Original Article

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DOI: 10.4103/jeca.jeca_10_19

Testosterone propionate ameliorates oxidatve stress and inflammation in nicotine-induced testicular toxicity

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Abstract:

BACKGROUND: Nicotine (NICO) is a major constituent of cigarette smoke and has been associated with adverse effects on the testes and male reproductive profile.

AIMS AND OBJECTIVES: This study was initiated to investigate the effects of testosterone (TES) propionate in NICO-induced testicular toxicity in rats by investigating the quantitative localization and intensity of immune expression of cyclooxygenase-2 (COX-2) and Ki-67.

MATERIALS AND METHODS: Eighteen adult Wistar rats were randomly divided into three groups as follows: Group A: NICO only; Group B: NICO+TES propionate (NICO+TES); and Group C: Normal Control. 0.8 mg/kg body weight of NICO and 2.5 mg/kg of TES propionate were administered, respectively, for 30 days after which the rats were sacrificed, and the testes were processed for antioxidant enzyme assay and immunohistochemical analysis.

RESULTS: Immunohistochemical study showed elevated COX-2 immunoexpression in the germinal epithelium of the NICO group relative to the NICO+TES and control groups. Ki-67 was expressed in the spermatozoa of all experimental groups. The primary spermatocytes of NICO+TES and control groups additionally tested positive for Ki 67. The results also showed a higher level of oxidative stress markers in the NICO group compared to the NICO+TES and control groups.

CONCLUSION: These findings indicate that NICO toxicity in the testes is mediated through inflammation and apoptosis as well as induction of oxidative stress; and that TES propionate ameliorates the severity of toxicity induced by NICO in rat testes by reducing the inflammation and oxidative stress.

Keywords:

Apoptosis, germplasm, immunoreactivity, infertility, oligospermia, proliferation

Introduction

Nicotine (NICO) is the active metabolite in cigarette smoke (Seema *et al.*, 2007). The consumption of NICO has been associated with negative parameters on the testes and reproductive hormones (Kavitharaj and Vijayammal, 1999; Aydos *et al.*, 2001). Severe inhibition of antioxidant enzymes glutathione peroxidase (GPx), glutathione (GSH) reductase, and glucose-6-phosphate dehydrogenase has been reported in rats chronically exposed to NICO (Jana *et al.*, 2010).

Androgens, produced by the testis and the adrenal glands, play a pivotal role in male reproductive and sexual function. The main testicular androgen, testosterone (TES), is produced by Leydig cells under the stimulation of pituitary luteinizing hormone (LH). LH secretion, in turn, is regulated by hypothalamic gonadotropin-releasing hormone, the most

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How to cite this article: Ukwenya VO. Testosterone propionate ameliorates oxidatve stress and inflammation in nicotine-induced testicular toxicity. J Exp Clin Anat 2019;18(1):74-78.

proximal effector of the hypothalamic-pituitary-gonadal axis. LH, together with testicular auto and paracrine factors is responsible for the regulation of balanced sex hormone production and gametogenesis.

The treatment of hypogonadism consists of TES replacement to reverse the symptoms of androgen deficiency while achieving normal range TES levels. In 1960s, androgens were used as stimulatory or rebound therapy for idiopathic oligo/asthenozoospermia (McCullagh, 1939). The treatment consisted of two different strategies: first, androgens were administered in a form and dose that did not influence pituitary gonadotropins secretion to stimulate the spermatogenesis or influence the sperm transport and maturation through an effect on the epididymis, ductus deferens, and seminal vesicles; second, androgens were used to suppress gonadotropins and spermatogenesis with a rebound effect after stopping the therapy (Dohle *et al*. 2003).

TES propionate is an androgen and anabolic steroid medication which is used as hormonal therapy in men with low-TES levels (Nieschlag, 2010; Llewellyn, 2010). Its other use include the treatment of breast cancer in women (Bolour and Braunstein, 2005).

There is a dearth in literature on the effects of NICO on key immune markers of inflammation and cell proliferation, *viz a viz* cyclooxygenase-2 (COX-2) and Ki-67. This research was done to investigate the differential effects of NICO and TES on COX-2 and Ki-67 and antioxidant defense system in rats.

Materials and Methods

Eighteen healthy male Wistar rats were used for this study. They were housed in plastic cages and maintained in an adequately ventilated room with 12 h-light and 12 h-dark cycle, relative humidity 50%–55%, and a temperature range of 26°C–28°C. The animals were fed rat chow and tap water *ad libitum.* After 2 weeks of acclimatization, they were divided randomly into three groups of six rats each as follows:

- Group A – NICO only
- Group B – NICO + TES propionate (NICO + TES) and
- Group C – Normal control.

0.8 mg/kg body weight of NICO (Merk) and 2.5 mg/kg of TES propionate (Merk) were administered intraperitoneally and intramuscularly, respectively, for 30 days after which the rats were sacrificed, and the testes were homogenized in icecold 0.1M Tris-HCl buffer (pH 7.4) and centrifuged at 10,000 × *g* in 4°C for 10 min, and the supernatant was collected. Superoxide dismutase (SOD), GPx, and malondialdehyde (MDA) were assayed as described below.

Anti-oxidant assays

Lipid peroxidation

Lipid peroxidation was evaluated according to the method of Buege and Aust. Thiobarbituric acid reactive substances (TBARS) assay is based on the reaction of TBARS with TBA to form a pink-colored product (Okawa *et al*., 1979). The optical density of the pink-colored product measured at 535 nm is directly proportional to TBARS concentration in the sample.

Superoxide dismutase

The activity of SOD was assayed using the method described by Paoletti and Mocali. This method consists of a purely chemical reaction sequence, which generates superoxide from molecular oxygen in the presence of ethylenediaminetetraacetic acid, manganese (II) chloride, and mercaptoethanol. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidation is linked to the availability of superoxide anions in the medium. The decrease in absorbance at 340 nm was monitored for 20 min at 5-min interval in a spectrophotometer. In this assay system, one unit of SOD activity is defined as the amount of enzyme required to inhibit the rate of NADPH oxidation of the control by 50%.

Glutathione peroxidase

The activity of GPx was determined by the method of Rotruck *et al.*, 1973 GPx converts reduced GSH into the oxidized form using hydrogen peroxide during its reaction. The amount of GSH utilized is estimated by measuring it in the assay mixture before and after the enzyme activity. GSH reacts with 5,5′-dithiobis (2-nitrobenzoic acid) to give a yellow color, which was then measured at 412 nm.

Enzyme immunohistochemistry

Ki-67 protein

Paraffin sections were mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma, Mu¨nchen, Germany). After deparaffinization and rehydration, sections were stained with the monoclonal antibodies PC10 (Dakopatts, Hamburg, Germany) against Ki-67 protein using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method (Cordell *et al*., 1984) after the sections were pretreated by the antigen retrieval technique using microwaves. Sections were then microwaved for 25 min at 1000W in sodium citrate buffer (pH 6.0), and after cooling, sections were incubated with the undiluted primary antibody for 1 h. Sections were then exposed twice to the secondary antibody (alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin G, 1:25; Dakopatts, Hamburg, Germany), followed by APAAP (1:50; Dakopatts) for 30 min each (first incubation), and for 10 min each (second incubation). After each incubation, sections were thoroughly washed with Tris-buffered saline (pH 7.4).

Cyclooxygenase-2

Sections were incubated in 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity after undergoing deparaffinization and rehydration. Maximum heat was applied in a microwave for 10 min in citrate buffer solution to reveal antigens after being washed with phosphate buffer saline (PBS). Blocking solution A (Invitrogen Histostain plus Broad Spectrum [3-Amino-9-Ethylcarbazol [AEC]) was dripped onto the sections to reduce nonspecific binding of antibodies. Sections were then incubated with primary and anti-COX-2 (M-19, sc-1747, Santa Cruz) diluted to 1:200 in PBS at room temperature for 1 h. Only, PBS was dripped on the tissues of the negative control group. Streptavidin-biotin peroxidase technique was used after primary antibody incubation. For this purpose, a broad-spectrum antibody (Invitrogen Histostain-plus broad spectrum [AEC]) antibody, counter to the type produced by the primary antibody, was added onto sections, and left at room temperature for 15 min. Streptavidin-horseradish peroxidase (Invitrogen Histostain-plus broad spectrum [AEC]) was dripped on the sections, and then incubated at room temperature for 15 min. After adding AEC staining kit solution, the sections were placed under a light microscope to check for immunoreactivity; the reaction was inactivated with distilled water in accordance with the immunoreactivity status. It was then counterstained in Gill's hematoxylin for 10 s.

The slides were examined and photomicrographed with a high-definition digital camera Leica ICC50 (Leica Microsystems) mounted on a microscope Leica DM750 (Leica Microsystems).

Results

Cyclooxygenase-2

Sections from NICO group showed intense immunoreactivity within the germplasm containing the spermatogonia and the germinal epithelium containing cells of the spermatogenic series [Figure 1]. The NICO+TES group showed moderate immunolabeling comparable to the normal control group.

Ki67

Ki-67-positive immunostaining was restricted to the peribasal cells in the NICO group, whereas in NICO+TES and control groups, both spermatogenic cells and primary spermatocytes stained positive [Figure 2]. Overall, the plate sections of the NICO group presented fewer Ki-67 cells when compared to the control and NICO+TES groups. Furthermore, the germinal epithelium in the NICO group is eroded and not of constant thickness across sections when compared to the control group.

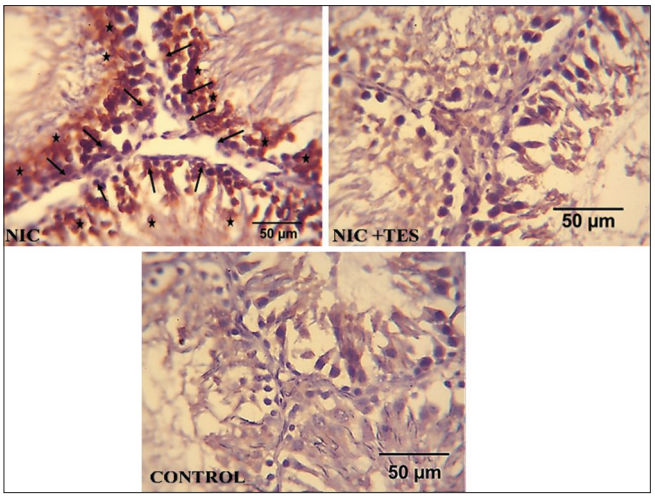

Figure 1: Sections showing localization and intensity of cyclooxygenase-2 in the seminiferous tubules of experimental rats. Observe the positive immunoreactivity in the germplasm of the nicotine only group, with the spermatozoa, spermatocytes, and spermatids staining intensely for cyclooxygenase-2 (arrows and stars). There is reduced immunoreactivity in the control and NICO + TES groups compared to the NICO group. NICO - Nicotine, TES - Testosterone

Markers of oxidative stress

Data on MDA levels of experimental rats are presented in Figure 3. NICO group featured elevated level of MDA that was significantly different from the NICO+TES and control groups at $P < 0.05$. The MDA assayed in the NICO+TES was lower compared to the NICO group but still significantly higher compared to the control group.

Data on SOD levels are presented in Figure 4. The SOD level in the NICO group was significantly lower compared to the NICO+TES and control groups at $P < 0.05$. There was no statistically significant difference between the SOD levels of NICO+TES and control groups at $P < 0.05$.

Figure 5 shows the data on GPx levels of experimental rats. The level of GPx in the NICO group was significantly lower than the NICO+TES and control groups at $P < 0.05$. There was no statistically significant difference between the GPx levels of NICO+TES and control groups at $P < 0.05$.

Discussion

This study investigated the impact of NICO on the key enzyme markers of inflammation and cell proliferation. Results indicated that NICO-treated rats showed intense positive immunoreactivity to COX-2 in the germplasm. The spermatogonia and primary spermatocytes stained positive to COX-2. Only, the peritubular spermatogonia of the testes of NICO-treated rats-stained positive to Ki-67. Slides showed tissue with eroded epithelium and paucity of cells in the germinal epithelium. These findings are proof that NICO induces inflammation

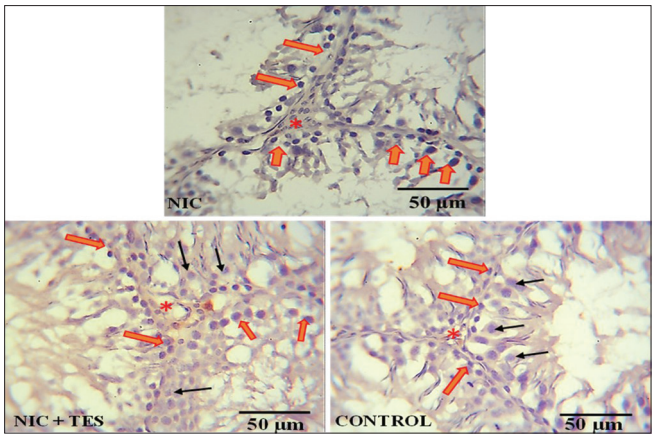

Figure 2: Sections from the seminiferous tubules of experimental rats showing the quantitative distribution pattern of Ki-67 protein. Ki-67 is expressed in the nucleus of spermatogonia of all groups (block arrows). Primary spermatocytes were additionally stained in the NICO + TES and control groups (arrows). NICO - Nicotine, TES - Testosterone

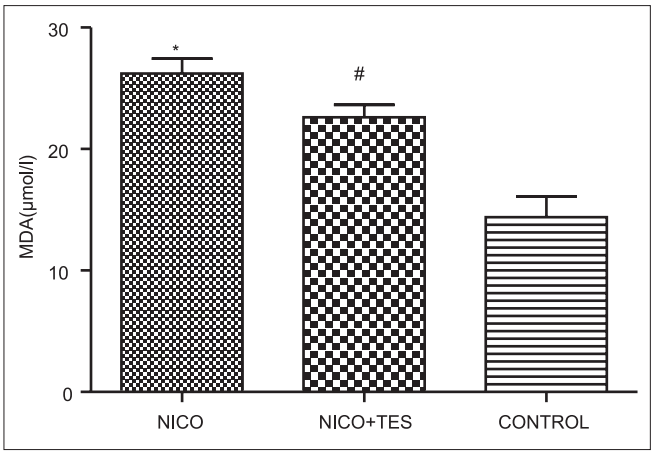

Figure 3: Effects of nicotine and testosterone on the MDA levels of experimental rats. Data are expressed as the mean ± standard error of the mean; *n* = 5 rats. NICO = Nicotine only group; NICO + TES = Nicotine + Testosterone group. *Significantly different at *P* < 0.05 versus NICO + TES and Control. + TES and control. #Significantly different at *P* < 0.05 versus Control. MDA - Malondialdehyde, NICO - Nicotine, TES - Testosterone

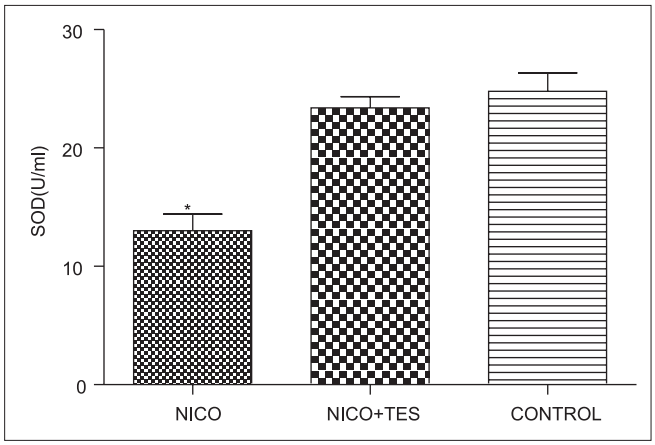

Figure 4: Effects of nicotine and testosterone on the SOD levels of experimental rats. Data are expressed as the mean ± standard error of the mean; *n* = 5 rats. NICO = Nicotine only group; NICO + TES = Nicotine + Testosterone group. *Significantly different at *P* < 0.05 versus NICO + TES and Control. SOD - Superoxide dismutase, NICO - Nicotine, TES - Testosterone

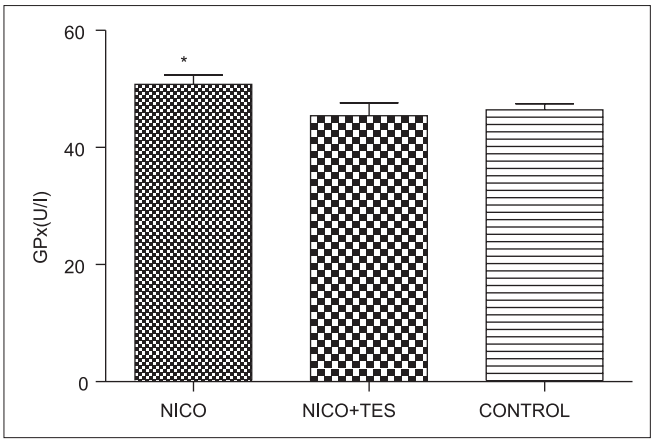

Figure 5: Effects of nicotine and testosterone on the GPx levels of experimental rats. Data are expressed as the mean ± standard error of the mean; *n* = 5 rats. NICO = Nicotine only group; NICO + TES = Nicotine + Testosterone group. *Significantly different at *P* < 0.05 versus NICO + TES and Control. GPx - Glutathione peroxidase, NICO - Nicotine, TES - Testosterone

in the germinal epithelium of the testes and induces apoptosis in spermatogonia cells resulting in fewer cells/discontinuation of the spermatogenic series and attenuation of the epithelium, resulting in the eventual widening of the lumen. These might account for the oligospermia associated with NICO toxicity. These corroborate report that NICO significantly increased COX-2 expression and led to the activation of inflammatory mediators in the rat model of acute nephritis (Jaimes *et al*., 2009). Similar findings have also been reported by Kushwaha and Jena who stated that NICO significantly increased the levels of nuclear factor-κB, COX-2, and tumor necrosis factor-α expression in diabetic testis.

The coadministration of TES propionate produced improved germinal features that were comparable with the control. They stained less intensely positive for COX-2. The spermatogonia and primary spermatocytes of NICO + TES rats both stained positive for Ki-67, thereby demonstrating the proliferative activity of the spermatogonia and ongoing spermatogenesis in this group.

NICO administration has been associated with oxidative stress in rats. In this study, the tissue of the NICO group had a significantly higher level of MDA compared to the NICO + TES and control groups. Notably, the SOD and GPx levels of NICO only group were significantly lower compared to the NICO + TES and controls. This is in agreement with report that chronic NICO treatment decreases the level of cytochrome P450IIE1, increases free radical formation, and decreases antioxidant

systems, which leads to tissue oxidative damage in rats (Erat *et al*., 2007). It has been adduced that increased oxidative stress status of rats treated with NICO would be expected to lead to affect the sperm cells since they are rich in polyunsaturated fatty acids which renders them vulnerable to attack by reactive oxygen species (Ates *et al*., 2004).

Conclusion

This study evinces evidence that NICO administration induces oxidative stress in the testes of rats, inflammation in the germinal epithelium of rat testes, and apoptosis in the peritubular stem cells (spermatogonia). This study also shows that oxidative stress and inflammation are correlated in NICO-induced testicular toxicity and that co-administration of TES propionate resulted in the amelioration of the harmful effects of NICO. This study supports the use of TES as hormonal replacement therapy in men with hypogonadism.

Financial support and sponsorship

This research work was financially supported by the 2014/2015 TETFund IBR Grant.

Conflicts of interest

There are no conflicts of interest.

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