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Research Article

Effects of Ascorbic Acid on Reproductive Functions of Male Wistar Rats Exposed to Nicotine

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ABSTRACT
Nicotine is a pharmacologically active and addictive alkaloid component of the cigarette smoke, and its effects on male reproductive system and fertility are well documented. Influence of ascorbic acid on reproductive functions of male wistar rats exposed to nicotine was examined in this study. Thirty-two adult male rats of 180 ± 15 g weight were used and grouped into control, nicotine, ascorbic and nicotine with ascorbic acid. The drugs were orally administered for thirty-five days. Plasma levels of FSH, LH and testosterone were significantly reduced in nicotine exposed rats when compared with the control ($p<0.05$), both FSH and LH plasma levels were significantly increased in rats exposed to ascorbic acid ($p<0.05$) relative to the control, while ascorbic acid also increased the level of these hormone in nicotine treated group ($p<0.05$). The cytoarchitecture of the seminiferous tubule shows high level of degeneration in the nicotine only treated group and this was reversed in the ascorbic acid treated group. There was a significant decreased in sperm motility, counts, percentage viability and morphology ($p<0.05$) in nicotine treated group relative to the control, while there was a significant improvement in these sperm parameters in co-administered ascorbic acid with nicotine when compared with the rats treated with nicotine only. In conclusion, ascorbic acid supplement may suppress nicotine toxic effects on reproductive functions in male rats.

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INTRODUCTION

Nicotine constitutes approximately 0.6ï 3.0% of the dry weight of tobacco derived from *Solanaceae* plants. It is the psychoactive substance in cigarette that acts as a stimulant and account for the highly addictive to cigarette smoking (Villégier *et al*., 2003). Averagely, cigarette yields about 1 mg of absorbed nicotine (Bose *et al*., 2007). Nicotine is rapidly absorbed by the brain and metabolized to cotinine by the cytochrome P450 enzymes of the liver, it has half-life of 2 hours and 20 hours for it metabolite (Reddy *et al*., 1995; Binnie *et al*., 2004).

Extensive studies have been reported on the male reproductive effect of cigarette smoking and its alkaloid active constituent (nicotine) in both human and experimental animals. For instance, cigarette smoking affects male fertility (Ramlau-Hansen *et al*., 2006; Gaur *et al*., 2007), it has been associated with decreased sperm count, alteration in motility of the sperms, and overall increase in the number of abnormal sperms in humans (Jorsaraei *et al*., 2008). Nicotine affects spermatogenesis, sperm count, motility, and the fertilizing potential of sperms (Aydos *et al*., 2001; Dhawan and Sharma, 2002; Oyeyipo *et al*., 2011). It also inhibits pulsatile luteinizing hormone (LH) secretion (Funabashi *et al*., 2005), decreased plasma gonadotropin level (Jana *et al.,* 2010; Heidary *et al.,* 2012) and decreased the plasma level of testosterone

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(Sarasin *et al*., 2003) through an inhibition in the multiple steps of testosterone biosynthesis in the rats and the mouse (Yamamoto *et al*., 1999). It has also been reported to cause testicular degeneration in rats (Azza *et al*., 2010; Oyeyipo *et al.,* 2010).

Ascorbic acid is a six-carbon keto-lactone, synthesized from glucose via several intermediates (Davis *et al.,* 1991). It is an anti-oxidant vitamin (Padayatty *et al*., 2003), with three biological actions of particular relevance to reproduction, each dependent on its role as a reducing agent: it is required for the biosynthesis of collagen, steroid and peptide hormones, it also prevent or reduce the oxidation of biomolecules that cause great damage to cell structures such as DNA and proteins (Luck *et al.,* 1995). It has also been reported to prevent passive smokers and sperm from oxidative damage (Dietrich *et al.,* 2003), improves the quality of sperm in smokers, reduces sperm agglutination and increased fertility in men (Dawson *et al.,* 1992; Sonmez *et al.,* 2005). Also, ascorbic acid has been reported to improve sperm parameters and chromatin quality in mice (Mangoli *et al*., 2012). Its low level has been associated with increased numbers of abnormal sperm, low counts reduced motility and agglutination of sperm (Wilson, 1954).

In the absence of study on the effects of ascorbic acid on the pituitary-testicular axis in male rats exposed to nicotine despite the widely reports on ascorbic acid and male reproductive functions in smokers, this study therefore aimed to evaluate the role of ascorbic acid in pituitary-testicular axis, sperm quality and testicular histology in male rats exposed to nicotine.

Materials and Methods

Nicotine Preparation

Nicotine hydrogen tartrate with product number 26140 (95% nicotine) used for this study was a product of BDH chemical Ltd Poole England. It stock solution was prepared at concentration of 1mg/ml and was stored in foil-wrapped glass bottle at 4^0C for not more than 7 days (Oyeyipo *et al.,* 2010).

Experimental Animals

Thirty-two (32) male albino wistar rats weighing between 170g to 200g were used. They were kept in clean environment with standard laboratory temperature (25^0C) and 12 hours light and dark cycle in accordance with U.S National Institute of Health (NIH) on the care and use of laboratory animals. The rats were fed with standard laboratory chow and had free access to water *ad libitum*.

After one week of acclimatization, the animals were randomly divided into four groups with eight rats in each group. The grouping was as follows;

1. Control group ï the rats in this group were given normal saline.
2. Nicotine group ï the animals were given 1.0mg/kg of nicotine.
3. Ascorbic acid group ï this group was treated with 100mg/kg of ascorbic acid.
4. Nicotine and ascorbic acid group ï the animals in this group were co-treated with both 1.0mg/kg of nicotine and 100mg/kg of ascorbic acid.

The treatments in all the groups were done orally using oral cannula for 35 days consecutively.

Experimental Procedure

After 35 days of treatment, blood samples were collected from thiopental anesthetized animals through cardiac puncture, centrifuged at 4000 rpm for 15 minutes and the plasma samples were used for follicle stimulating hormone (FSH), leutenizing hormone (LH) and testosterone assays.

The animals were then sacrificed and the caudal epididymides were immediately dissected from the testes for sperm motility, counts, viability and morphology assessments respectively. The testes were fixed in 10% formalin for testicular histology.

Hormones Assay

The plasma levels of FSH, LH and testosterone were measured by enzyme immunoassays method. The hormonal kits used for the assay was a product of Monobind Inc Lake forest, CA 92630, USA.

Testicular Histology

The testes of all the rats were fixed in 10% formalin, dehydrated stepwise in graded ethanol, cleared in xylene and then embedded in paraffin wax. A section of 5µm thick paraffin section of each testicular tissue was stained with hematoxylin and eosin, followed by examination under a light microscope at 200 magnification and micrographs taken (Bancroft and Stevens, 2002).

Sperm Motility

One drop of caudal epididymis sperm was dropped into a slide and diluted with few drop of normal saline at room temperature. The slide was examined under compound microscope. The number of motile and non-motile sperm was counted in ten random fields. The number of motile sperm was then expressed as a percentage of the total number of sperm (Cheesbrough, 2006).

Sperm Counts

Sperm count was performed as described in Cheesbrough laboratory manual (Cheesbrough, 2006) with modifications. The caudal epididymis was carefully separated from the testis and homogenized in 2 ml of normal saline and the suspension was obtained. The suspension was diluted with sodium bicarbonate-formalin in ratio 1 to 20. The improved Neubauer hemocytometer chamber was filled with well diluted sperm then the sperm were counted in 2 sq mm of Neubauer hemocytometer chamber. The sperm counts were calculated in 1 ml of fluid multiplied the number counted by 100,000.

Sperm Viability

The caudal epididymis sperm was dropped on the slide and mixed with a drop of 0.5% eosin solution. After 2 minutes, the slide was examined under compound microscope with 40X objective lens to count the percentage of viable (unstained) and non-viable sperm (stain red) (Cheesbrough, 2006).

Sperm Morphology

A drop of sperm suspension was smeared on a glass slide, fixed with 95% ethanol for 10 minutes and was allowed to air-dry. The smear was washed with sodium bicarbonate formalin solution to remove any mucus and then rinsed with several changed of water. The smear was covered with diluted (1 in 20) carbon fuchsin and allowed to stain for 3 minutes. The stained was washed off with water and counter stained with covered smear with diluted (1 in 20) Loefflerôs methylene blue for 2 minutes. The normal sperm which were stained (nucleus of hand ï dark blue; cytoplasm of head ï pale blue; middle piece and tail ï pink red) were counted and expressed in percentage (Cheesbrough, 2006).

Statistical analysis

The results were expressed as mean and standard error of mean (Mean ± SEM). Statistical significance between the groups was assessed by one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. $p<0.05$ was considered significant. Statistical Package for Social sciences 16 was used for the analysis.

Results

FSH, LH and Testosterone

Table 1 showed that FSH and LH were significantly reduced in groups treated with nicotine and nicotine with ascorbic acid relative to the control group ($p<0.05$), while these levels were significantly

FSH, LH and Testosterone

Table 1 showed that FSH and LH were significantly reduced in groups treated with nicotine and nicotine with ascorbic acid relative to the control group ($p<0.05$), while these levels were significantly increased in the ascorbic acid treated group when compared with the control ($p<0.05$). Also, the levels of FSH and LH were significantly increased ($p<0.05$) in nicotine and ascorbic acid group relative to nicotine group. Testosterone level was significantly reduced in nicotine, ascorbic acid, and nicotine with ascorbic acid groups when compared with the control group ($p<0.05$) (table 1).

Testicular Histology

The transverse section of the testes of control and ascorbic acid groups showed normal testicular architecture, the outer capsule of fibroblastic connecting tissues bound the seminiferous tubules with narrow lumen and sperm cells were normal (figure 1 A & C). There was disorganization in both the outer capsule of fibroblastic layers of the seminiferous tubule and seminiferous tubule with reduced sperm cells in the testes of rats treated with nicotine (figure 1 B). There was reorganization in the seminiferous tubule and the outer capsule of fibroblastic connecting tissues surrounding the tubule was normal in the testis of rats that were co-treated with nicotine and ascorbic acid (figure1 D).

Sperm Motility, Counts, Viability and Morphology

Table 2 showed the results of the effect of ascorbic acid on sperm motility, counts, viability and morphology of the male rats exposed to nicotine. The results revealed that the percentage forward progressive sperm motility was significantly reduced in nicotine and nicotine with ascorbic acid groups relative to the control group ($p<0.05$), while the progressive sperm motility was significantly improved ($p<0.05$) when ascorbic acid was co-administrated with nicotine relative to nicotine group. Sperm counts was significantly decreased in nicotine and nicotine with ascorbic acid treated rats relative to control group ($p<0.05$). The sperm count was significantly increased by ascorbic acid ($p<0.05$) when co-administrated with nicotine when compared with nicotine group. Percentage sperm viability in rats exposed to nicotine and nicotine with ascorbic acid showed significant decrease when compared with rats in control group ($p<0.05$). Also, the percentage normal

Table 1: Effect of ascorbic acid on Follicle stimulating hormone (FSH), Luteinizing hormone (LH) and testosterone of male rats exposed to nicotine

Groups	FSH (miu/ml)	LH (miu/ml)	Testosterone (ng/ml)
Control	9.11 ± 0.74	13.01 ± 0.98	12.94 ± 0.050
Nicotine	0.67 ± 0.02[a]	2.60 ± 0.18[a]	12.60 ± 0.021[a]
Ascorbic acid	10.88 ± 0.68[a]	21.56 ± 0.83[a]	12.79 ± 0.025[a]
Nicotine+Ascorbic acid	3.44 ± 0.26[a,b]	5.35 ± 0.26[a,b]	12.65 ± 0.021[a,b]

Data are expressed in Mean ± SEM of 8 rats, [a, b] are Mean significant difference relative to control and nicotine groups respectively at $p<0.05$

Table 2: Effect of ascorbic acid on sperm motility, counts, viability and morphology of male rats exposed to nicotine

Groups	Sperm motility (%)	Sperm counts (million/ml)	Sperm viability (%)	Sperm morphology (%)
Control	68.4 ± 1.33	47.4 ± 2.79	76.6 ± 2.00	79.0 ± 2.86
Nicotine	32.2 ± 2.60[a]	23.0 ± 2.56[a]	46.6 ± 1.92[a]	55.2 ± 2.15[a]
Ascorbic acid	72.6 ± 3.57	44.8 ± 2.48	79.6 ± 2.81	80.0 ± 1.00
Nicotine + Ascorbic acid	55.2 ± 2.06[a,b]	33.6 ± 1.54[a,b]	66.2 ± 2.65[a,b]	71.8 ± 1.36[a,b]

Data are expressed in Mean ± SEM of 8 rats, [a, b] are Mean significant difference relative to control and nicotine groups respectively at $p<0.05$

sperm morphology was significantly reduced both in nicotine and nicotine with ascorbic acid treated animals ($p<0.05$). The percentage sperm viability and morphology were significantly improved in nicotine and ascorbic acid group ($p<0.05$) when compared with nicotine group.

Discussion

Pituitary-testicular axis is immensely important in regulation of male reproductive functions. Testosterone which is regulated by LH from anterior pituitary gland plays a vital role in final maturation of spermatozoon and while FSH is needed for the maintenances of the gametogenic function of the testis (Barrett *et al*., 2011). The results of this study showed significant reduction in FSH, LH and testosterone in rats that were exposed to nicotine, although, Zavos and Zarmakoupis-Zavos (1999) reported high plasma FSH level in male smokers. The possibility of the low levels of plasma FSH and LH concentration following nicotine exposure has been reported to be probably due to elevation of glucocorticoid and corticosterone secretion from adrenal gland (Jana *et al.,* 2010), glucocorticoid may suppress the sensitivity of the gonadotroph cells to gonadotropin-releasing hormone and, therefore, may prevent gonadotropin secretion (Kamel and Kubajak, 1987). Inhibition of FSH and LH by nicotine may be as a result of its negative effect on central nervous system that can inhibit the neural stimulus essential for the release of pituitary gonadotrophins (Reddy *et al.,* 1995), which lead to a lack of pituitary gonadotrophins essential for initiating and completing spermatogenesis and steroidogenesis in the testis (Aydos *et al.,* 2001).

Fávaro *et al.* (2006) and Ahmadnia *et al.* (2007) reported many alterations attributed to the direct cytotoxic effects of nicotine leading to decrease testosterone synthesis.

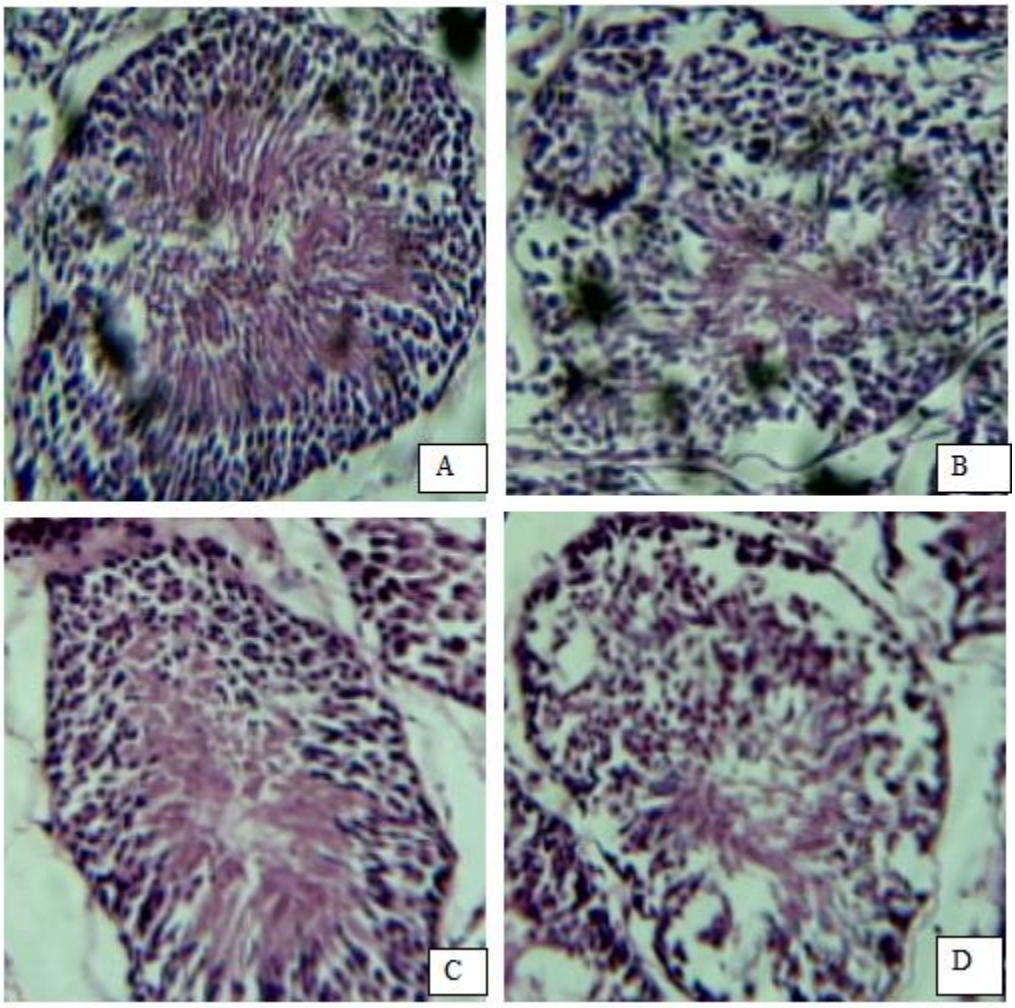

Fig.1:
Histology micrograph of transverse testes section of rats (H&E, 200X): (A) Control group shows normal testis structure with seminiferous tubule; (B) Nicotine treated group showed disarray architectural of seminiferous tubule; (C) Ascorbic acid treated group with normal testis structure and seminiferous tubule; (D) Nicotine with Ascorbic acid treated group shows re-organization of seminiferous tubule

The results of this study revealed that ascorbic acid significantly increased the plasma level of FSH, LH and testosterone both in ascorbic acid group and when supplemented in nicotine treated group. The significant increase in testosterone level observed in this study may be as a result of ascorbic acid roles in the synthesis of testosterone (Sonmez *et al.,* 2005). The elevation in FSH and LH by ascorbic acid supplement in rats exposed to nicotine may be related to the fact that ascorbic acid can be a vitaminergic transmitter that activates the release of LH and FSH from the anterior pituitary gland (Karanth *et al.,* 2001). The ameliorating action of ascorbic acid in elevating FSH, LH and testosterone in nicotine treated rats may also be attributed to its anti-oxidant property (Padayatty *et al*., 2003) which may prevent the depressive effects of nicotine on the hypothalamus that secrete gonadotrophin that control pituitary gonadotrophins. These results were in agreement with Fernandes *et al*. (2011); Obianime and Roberts (2009), who earlier reported that ascorbic acid supplement revert FSH, LH and testosterone secretion in hyperglycemic and cadmium induced toxicity in male rats.

The structural architecture of the testis was intact with a normal spermatogenic process in the control rats of this study, while there was a destruction of both fibrous connective tissue surrounding the seminiferous tubule and spermatogenesis process in nicotine exposed rats. This may account for the reduction in sperm motility, counts and viability and plasma testosterone observed in this study. The disarray in testicular histology observed was gradually reorganized in an

ascorbic acid supplemented group. This further provides evidence to buttress both the detrimental effects of nicotine and ameliorative effects of ascorbic acid on sperm parameters.

From this study, results indicated that nicotine induces significant reduction in sperm motility, counts, viability and increased the percentage of abnormal sperm, an observation which was in accordance with earlier reports on the detrimental effect of nicotine on sperm parameters (Oyeyipo *et al*., 2011; Jana *et al.,* 2009). It has been proven that nicotine increased the production of reactive oxygen species (ROS) by increased generations of testicular H_2O_2 and hydroxyl radicals in experimental rats (Bandopadhyay *et al.,* 2008).

All these observations were significantly improved in ascorbic acid supplemented rats following exposure to nicotine. Low ascorbic acid levels have been reported to be associated with low sperm counts, increased number of abnormal sperm, reduced motility and agglutination (Wilson, 1954). Also, it has been reported that dietary supplement of ascorbic acid improved sperm quality (Luck*et al.,* 1995). The beneficial effects of ascorbic acid seen in this study may result from its antioxidant activity in mopping nicotine induced free radicals (Dietrich *et al.,* 2003). The elevation of FSH and testosterone secretion in rats that were exposed to both ascorbic acid and nicotine in this study may be used to support the improved level of sperm motility, count, viability and normal morphology that were observed in this group, since both hormones are essential in early and final maturation of sperm cells (Barrett *et al.,* 2011).

We therefore suggested that the nicotine induced degenerative changes in the testicular structure, reduction sperm parameters and inhibition of testosterone production acts primarily at the level of hypothalamic-pituitary axis to inhibit the release of gonadotropins and ascorbic acid supplementation could ameliorate the toxic effects of nicotine on male reproductive functions in rats.

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