].
Nicotine has been reported to inhibit follicular stimulating hormone (FSH) and luteinizing hormone (LH) release from pituitary gland[5].Studies have also shown that nicotine affects spermatogenesis[6], significantly reduce semen quality and adversely affects hypophysis-gonadal hormone axis[7]. Other researches have also shown that both nicotine and its metabolite, cotinine decrease rostenedione and testosterone concentrations in rats by competitive inhibition of multiple stages in testosterone biosynthesis[8].
The use of nicotine especially through smoking poses serious health problems notable among which is reduced fertility[9]. This study was conducted to investigate the differential effects of testosterone on testicular histology and reproductive hormone profiles of rats secondary to induced nicotine toxicity.

MATERIALS AND METHODS

Eighteen (18) healthy male Sprague-Dawley rats were used for this study. The rats, weighing between 160-220g were housed in standard well-ventilated metal cages (5 animals per cage), in the rat control room of the Department of Anatomy. They were exposed to 12 hour-light and 12 hour-dark cycle, relative humidity 50-55% and a temperature range of 26 to 28^{0}C. The animals had access to rat chow (Livestock feeds Plc. Lagos, Nigeria) and tap water *ad libitum*.They were allowed two weeks of acclimatization after which the animals were divided randomly into 3 groups of 6 rats each as follows: Group A: Nicotine only; Group B: Nicotine + Testosterone and Group C: Normal control. 0.8 mg/kg body weight of nicotine (Merk) and 2.5 mg/kg of testosterone (Merk) were administered respectively were administered intraperitoneally and testosterone for a period of 30 days after which the rats were killed and the testes fixed for histological analysis. All procedures involving animals in this study conformed to the guiding principles for research involving animals as recommended by the Guiding Principles in the Care and Use of Animals[10].

TISSUE PROCESSING

At the end of the experiment, the rats were killed and cut open by abdominopelvic incision. Thereafter, the testes were excised, trimmed of all fat and blotted dry. They were then weighed using a sensitive scale and thereafter fixed in 10% formo-saline. The fixed tissues were transferred to

graded series of ethanol and then cleared in xylene. The tissues were then infiltrated in molten paraffin wax in the oven at 58^0C. Serial sections of 5 µm thick were obtained from a solid block of tissue, fixed on clean slides and stained with haematoxylin and eosin stains.

HISTOMORPHOMETRY

Histomorphometric analysis of the testes was carried out on the H&E slides at magnification of x 200 using the computer assisted image analysis programme (Image J). For each testis, five sections from were sampled over the whole specimen, using a systematic random scheme[11]. The underlisted morphometric parameters were taken:

1) Diameter of seminiferous tubules: The mean diameter (*D*) was derived by taking the average of two diameters, D_1 and D_2 and D_1 and D_2 were taken only when $D_1/D_2 \geq 0.85$.*D* was obtained by dividing the obtained diameter by the magnification[12]

2) Cross-sectional area (A_c) of the seminiferous tubules: Cross-sectional area (A_c) of the seminiferous tubules $=\pi D^2/4$, where π is equivalent to 3.142 and D is the mean diameter of the seminiferous tubules [12]

3) Germinal epithelium thickness: the thickness of the germinal epithelium was measured from its base to its free surface[13] and was obtained by dividing the measured epithelial thickness by the magnification.

4) Number of profiles of seminiferous tubules in a unit area (N_A): The number of profiles of seminiferous tubules per unit area was determined by using the unbiased counting frame concept[11].Using this frame in addition to counting profiles completely inside the frame, all profiles inside the frame, provided they did not touch or intercept the forbidden line or exclusion edges, were counted.

5) Numerical density (N_v) of seminiferous tubules: This is the number of profiles per unit volume and was determined by using the modified Floderus equation: $N_v=N_A/(D+T)$, where N_A is the number of profiles per unit area, D is the diameter and T is the average thickness of the section[14]

6) Volume, *V* of the seminiferous tubules was determined by the Cavalierian principle which states that the volume of a serially sectioned structure is equal to the product of the slice areas and slice thickness[15] Hence $V=A \times T$, where T = 5 microns.

HORMONAL ASSAY

Plasma FSH, LH and serum testosterone were determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (AccuBind, USA). Absorbance was read at 450 nm in a Plate Reader (Biotek, China).

RESULTS

Histology

Histological sections of the testes of N rats revealed desquamated cells, atrophic germinal epithelium, thickened basement membrane, congested blood vessels, depleted lumina and reduced tubular diameter compared to the normal control. The testes of NT treated rats showed features that were comparable to the normal control: intact basement membrane, orderly array of spermatogenic cells that constitute the germinal epithelium, abundant luminal spermatozoa.

Histomorphometry

Data on histomorphometric analysis of the seminiferous tubules are presented in Table 1.

NT and Control groups were significantly wider in tubular diameter (229.45 ± 5.03 µm, 235.10 ± 6.05 µm) compared with the N group (180.35 ± 5.19 µm) at $P<0.05$ (Table 1).

The germinal epithelia thickness of NT and Control rats were significantly different from the N group at $P<0.05$ (55.10 ± 0.04 µm, 55.40 ± 2.08 µm vs 23.06 ± 6.10 µm).

The cross-sectional area A_C in the Nicotine (N) group (24.31± 1.01 μm^2) was significantly smaller ($P< 0.05$) compared to those recorded for the NT and control groups (40.16 ± 0.46 μm^2 and 42.51 ± 1.10 μm^2). The volume *V* for the control (131.80± 6.45 μm^3) and treated groups A and B (114.60 ± 9.42 μm^3 and 138.74 ± 5.75 μm^3) was also significantly higher ($P< 0.05$) compared to the N group (76.10± 4.20 μm^3).

However, the number of profiles per unit area (N_A) and the numerical density (N_v) recorded in the N group were significantly higher ($P< 0.05$) than observed in the two other groups (Table 1).

Hormone Profile

Data on reproductive hormones are presented in Table 2. The concentration of FSH in the N group (2.90 mIU/ml), was significantly lower than recorded for the NT (8.10 mIU/ml) and control groups at $P < 0.05$ (8.05 mIU/ml).

The concentration of LH level was also significantly lower in the N group (3.71 mIU/ml) compared to NT (4.90 mIU/ml) and control group (5.41 mIU/ml) at $P < 0.05$.

There was a statistical difference in the testosterone concentration of group N compared to the NT and control group. However, the testosterone concentration of the NT group was significantly higher than observed in the N group and Control at $P < 0.05$.

Table 1: Effects of administration of nicotine and testosterone on the size of the seminiferous tubules of Wistar rats

Parameters	N	NT	Control
D (µm)	180.35 ± 5.19*	229.45 ± 5.03	235.10 ± 6.05 $^{+}$
G_E (µm)	23.06 ± 6.10*	55.10 ± 0.04	55.40 ±2.08^{+}
Lumen (µm)	131.01 ± 8.46*	114 ± 2.95 [a]	117.10±3.41^{+}
A_C (μm^2) x 10^3	24.31± 1.01*	40.16 ± 0.46	42.51± 1.10
V (μm^3) x 10^{-3}	76.10± 4.20*	136.85 ± 8.34	131.8± 5.75^{+}
N_A (μm^{-2}) x 10^{-2}	5.17 ± 0.01*	1.58 ± 0.05	1.11± 0.01^{+}
N_V (μm^{-3}) x 10^{-3}	14.2 ±0.6*	4.70 ± 0.25	5.25±0.14^{+}

Values are mean ± SEM, n= 6
**:significantly different from Group NT and Control (P<0.05)*
+:significantly different from NT(P<0.05)

D: mean diameter of seminiferous tubule

G_E: germinal epithelium

A_C: cross-sectional area

V: volume

N_A: numerical area

N_V: numerical volume

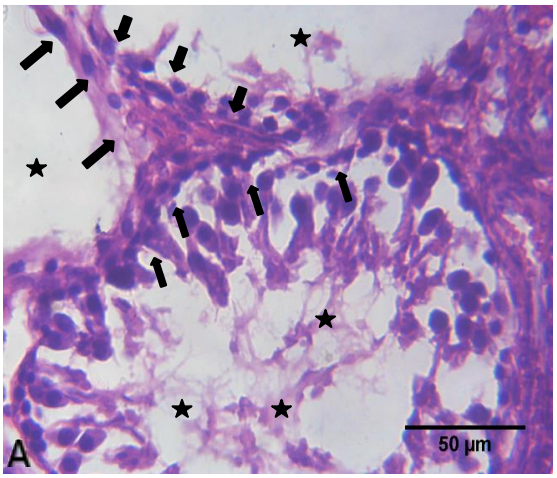
Table 2: Effects of nicotine and testosterone on the reproductive hormones of experimental rats

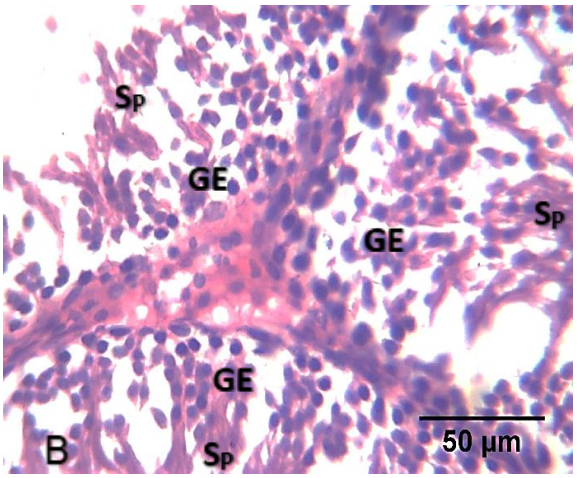
Groups	FSH (mIU/ml)	LH (mIU/ml)	Testosterone (ng/ml)
N	2.90*	3.71*	0.74*
NT	8.10	4.90	1.5+
Control	8.05	5.41	0.91

Values are mean ± SEM; n=6 in each group

**Significantly different from Group NT and Control at P <0.05*

+Significantly different from N and Control at P<0.05





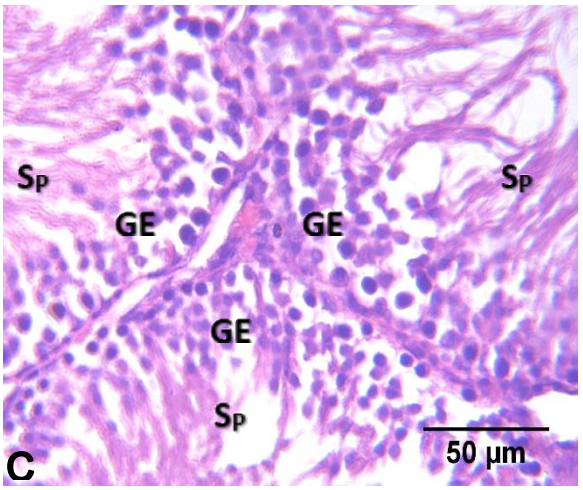


Figure 1. Micrographs of histological sections of experimental rats. Stain: H&E. Magnification: 50 µm.
1A: Stars indicate widened luminal diameter; arrows indicate atrophied germinal epithelium in N rats. 1 B & C: Observe the thick germinal epithelium (GE) in NT and Control (C) rats as well as abundant spermatogonia (Sp) in the lumina.

DISCUSSION

This study showed that the administration of nicotine had negative impacts on the structure and function of the seminiferous tubules. Nicotine-treated rats featured desquamated cells, atrophic germinal epithelium, thickened basement membrane, congested blood vessels.

Moreover, cross-sections of the seminiferous tubules in the nicotine-only group exhibited a severe edema in interstitial connective tissue, decreased Leydig cells distribution and reduced spermatid volume (Table 1 and Figure 1A). Histomorphometric analyses showed that nicotine resulted in tubular depletion and germinal epithelium dissociation, depleted lumina and reduced tubular diameter as well as increased luminal diameter compared to the normal control (Figure 1A).

In this study, nicotine-only (N) rats showed wide, empty lumina with very few spermatids. This could result to infertility. Infertility is a serious health challenge among couples of child-bearing age and approximately half of known causes of primary infertility are now attributes to male factor. Recently, tobacco consumption has been documented to act as an endocrine disruptor on the male hormone profile, specifically on LH, testosterone, and prolactin levels[16]

The few spermatids observed in this study as well as the reduction in the plasma testosterone concentration of nicotine-treated rats indicates that nicotine has an adverse effect on spermatogenesis by reducing the role of testosterone. This agrees with previous studies that indicate the nicotine-reduced gonadotropins secretion is partially responsible for sharp reduction in testosterone level [17,18]

The germinal epithelium is the site of spermatogenesis and is sensitive to a variety of toxic agents, making it a major target for cytotoxic substances. Degenerating cells and the formation of multi-nucleate giant cells by coalescence of spermatids has been reported to be a prominent feature of these toxic effects [19] and is an indication of spermatid failure.

Similar reduction in tubular diameter was reported in adult STZ-diabetic wistar rats [20]prepubertal STZ-diabetic rats [21] and in the offsprings of diabetic female rats [22]

Reduction in the germinal epithelium impaired the process of spermatogenesis, resulting in a fewer luminal spermatozoa and a wider tubular lumen as seen in this work. It is noteworthy that whereas spermatogonia form the peribasal cells of hyperglycaemic testes, the epithelia and lumen still featured scanty spermatozoa compared to the treated and control groups. This suggests that the deleterious effect of hyperglycaemia on the testes of this group was not a complete cessation of spermatogenesis but rather suspension of spermatogenesis. This agrees with the report of a major reduction in the germinal epithelial thickness, dispersion of the germinal cells as well as spermatogenesis arrest in prenatal ethanol-exposed rats[23].

The administration of testosterone was associated with improved reproductive hormone profile and seminiferous tubule cytoarchitecture in nicotine-treated rats. Histological sections and Histomorphometric features of the NT rats were similar to the control, exhibiting a general improvement over the N rats.

The study showed that exogenous testosterone ameliorates the negative effects of nicotine toxicity in the male rat.

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