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Evaluation of andrological indices and testicular histology following administration of varied doses of nicotine

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Abstract

This study is aimed at determining the effect of Nicotine on male fertility by evaluating some andrological parameters of male Wistar rat such as sperm parameters (spermatozoa count and motility), serum concentration of testosterone and testicular weight. Histopathology of the testis was also carried out on the effect of nicotine on testicular microstructure. 20 adult male rats were randomly divided into four groups, the test groups were administered with 0.2mg/100g, 0.4/100g and 0.6/100g body weight daily for 30 days while the control were administered with 2mls 0.9% physiological saline. Nicotine caused a significant reduction ($P < 0.05$) and ($P< 0.01$) in the mean values of sperm count, serum testosterone concentration and testicular weight in the test when compared with the control. Also, in the test group, the deleterious effect of nicotine on the sperm parameters and testosterone concentration was corroborated by histopathology which revealed a marked degeneration of germ cell layers in the seminiferous tubule and disruption of interstitial cells of the testis thereby interfering with spermatogenesis and testosterone secretion while there was no visible change in the control group. It was concluded that nicotine exerted toxic effect on the germ cell layers in seminiferous tubule with concomitant reduction in reproductive potentials of the male rat whilst showing no significant change in sperm motility. Nicotine and nicotine based products should therefore be taken with caution in cases of infertility.

Key words: *germ cells*, testes, testosterone, fertility, spermatogenesis.

INTRODUCTION

Nicotine is an alkaloid found in the nightshade family of plants (Solanaceae); biosynthesis takes place in the roots and accumulation occurs in the leaves. It constitutes approximately 0.6–3.0% of the dry weight of tobacco (NIH) and is present in the range of 2–7 µg/kg of various edible plants.[2] It functions as an antiherbivore chemical; therefore, nicotine was widely used as an insecticide in the past(Rodgman et al, 2009) and nicotine analogs such as imidacloprid are currently widely used. In low concentrations (an average cigarette yields about 1 mg of absorbed nicotine), the substance acts as a stimulant in mammals, while high concentrations (30–60 mg) can be fatal (IPCS INCHEM). This stimulant effect is the main factor responsible for the

dependence-forming properties of tobacco smoking (genetic science learning centre Utah). According to the American Heart Association, nicotine addiction has historically been one of the hardest addictions to break, while the pharmacological and behavioural characteristics that determine tobacco addiction are similar to those determining addiction to heroin and cocaine (conolly et al, 2007). As of 2002, about twenty percent of young teens (13-15) smoke worldwide. 80,000-100,000 children begin smoking every day. Half of those who begin smoking in adolescent years are projected to go on to smoke for 15 to 20 years (WHO, 2002). In the developing world tobacco consumption is rising by 3.4% annually (WHO, 2002).

While the association between inhalation of mainstream smoke and cardiovascular disease, respiratory disease and cancer has been established for many years, the impact of smoking on reproduction is recognized, but less well characterized and less well known a report on the health consequences of smoking by the centre for disease control Washington stated. Cigarette smoking has a negative impact on the ability to become pregnant and carry a pregnancy to term. Virtually all scientific studies support the conclusion that smoking has an adverse impact on fertility. The prevalence of infertility is higher, and the time it takes to conceive is longer, in smokers compared to nonsmokers. Active smoking by either partner has adverse effects, and the impact of passive cigarette smoke exposure is only slightly smaller than for active smoking. Research indicates that cigarette smoking is harmful to a woman's ovaries, and the degree of harm is dependent upon the amount and the period of time a woman smokes. Smoking appears to accelerate the loss of eggs and reproductive function and may advance the time of menopause by several years.

Components in cigarette smoke have been shown to interfere with the ability of cells in the ovary to make estrogen and to cause a woman's eggs (oocytes) to be more prone to genetic abnormalities. Smoking is strongly associated with an increased risk of spontaneous miscarriage and possibly ectopic pregnancy as well. Pregnant smokers are more likely to have low birth weight babies and premature birth. The incidence of sudden infant death syndrome (SIDS) also increases in households where someone smokes (American society for reproductive medicine, 2003).

Men who smoke cigarettes have a lower sperm count and motility and increased abnormalities in sperm shape and function. The effect of smoking on male fertility, however, is more difficult to discern because it is difficult to create studies to address that question. Although the effects of cigarette smoking on male fertility remain inconclusive, the harmful effect of passive smoke on the fertility of female partners and the evidence that smoking adversely affects sperm quality suggest that smoking in men should be regarded as an infertility risk factor (American society for reproductive medicine).

The rising incidence in tobacco smoking particularly in the developing world as well as the little knowledge of the effect of tobacco on male fertility in comparison to the females necessitates studies such as these to determine the effect nicotine on spermatogenesis and the cytoarchitecture of the testis, using the male albino wistar rat as a model.

MATERIALS AND METHODS

Nicotine salt

Nicotine hydrogen tartrate salt ($C_{10}H_{14}N_2 2C_4H_6O_6$) purchased from sigma Aldrich catalog number **N5260-25G.**

Sample preparation

Nicotine base is ($C_{10}H_{14}N_2$) which by weight is 35.06% of Nicotine hydrogen tartrate salt.

1. Determination of percentage weight of nicotine in nicotine salt
   molecular weight of nicotine $C_{10}H_{14}N_2$ = (12×10 +14 ×1 +14 ×2) = 162g/mole
   molecular weight of nicotine salt is given as 462.41g/mol
   thus
   $\%\ nicotine\ base = (molecular\ mass\ of\ nicotine \div molecular\ mass\ of\ nicotine\ salt) \times 100\%$
   (162÷ 462.41) x 100 = 35.06%
2. Preparation of solution.
   First dose level =0.2mg/100g body weight of rats
   Total volume prepared = 2 litres
   Concentration of solution= 0.2mg/2ml
   If 0.2mg = 2ml then X= 2000ml
   (2000÷2) ×0.2 = X = 200mg. This implies that 200mg of nicotine base was dissolved in 2 litres of normal saline to get the concentration of 0.2mg/ml

   Weight of nicotine salt equivalent to 200mg base is calculated as follows
   If 200mg = 35.06% then Xmg = 100% thus Xmg = 100÷35.06 ×200 =570.45mg
   Thus 570.45mg of nicotine tartrate was dissolved in 2L of normal saline to yield a concentration of 0.2mg nicotine base in 2ml of normal saline

   Second dose level = 0.4mg/100g body weight of rats
   Total volume prepared = 2 litres
   Concentration of solution= 0.4mg/2ml
   If 0.4mg = 2ml then X= 2000ml
   (2000÷2) ×0.4 = X = 400mg. This implies that 400mg of nicotine base was dissolved in 2 litres of normal saline to get the concentration of 0.4mg/ml

   Weight of nicotine salt equivalent to 400mg base is calculated as follows
   If 400mg = 35.06% then X mg = 100% thus X mg = 100÷35.06 ×400 = 1.141g of nicotine salt
   Thus 1.141g of nicotine tartrate was dissolved in 2L of normal saline to yield a concentration of 0.4mg nicotine base in 2ml of normal saline.

   Third dose level =0.6mg/100g body weight of rats
   Total volume prepared = 2 litres
   Concentration of solution= 0.6mg/2ml

If 0.6mg = 2ml then X= 2000ml

(2000÷2) ×0.6 = X = 600mg. This implies that 600mg of nicotine base was dissolved in 2 litres of normal saline to get the concentration of 0.2mg/ml

Weight of nicotine salt equivalent to 600mg base is calculated as follows

If 600mg = 35.06% then X mg = 100% thus X mg = 100÷35.06 ×600 = 1.711g of nicotine salt.

Thus 1.711g of nicotine tartrate was dissolved in 2L of normal saline to yield a concentration of 0.6mg nicotine base in 2ml of normal saline.

Animals

20 male Wistar rats weighing 220 - 280 g were used in the study. All animals were kept in the animal house of the University of Jos. They were maintained at room temperature and 12 hours light/dark cycle. All the experimental procedures were done following the experimental guidelines of Institutional Animal Ethics Committee (IAEC).

Experimental protocol

Four groups with 5 rats selected randomly in each group were formed . Groups 1-3 were the test groups, whilst group 4 was used as the control, each rat in group 1 was treated with 0.2 mg/100g body weight /p.o of nicotine daily for 30 days, groups 2 and 3 were given 0.4mg/ 100g, and 0.6mg/ 100g body weigt of nicotine per os daily for 30 days respectively. While group 4 (control group) was given 2ml 0.9% physiological saline solution for the same period of exposure.

Chemicals

Testosterone EIA kit ways purchased from Monobind Inc. Lake forest U.S.A.

Other chemicals were of analytical grade and procured locally.

Sample collection

The rats were anaesthetized using ether and afterward sacrificed by cervical dislocation and blood sample collected by cardiac punc-ture. Orchidectomy was performed by open castration method. A midline or pre-scrotal incision was made and the testicles were milked out of the incision site and weighed with the aid of OHAUS electric weighing balance.

The testicles were exposed by incising the tunica vaginalis. The spermatic cord was exposed, ligated and incised. Semen samples were thereafter collected from the caudal epididymis. The methods of collection were similar to that described by Akusu et al. (1985), Oyeyemi and Ubiogoro (2005) Oyekunle and Omope(2010). The samples were analyzed immediately after collection.

Sperm count and motility assay

Sperm motility was assessed by the method described by Zemjanis (1977). The spermatozoa were counted by hemocytometer using the improved Neubauer (Deep 1/10 mm, LABART, Germany) chamber as described by Pant and Srivastava (2003).

Testosterone assay

This was carried out with the use of Testosterone EIA Kit (product code:3725-300 ELISA microwells and microplate immunoassay using Statfax-2100 microplate reader) obtained from Monobind Inc. Lake forest U.S.A.

Description of ACE competitive EIAs

This assay is based on the competition between Testosterone and a Testosterone acetylcholinesterase (AChE) conjugate (Testo-sterone Tracer) for a limited amount of Testosterone Antiserum. The concentration of the Testosterone Tracer is held constant while the concentration of Testosterone varies; the amount of Testo-sterone that is able to bind to the Testosterone Antiserum will be inversely proportional to the concentration of Testosterone in the well. This Antiserum-Testosterone complex binds to mono-clonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagent and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorb strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of the Testosterone Tracer bind to the well, which is inversely proportional to the amount of free Testosterone present in the well during the incubation; or Absorbance α [Bound Testosterone Tracer] α 1/[Testosterone].

Histological procedures

After the extraction of the testis from the animal's body, the organ was promptly and adequately treated with 10% formal saline (fixa-tion) in order to preserve its structure and molecular composition. After fixation, the piece of organ was dehydrated by bathing it successfully in graded mixture of ethanol and water (70 - 100%). The ethanol was then replaced with a solvent miscible with the embedding medium (xylene). As the tissues were infiltrated with xylene, it became transparent (clearing). Once the tissue has been impregnated by xylene it was placed in melted paraffin in an oven maintained at 58 - 60°C (embedding). The heat caused the solvent to evaporate and the spaces within the tissues become filled with paraffin. The tissue together with its impregnating paraffin hardens after been taken out of the oven this was done with the aid of the Leica TP120 automatic tissue processor. The hard block containing the tissue was then taken to the microtome and sectioned by the microtome steel. The sections were then floated on water and transferred to a glass slide and stained with heamatoxylin and eosin stains with the aid of the leicaauto stainer XL . The slides were then viewed under light microscope with medium magnification.

Statistical analysis

Statistical analysis was done using graphpad instat3 tool to conduct one way analysis of variance (ANOVA).

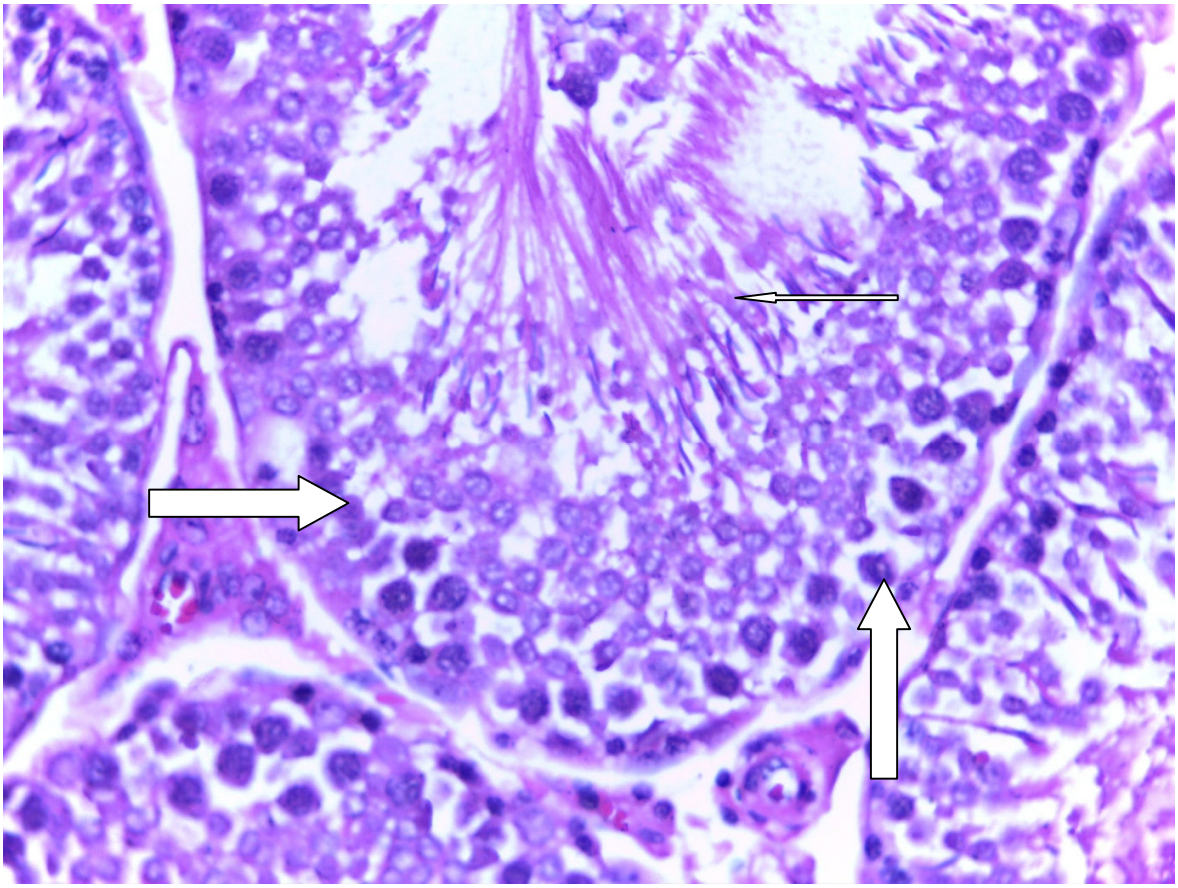
Results

Are presented as the mean ± standard error of mean.

Parameters	Control(grp 4)	Group1	Group2	Group 3
Sperm motility	40.4±0.75	36.2±2.31	42.8±1.31	38.2±2.0
Sperm count($\times 10^6$/ml)	32.5±1.70	27.7±6.81*	22.6±6.96**	22.4±1.02**
Testicular weight(g)	1.48±0.07	1.31±0.05	1.2±0.05**	1.1±0.05**
Serum testosterone (nmol/l)	7.4±0.69	4.8±0.58*	4.0±1.0**	0.6±0.28**

P<0.5, significant. *P<0.01 highly significant.** *P<0.001 extremely significant

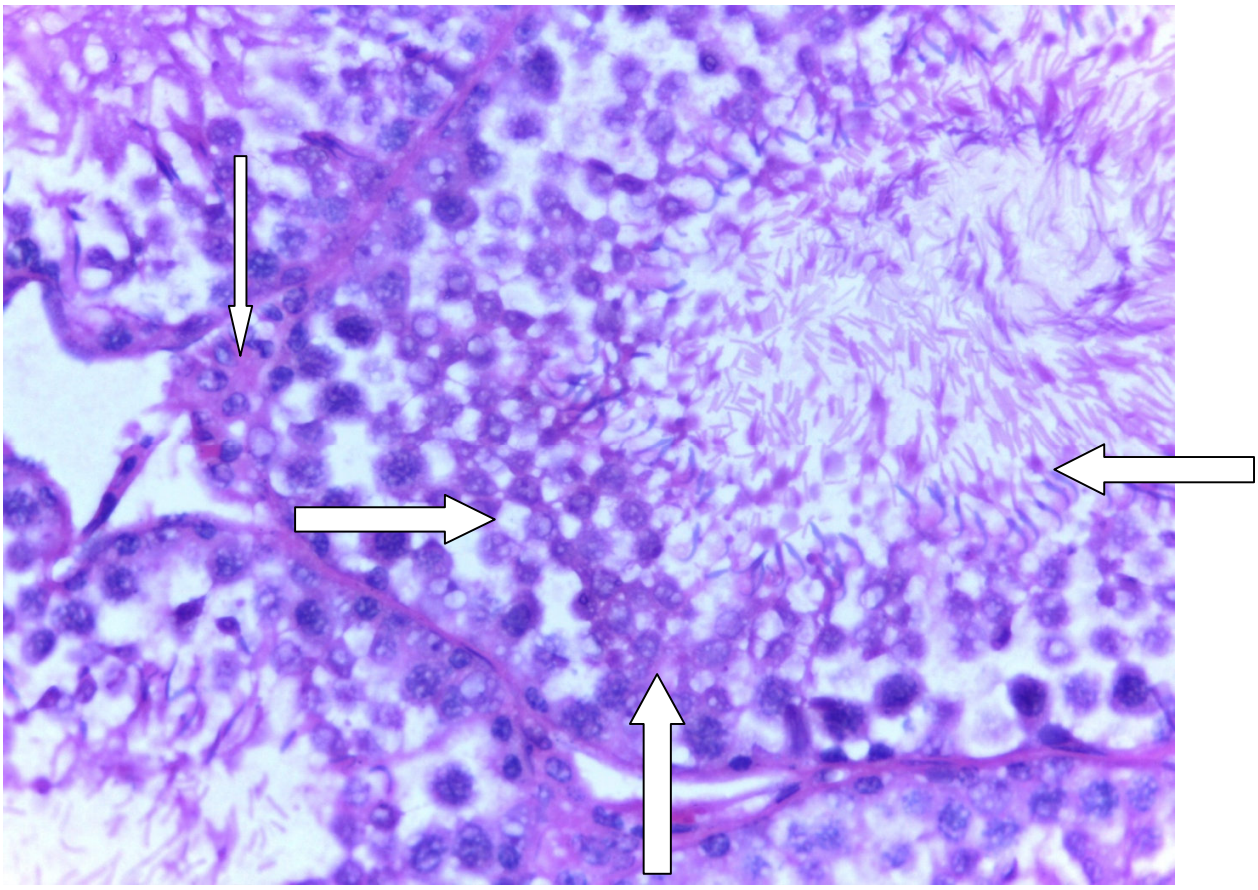
The results showed a significant decrease in sperm count, testicular weight and a corresponding decrease in serum testosterone when compared with the control which suggests that ingestion of nicotine particularly at doses greater than 0.2mg /100g for a period of thirty days or more may adversely affect fertility.



×40 magnification.

Plate 1: showing histological slide of semineferous tubule of control (2ml of normal saline)

Right arrow showing multiple layers of developing germ cells.
Left arrow showing spermatid.
Upward pointing arrow showing intact membrane of seminiferous tubule.



×40 magnification

Plate 2: showing seminiferous tubule of group 1 (2mg/100g)

Arrow up showing sertoli cells.

Left arrow showing spermatids.

Right arrow showing developing germ cells.

Downward pointing arrow showing interstitial cells

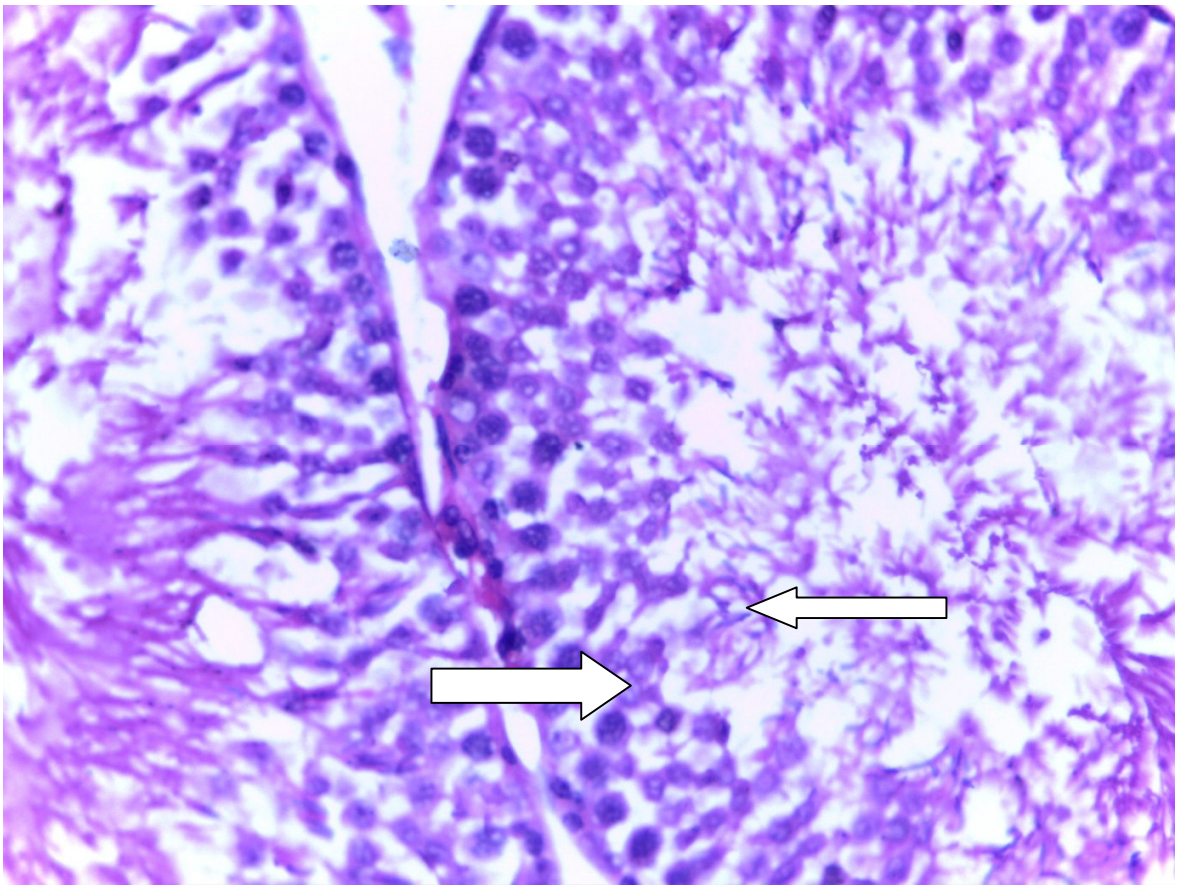


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Plate 3 : showing histological slide for group 2 (0.4mg/100g)
Right arrow showing developing germ cells.
Left arrow showing scanty stage 7 spermatids.

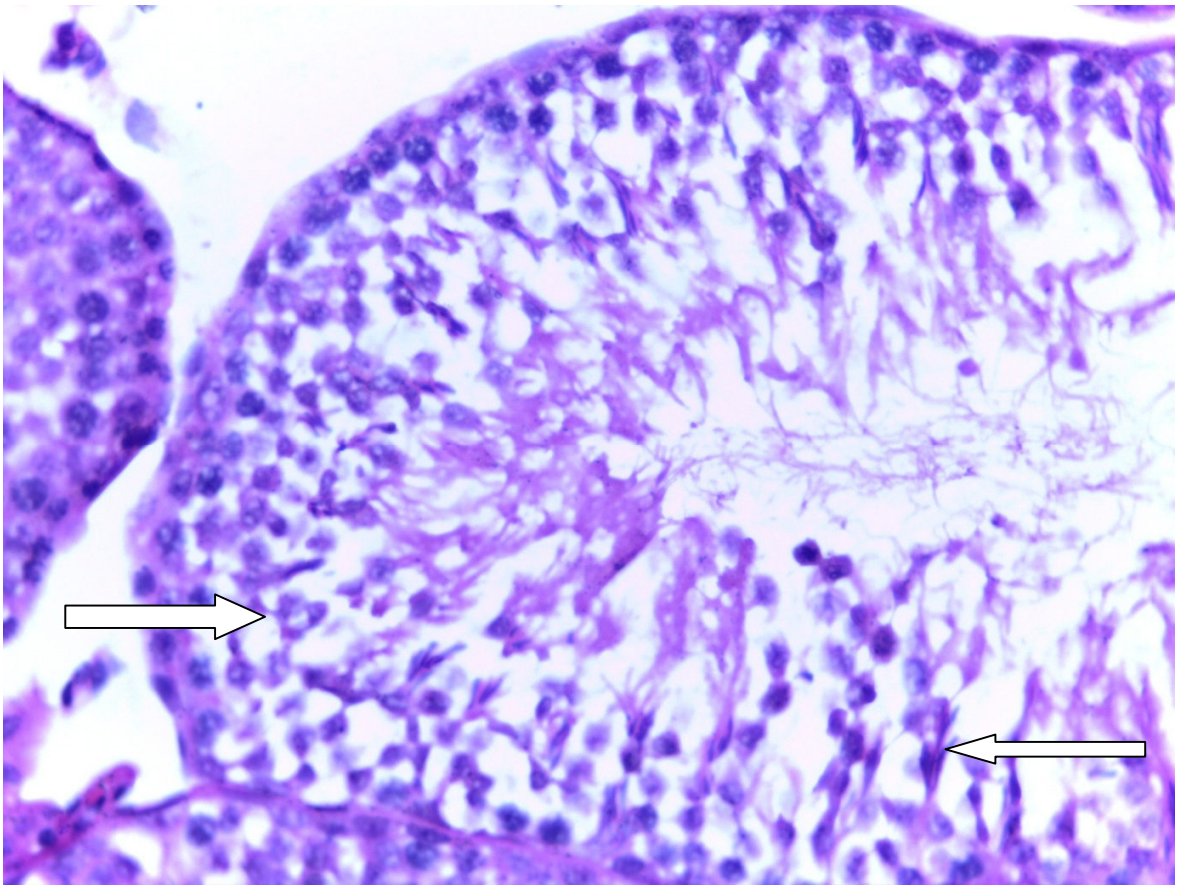

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Plate 4: showing group 3 semineferous tubule (0.6mg/100g)

Right arrow showing scanty germ cell layer.

Left arrow showing stage 7 spermatids.

The histology showed normal semineferous tubules with decrease spermatogonic cells suggesting a probable decrease in spermatogenesis when compared to control. This in concert with the decreasing sperm count, testosterone and testicular weight basically suggest an adverse effect of nicotine on spermatogenesis and probably fertility.

DISCUSSION

The sperm cell count, sperm cell motility, testicular weight and serum testosterone concentration were used in this study to evaluate the effect of administration of varied doses nicotine over a period of thirty days on male reproductive system using the male Wistar rat as animal model. These andrological parameters such as sperm count, motility and serum testosterone as well as life and dead ratio and morphology (not used in this study) are usually evaluated to determine the fertility of a male subject. When critical percentage of sperm cell abnormalities is present in the semen, the male subject is usually considered infertile (Zemjanis, 1977). In this study, the sperm cell count was observed to be significantly reduced ($P < 0.05$) for the dose of 0.2mg/100g body weight of the rat and at ($P<0.01$) for the doses of 0.4mg/100g and 0.6/100g body weight of

the rats respectively. The decrease seen in the germ cells and stage 7 spermatids on the histogical slides showing the seminiferous tubules, suggest a decrease in spermatogenesis which is similar to the findings of Nesseim et al (2011), though there were no thickening of basal lamina or damaged basement membrane observed. The decrease in testicular weight recorded of $P<0.05$ and $P<0.01$ for group 1 and group 2 and 3 respectively may probably be as a result of an overall decrease in weight associated with a decreased appetite and increased metabolism as suggested by Orsini (2001) or probably as a direct cytopathic effect on the testis but this could not be ascertained as the weight of the rats were not taken before they were anaesthesized and samples collected.

There was a dose dependent decrease in testosterone concentration at $P<0.05$ for group 1 and $P<0.01$ for group 2 and 3 respectively which is in concert with the findings of Briggs (1973) and Sharawy and Mahmoud, (1982). Other studies have shown inconsistencies on the serum level of testosterone in nicotine exposed individuals or animal models this may be due to the circardian rythm of testosterone or its binding ability to sex hormone binding globulin (Kapoor and Jones, 2005). In this study however the decrease may be associated the decrease testicular weight which may be proportional to the amount of functioning interstitial cells of leydig.

Conclusion

The outcome of this study showed that administration of varied doses of nicotine caused a dose dependent reduction in mean values of andrological parameters as a result of a decrease in germ cell development as shown in the scanty germ cells seen in the histological slides of the semineferous tubules with increase dose of nicotine base, whilst showing no significant change in sperm motility. This indicates that prolonged consumption of nicotine and products containing nicotine at doses above 0.2mg/100g could induce infertility in males. Thus consumption of nicotine and nicotine based products should be taken with caution particularly in cases of infertility.

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