

# The Effect of Prenatal Exposure to Nicotine on Pituitary –Testicular Axis and Diminishing Steroidogenic Acute Regulatory Protein Expression in Albino Rats

Surya .V.

Dept. of Biotechnology and Biochemical Engineering, Mohandas College of Engineering & Technology, Anad

---

**Abstract:**-Cigarette smoking prevalence is still increasing in the developing world especially among men mainly in central Asia, east Europe and Africa. Furthermore, maternal cigarette smoking during pregnancy has multiple deleterious effects on the offspring, which may persist into adulthood. The present study was done to evaluate the pituitary-testicular activities of rats subjected to chronic nicotine treatment. The testicular key androgenic enzymes activities, plasma and intratesticular testosterone (ITT) concentrations, and plasma concentration of gonadotropin were significantly reduced by nicotine treatment along with the decreased sperm counts and the disruption of spermatogenesis indicated by significant reduction in the number of different generations of germ cells at stage VII of the spermatogenesis cycle with increased sperm head abnormalities. The Western blot and the reverse transcriptase-PCR analysis revealed that the nicotine induced a marked decrease in the expression of testicular steroidogenic acute regulatory (StAR) protein, which helps in the transfer of cholesterol in mitochondria for the testosterone biosynthesis. Human chorionic gonadotropin or taurine supplementation with nicotine prevented the degeneration of germ cells to some extent, restored spermatogenesis moderately with decreased sperm head abnormalities, and enhanced sperm counts, accompanied with increase in plasma and ITT concentrations, testicular StAR gene expression, and key androgenic enzymes activities. The results indicated that nicotine caused testicular toxicity by germ cell degeneration, inhibition of StAR gene expression along with androgen production in adult male rats probably by affecting pituitary gonadotropin,

**Keywords:** Gonadotrophs, Intratesticular testosterone, Nicotine, spermatogenesis, St A Rgene)

## 1. INTRODUCTION

Smoking is an important cause of increased mortality and morbidity in the developed countries. Its prevalence is still increasing in the developing world especially among men mainly in central Asia, east Europe and Africa . Cigarette smoking is not only a potent cause of lung cancer but also has been associated with low birth weight, preterm delivery and abortion in women who are addicted to it. Maternal cigarette smoking during pregnancy has multiple deleterious effects on the offspring, which persist into adulthood .

The male reproductive system is known to be highly sensitive to many chemicals . Growing evidence indicates that over 4000 chemical compounds are usually concentrated and condensed into tobacco mixture . Nicotine has always been identified as the most powerful toxin of cigarette smoking .

The present study was done to evaluate the pituitary-testicular activities of rats subjected to chronic nicotine treatment. The testicular key androgenic enzymes activities, plasma and intratesticular testosterone (ITT) concentrations, and plasma concentration of gonadotropin were significantly reduced by nicotine treatment along with the decreased sperm counts and the disruption of spermatogenesis indicated by significant reduction in the number of different generations of germ cells at stage VII of the spermatogenesis cycle with increased sperm head abnormalities. The Western blot and the reverse transcriptase-PCR analysis revealed that the nicotine induced a marked decrease in the expression of testicular steroidogenic acute regulatory (StAR) protein, which helps in the transfer of cholesterol in mitochondria for the testosterone biosynthesis. Human chorionic gonadotropin or taurine supplementation with nicotine prevented the degeneration of germ cells to some extent, restored spermatogenesis moderately with decreased sperm head

abnormalities, and enhanced sperm counts, accompanied with increase in plasma and ITT concentrations, testicular StAR gene expression, and key androgenic enzymes activities.. The results indicated that nicotine caused testicular toxicity by germ cell degeneration, inhibition of StAR gene expression along with androgen production in adult male rats probably by affecting pituitary gonadotropin, and/or modulating the extent of testicular antioxidant status.

## 2.EFFECT OF EXPOSURE TO NICOTINE – HOW IT ACTS ??

Nicotine, a pharmacologically active and addictive alkaloid component of the cigarette smoke, and its effects on male reproductive system and fertility have been reported previously(Aydos et al., 2001). Within the body, nicotine is oxidized to its metabolite cotinine, which has a long half-life, and both the nicotine and the cotinine adversely affected spermatogenesis, epididymal sperm count, motility, and the fertilizing potential of sperms). Cigarette smoking has also been associated with decreased sperm count, alteration in motility of the sperms, and overall increase in the number of abnormal sperms in humans(Aydos et al., 2001). .Most of the research findings have indicated that nicotine decreases the level of testosterone through the inhibition in the multiple steps of testosterone biosynthesis in the rats and the mouse It has also been established that nicotine administration decreases the testicular androgenic enzymes along with plasma testosterone and sperm counts in mature male albino rats (Kalikauskas et al., 1985)..Although several reports have been shown consistently in human chronic smokers that nicotine increases the circulatory levels of cortisol, growth hormone, and prolactin ,yet either increasing influence or no influence has been reported for luteinizing hormone (LH). However, recently, it has been established that nicotine inhibits pulsatile LH secretion in human males but not in human females(Sarasin et al., 2003). Despite having increased knowledge of the adverse reproductive effects of nicotine, it is still unclear whether nicotine affects the pulsatile gonadotropin secretion or not. The actual molecular events resulting in male reproductive toxicity from nicotine treatment also remains unclear(Patterson et al., 1990).. Nowadays, it is clear that the deleterious toxic effects of nicotine are, at least in part, because of the increased

production of reactive oxygen species (ROS) .ROS damages DNA, proteins, carbohydrates, and lipids and affects enzyme activity and cellular genetic machinery However, the biological systems possess a number of mechanisms to remove ROS, such as the integrated antioxidant defense systems. Indeed, it has been shown that ROS inhibits steroidogenesis by interfering with cholesterol transport to mitochondria and/or catalytic function of P450 enzymes(Yamamoto et al., 1998).. Moreover, ROS also inhibits steroidogenesis during cholesterol transfer by suppression of the steroidogenic acute regulatory (StAR) protein expression in the MA-10 tumor Leydig cells The enzymatic scavengers of ROS, such as reduced glutathione (GSH) metabolism–regulating enzymes, i.e.,  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT), glutathione peroxidase (GPx), glutathione reductase (GR), and glucose-6-phosphate dehydrogenase (G-6-PDH), may protect the cellular system from various deleterious effects of the free radicals induced by xenobiotics in testis. The nonenzymatic antioxidant, GSH, scavenges and quenches free radicals, gets oxidized, and inactivates the process of free radical–mediated damage .It has also been reported that chronic nicotine treatment decreases the level of cytochrome P450 IIE1, increases free radical formation, and decreases antioxidant systems, which leads to tissue oxidative damage in rats (Yamamoto et al., 1998).

Therefore, it is assumed that the application of antioxidants as the possible preventive or counteractive agents can be targeted as therapeutic means for nicotine-induced testicular abnormalities(Vine, 1996). Taurine (2-amino ethane sulfonic acid) is a sulfur-containing amino acid, which is the most abundant free  $\beta$ -amino acid in the male reproductive system .Taurine acts as an antioxidant to prevent the oxidative damage of sperms (Alvarez and Storey, 1983). Also, taurine supplementation effectively counteracts the toxic effects of chronic nicotine administration on heart, urinary bladder, and kidney functions and attenuates the oxidative damage possibly by its antioxidant effects .Besides, the human chorionic gonadotropin (hCG), which stimulates the pituitary to release follicle-stimulating hormone (FSH), has also LH-like activity in the mammals In addition, hCG-mediated induction of steroid production correlates with increased StAR protein expression, a well-documented phenomenon in the steroidogenic Leydig cells(Hales et al., 1999).

In this study, therefore, the changes in enzymatic and hormonal milieu after nicotine, with or without hCG and taurine treatments, were investigated to find out the possible mode of influence of nicotine on pituitary-testicular activities with special emphasis on testicular spermatogenesis, steroidogenesis, and StAR gene expressions, along with ROS generation, pro- and antioxidant status (Stocco et al., 1993). Nicotine intake through cigarette smoking has become a very common occurrence and a serious health and economic issue in most societies. It is documented that tobacco usage, according to the World Health Organization, is associated with approximately one-third of the world's population older than 15 years of age.

Infertility which is a major health issue among couples of child-bearing age is also on the increase with approximately half of known causes of primary infertility attributed to male factor. Studies have implicated nicotine as one of the main pharmacological substances responsible for inducing infertility due to cigarette smoking (Erat et al., 2007). These studies have shown that nicotine affects sperm function by depressing the sperm count motility, viability, normal morphology and reduce the weight of the testis. Fertility studies also reveal a significant decrease in the libido of male rats treated with nicotine. However, the mechanism by which nicotine causes male infertility is poorly understood.

Increased oxidative stress results from excess generation of reactive chemical species called free radicals from a number of sources and/or from decreased enzymic and non-enzymic antioxidant defenses. Free radicals and other reactive species have been implicated in the progression of not <100 different diseases. (Lobo et al. 1987). Thus, the interest in free radicals and oxidative stress has grown in recent times.

Elevated oxidative stress has been associated with an imbalance in the activity of the cardiac autonomic nervous system and several other heart diseases (Alvarez et al. 1983). Oxidative stress is also associated with high frequencies of single- and double-strand deoxyribonucleic acid (DNA) breaks leading to decreased fertilization rates and poor embryo cleavage and quality since infertility cases have been associated with sperm samples containing a high frequency of damaged DNA.

Nicotine has been documented to alter the oxidant and antioxidant balance in rat lymphocyte in a dose and time dependent

manner and alters lipid peroxidation and antioxidant enzyme in plasma and ovaries of female rats (Sener et al., 2005a, 2005b). It is still uncertain if nicotine, the main pharmacological active substance in tobacco, is responsible for various deleterious effects due to free radical production because evidence has shown nicotine to have both antioxidant effects and pro-oxidant effects. However, no study has investigated the effect of nicotine on male reproductive organs oxidant and antioxidant balance in rats despite the involvement of oxidative stress in male infertility (Stocco, 2001; Stocco and Clark, 1996).

The present study was therefore designed to investigate the effects of nicotine on oxidant and antioxidant balance in some reproductive organs of male albino rats during treatment and withdrawal periods. The testicular key androgenic activities, plasma and intratesticular testosterone concentrations and plasma concentration of gonadotrophin were significantly reduced by nicotine treatment along with the decreased sperm counts and the disruption of spermatogenesis indicated by significant reduction in the number of different generations of germ cells of stage 7 of cycle with increased abnormalities.

### 3. Materials and methods

#### 3.1 Animal selection and care.

Inbred male albino rats (4 months of age, weighing  $120 \pm 10$  mg) were obtained. The rats were acclimatized under laboratory conditions prior to the experiment. The animals were housed in six to a polypropylene cage and provided with food and water ad libitum. The animals were maintained under standard conditions of temperature ( $22^\circ\text{C} \pm 2^\circ\text{C}$ ) and humidity ( $60 \pm 5\%$ ) with an alternating 12-h light/dark cycles. The National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23 revised 1985: U.S. Department of Health, Education and Welfare, Bethesda, MA) was followed throughout the experimental duration. The experimental protocol also met the National Guidelines on the Proper Care and Use of Animals in Laboratory Research. The animals were fed with standard pellet diet and was approved duly by the "Institutional Animal Ethics Committee of the Institute." The animal house and breeding facility were registered with Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

### 3.2 Chemicals.

Nicotine, hCG, rabbit monoclonal antibody against  $\beta$ -actin, androstenedione and dehydroepiandrosterone, and other fine chemicals were obtained. TRIZOL reagent was purchased from Invitrogen. Antiserum against a synthetic peptide consisting of amino acids sequence 88–98 of the mouse StAR protein generated in rabbit was obtained. Rabbit polyclonal anti- $3\beta$ -Hydroxysteroid dehydrogenase ( $3\beta$ -HSD) and  $17\beta$ -hydroxysteroid dehydrogenase ( $17\beta$ -HSD) were purchased. Specific  $\beta$ -actin antibody was purchased from Cell Signaling Technology. Horseradish peroxidase-conjugated goat anti-rabbit IgG were obtained and 5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazole carbocyanide iodide (JC-1) probe was obtained. All other chemicals and reagents were analytical grade.

### 3.3 Drug treatments.

The animals were randomly categorized into experimental and control groups and divided equally into four groups, with 12 animals per group and their initial body weights were recorded. The animals of each group were treated as follows. The control group received ip injection of 1 ml physiological normal saline as a vehicle. The nicotine-treated group was given ip injection of nicotine in physiological normal saline at a dose of 0.6 mg/kg body weight/day. The hCG-supplemented nicotine-treated group received sc injection of hCG at a dose of 5 IU/kg body weight/day before nicotine treatment (0.6 mg/kg body weight/day through ip route). The taurine-supplemented nicotine-treated group received taurine at a dose of 50 mg/kg body weight/day orally before nicotine treatment (0.6 mg/kg body weight/day through ip route). All the treatments continued for 12 weeks (the duration of 6 spermatogenesis cycle). Nicotine dilution was made such that 1 ml of normal saline contained the required dose of nicotine. The selection of nicotine, hCG, and taurine doses and the routes of treatments were based on the prior studies (Jana et al., 2006; Manna et al., 2008; Seema et al., 2007; Sener et al., 2005a, 2005b). The  $LD_{50}$  of nicotine in human is 0.5–1.0 mg/kg body weight and the cigarette smoke contains an average of 0.5–1.6 mg of nicotine per cigarette. So we used 0.6 mg nicotine/kg body weight to make it relevant with the human exposure, though the  $LD_{50}$  of nicotine is 50 mg/kg body weight in rat (Okamoto et al., 1994). The ip administration required higher doses to produce toxicity because

of the presystemic metabolism of nicotine in liver before it reached the systemic venous circulation. There is also a continued interest in the use of transdermal nicotine patches to aid smoking cessation in man. Thus, in this chronic study, we used ip route to make it relevant with the exposure in a human chronic moderate smokers or the transdermal patch users. Moreover, ip injection was also predominantly used in testing of drugs or administration of systemic drugs because of easy administrations compared with other parenteral routes.

### 3.4 Animal sacrifice, collection of blood, brain, and reproductive organs.

The experiment was terminated at the end of 12 weeks, and all the animals were kept in euthanasia on the following morning by decapitation, preceded by anesthesia after overnight fast within 0800 h and 1000 h, to avoid any diurnal fluctuation in the concentrations of hormones. The body weight was recorded at the end of the treatment. The blood was collected from abdominal aorta from each of the animal using heparinized syringe (22-gauge needles) as soon as possible. The plasma samples were separated by centrifugation, frozen, and stored at  $-20^{\circ}\text{C}$  until all the samples had been collected for the determination of the different hormonal parameters. The paired testes and epididymes were dissected out quickly and washed in 0.9% (wt/vol) cold normal saline, pat dried, and the wet weight was taken in an electrical monopan balance. For histological analysis, the testes from six animals from each group were immediately fixed in buffer formalin for 24 h. The testes from the rest of the animals in each group were snap frozen and stored in liquid nitrogen till they were assayed for intratesticular testosterone (ITT), androgenic key enzyme activities, antioxidant enzyme activities, and Western blot and reverse transcriptase (RT)-PCR analysis of StAR protein.

### 3.5 Testicular histology and quantitative study of spermatogenesis.

Formalin-fixed testicular tissues were dehydrated stepwise in the graded ethanol and embedded in paraffin wax. A section of 5  $\mu\text{m}$  thickness was taken from the mid portion of each testicular tissue and stained with hematoxylin and eosin, followed by examination under a light microscope. The quantitative study of spermatogenesis was carried out by counting the relative number of each variety of germ cells at stage VII of the spermatogenesis cycle, i.e., type

A spermatogonia (Asg), preleptotene spermatocytes (pLSc), mid pachytene spermatocytes (mPSc), and step 7 spermatids (7Sd), according to the method stated elsewhere (Jana et al., 2006). The nuclei of different germ cells (except step 19 spermatids, which cannot be enumerated precisely) were counted in 20 round tubules in rat, and the data were expressed as numbers of germ cells per tubular cross section. Stage VII spermatogenesis was analyzed as it is highly susceptible to testosterone deficiency reflecting the final stages of spermatid maturation and thus provides evidence of spermatogenesis as a whole (Jana et al., 2006).

### 3.6 Epididymal sperm count and sperm abnormalities.

The epididymal sperms were counted by trypan blue exclusion method as described previously. The sperm numbers were expressed as per milliliter of suspension. The abnormal sperms were recognized and recorded. per Wyrobek and Bruce (1975) For each suspension, 100 sperms were examined at 400-fold magnifications with blue-green filters so a total of 1200 sperms were thus examined for each group. The abnormal sperms were readily recognized as sperms having following traits: (1) lack of usual hook, (2) having a banana-like form, (3) amorphous morphology, (4) folding among themselves, and (5) posses two tails.

### 3.7 Assay of plasma LH and FSH.

The LH and FSH concentrations in the plasma were measured using a double-antibody radioimmunoassay (RIA) following the standard methods stated with the reagents supplied by the Rat Pituitary Distribution Programme and National Institute of Diabetes and Digestive Kidney Diseases (Bethesda, MD). Intrarun precision coefficient of variations in LH or FSH assay was 3.5%.

#### 3.7.1 Assay of plasma and ITT concentrations.

The testis was homogenized in 0.5 ml of water using Teflon homogenizer fitted into a microfuge tube chilled in ice. Each sample was centrifuged at  $10,000 \times g$  for 10 min. The supernatant was removed, frozen, and stored at  $-80^{\circ}\text{C}$  until the hormone assay (Jana et al., 2006) RIA of testosterone was carried out using a double-antibody ( $^{125}\text{I}$ ) RIA. Because chromatographic purification of the sample was not performed, each testosterone value was the

sum of testosterone and dihydrotestosterone. The intra-assay coefficient of variation was 6.5%.

### 3.8 Western blot analysis of testicular StAR.

The testicular tissue was homogenized in  $\sim 20$  volumes of buffer using Tissue Protein Extraction Kit and cleared by centrifugation at  $10,000 \times g$  for 10 min at  $4^{\circ}\text{C}$  to produce a protein extract. The protein content of the preparation was determined with BCA Kit (Pierce) using bovine serum albumin (BSA) as a standard. Equal concentration of each sample (100  $\mu\text{g}$  protein) was resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. The membrane was blocked for 1 h in TBST (0.05% Tween 20 in Tris-buffered saline, pH 7.6) with 5% not fat milk and then incubated with 1:100 specific primary antibodies (rabbit polyclonal StAR,  $3\beta\text{-HSD}$ , and  $17\beta\text{-HSD}$  antiserums) at  $4^{\circ}\text{C}$  overnight and subsequently exposed to horseradish peroxidase-conjugated anti-rabbit IgG goat polyclonal secondary antibody (Holland and Storey (1981). In between each step, the membrane was washed with TBST about 10 min for three times. The bands were visualized with the enhanced chemiluminescence system, according to the manufacturer's instructions. Finally, the developed blots were subjected to densitometry using the  $\beta\text{-actin}$  as an internal control.

### 3.9 RNA isolation and PCR coamplification.

The total RNAs were extracted from rat testis tissues with TRIZOL reagent according to the manufacturer's instructions (Invitrogen). The quantity of RNA was estimated by spectrophotometer at 260 nm. The complementary DNAs (cDNAs) were obtained from reverse transcription of 5  $\mu\text{g}$  of total RNAs using random hexanucleotides as primers (5  $\mu\text{mol}$ ) in the presence of dNTPs (0.2 mmol), dithiothreitol (10 nmol), and Muloney murine leukemia virus reverse transcriptase (10 U/ $\mu\text{l}$ ) for 1 h at  $37^{\circ}\text{C}$ . For PCR analysis, the target gene (StAR) was coamplified with the standard gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The stock reaction (20  $\mu\text{l}$ ), containing 0.02 U/ $\mu\text{l}$  Taq polymerase, 1.5 mmol  $\text{MgCl}_2$ , 200  $\mu\text{mol}$  dNTP, 1  $\mu\text{mol}$  StAR primers, 10 nmol GAPDH primers, and 2  $\mu\text{l}$  reverse transcription mixture (cDNA), was prepared in ice. The PCR conditions were  $94^{\circ}\text{C}$  for 5 min, followed by 25 cycles of  $94^{\circ}\text{C}$  for 60 s,  $58^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 60 s, and then  $72^{\circ}\text{C}$  for 5 min. After amplification,

the PCR products were separated by electrophoresis on 2% agarose gels containing 0.005% of ethidium bromide, and the bands were visualized by UV light by densitometric scanning using gel doc scanner (Bio Rad Laboratories Inc., Japan). Primers used for rat StAR were forward: 5' TTGGCATACTCAACAACCA 3' and

reverse 5' ATGACACCGCTTTGCTCA 3' (gene bank accession no. NM031558; product size: 389 bp) and GAPDH were forward: 5' AGACAGCCGCATCTTCTTGT 3' and reverse 5' CTTGCCGTGGGTAGAGTCAT 3' (gene bank accession no. NM017008; product size: 207 bp)

Treatment	Relative testicular weights (testicular weight and body weight ratio %)	Spermatogenesis at stage VII (Numbers of germ cells/tubular cross section of testicular tissue)				Epididymal sperm counts (numbers/ml)
		Asg	pLSc	mPSc	7Sd	
Control	1.36 ± 0.05	0.65 ± 0.008	15.34 ± 0.06	18.65 ± 0.56	58.30 ± 2.21	12736 ± 421
Nicotine	1.10 ± 0.03*	0.31 ± 0.002*	6.45 ± 0.03*	7.92 ± 0.42*	30.26 ± 1.14*	7132 ± 256*
Nicotine with hCG	1.34 ± 0.02	0.57 ± 0.003*	13.18 ± 0.04*	15.06 ± 0.38*	51.14 ± 2.15*	10954 ± 321*
Nicotine with taurine	1.32 ± 0.03	0.48 ± 0.005*	10.02 ± 0.06*	11.82 ± 0.45*	45.03 ± 1.64*	10015 ± 214*

#### 4. RESULTS

##### 4.1 Food Consumption and Body Growth and Relative Testis Weight

None of the animals treated with nicotine showed any signs of morbidity or mortality during the studies. No differences in food consumption were seen in any of the group of animals throughout the experimental schedule. A reduction in relative testis weight (testicular weight and body weight ratio) was noted in nicotine-treated animals in comparison with the control indicating testicular atrophy and damage. However, cotreatment of hCG or taurine with nicotine significantly improved relative testicular weight when compared with the rats treated only with nicotine

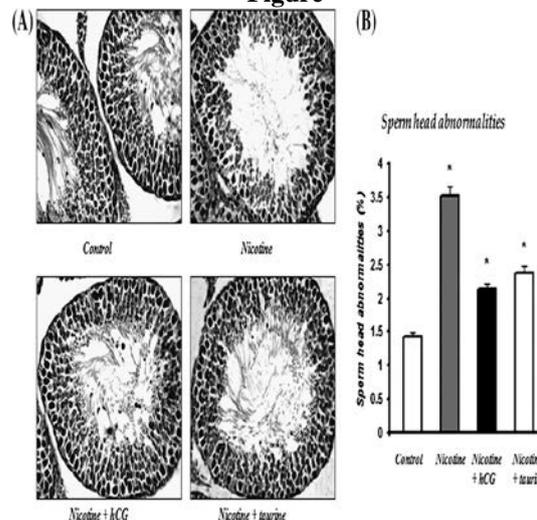
##### TABLE

The Changes in Relative Testicular Weights (Testicular Weight and Body Weight Ratio %), Quantitative analysis of spermatogenesis at stage 7 of spermatogenesis cycle (number of germ cells per tubular cross section of testicular tissue) and epididymis sperm count after repeated ip injections of normal saline (1ml/rat/day as control) or Nicotine at the dose of 0.6mg/kg body weight/day (nicotine with hCG) or Taurine at the dose of 5IU/kg body weight/day (nicotine with hCG) or Taurine at the dose of 50 mg/kg body weight/day for 12 weeks (about the duration of six spermatogenesis cycle) in mature male rats.

##### 4.2 EFFECT OF TESTICULAR HISTOLOGY

The histological study of the testicular section of the control rats demonstrated typical distinct cellular arrangements in seminiferous tubule with normal appearance of spermatogenesis. On the other hand, nicotine exposure caused seminiferous tubular derangement, sloughing of germ cells from germinal epithelium with disintegration of spermatocytes and spermatids resulting in disruption of spermatogenesis. hCG or taurine cosupplementation with nicotine illustrated complete restitution of seminiferous tubular histoarchitecture, moderately reestablished distinct cellular arrangements in seminiferous tubule along with restored spermatogenesis as a whole to some extent (Fig. 1A).

Figure



Representative photomicrographs of testicular sections (A) and the bar diagram of sperm morphological analysis (B) after repeated ip injections of normal saline (1ml/rat/day as control) or nicotine at the dose of 0.6mg/kg body weight/day(nicotine) along with hCG at the dose of 5IU/kg body weight /day (nicotine+hCG) or Taurine at the dose of 50mg/kg body weight /day(nicotine+taurine) for 12 weeks. Formalin fixed testicular tissues were stepwise dehydrated in graded ethanol and embedded in paraffin wax. The tissue of specific thickness were taken from the mid-portion of each testicular tissue and stained with haematoxylin and eosin and examined under a light microscope(400X). For sperm morphology analysis, sperms were collected from caudae of excised epididymis. Sperm smears were prepared, stained with 10% Giemsa diluted with Sorrensons buffer (pH6.8) and observed under microscope. Each value represents mean $\pm$ SEM, n=12; ANOVA followed by multiple two-tail test (p<0.05 as compared with respective control)

#### 4.3 EFFECT ON SPERMATOGENESIS

The quantitative study of spermatogenesis at stage VII revealed the detrimental effect of nicotine on the different generations of germ cells in the testicular spermatogenesis process. The number of counts of Asg, pLSc, mPSc, and 7Sd were significantly decreased in all the nicotine-treated animals in comparison with the control rats. However, hCG or taurine coadministration with nicotine protected the degeneration of different generations of germ cells to some extent and significantly increased the number of counts of Asg, pLSc, mPSc, and 7Sd toward control, although the counts showed a significant lower value when compared with the controls. Besides, the coadministration of hCG with nicotine to the rats showed a significant higher number of counts of pLSc, mPSc, and 7Sd when compared with the taurine plus nicotine-treated rats supporting that with respect to taurine, hCG has a strong stimulatory function on testicular spermatogenesis (Table 1).

#### 4.4 Effect on Epididymal Sperm Count and Sperm Head Abnormalities

The significant decrease of the epididymal sperm counts in all the nicotine-treated rats in comparison with the control supported the detrimental effects of nicotine on the spermatozoa. However, hCG or taurine coadministration with nicotine significantly restored this parameter toward the control level, though a significant

lower value was still persisting in comparison with the control. Moreover, the coadministration of hCG with nicotine demonstrated a significant higher count of epididymal sperms when compared with the taurine- and nicotine-treated rats, which confirmed the additional protective role of hCG on nicotine-induced testicular sperm degeneration in comparison with the taurine (Table 1). The sperm head abnormalities was monitored to ensure the detrimental effect of nicotine on spermatozoa. The sperm head abnormalities were increased significantly after nicotine treatment when compared with the controls. The folded and banana-like form of sperms were predominant following nicotine exposure. hCG or taurine supplementation significantly protected the sperms from the damaging effect of nicotine, though the hCG and the taurine, both were unable to protect the spermatozoa completely from the detrimental effects of nicotine as a higher percentage of sperm head abnormalities were evident in both the groups of animals treated with hCG or taurine along with nicotine (Fig. 1B).

#### 4.5 Effect on Plasma and Intratesticular Concentrations of Testosterone

The repeated exposure of nicotine significantly decreased the plasma and ITT concentrations in comparison with the control, suggesting the inhibitory role of nicotine on testicular androgenesis. However, hCG coadministration with nicotine significantly restored the plasma and ITT concentrations to the control level. Nevertheless, cotreatment of taurine with nicotine significantly augmented these parameters with respect to the animals treated with only nicotine. Moreover, significant lower values of these parameters were noted in the taurine with nicotine groups when compared with the control groups, suggesting that hCG has a stronger influence on testicular androgenesis than taurine (Figs. 2 and 3B).

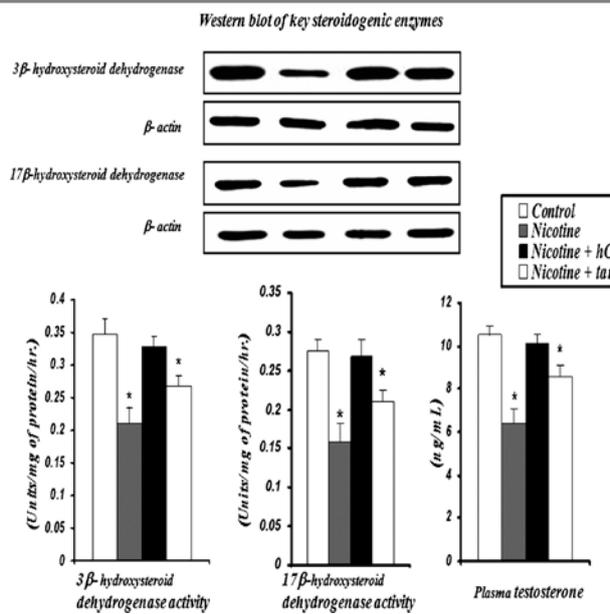


FIG. 2.

Western blot analysis in testicular androgenic enzymes (3β-HSD and 17β-HSD) expressions and diagrammatic representations of the changes in these enzymes activities (units per milligram of protein per hour) along with plasma concentrations of testosterone (nanogram per milliliter) after repeated ip injections of normal saline (1 ml/rat/day as control) or nicotine at the dose of 0.6 mg/kg body weight/day (nicotine) along with hCG at the dose of 5 IU/kg body weight/day (nicotine + hCG) or taurine at the dose of 50 mg/kg body weight/day (taurine + nicotine) for 12 weeks (about the duration of six spermatogenesis cycle) in mature male rats. Total protein was extracted from testis and 100 microgram of protein lysate was used for Western blot analysis. Each value represents mean ± SEM, n = 12; ANOVA followed by multiple two-tail t test (\*p < 0.05 as compared with respective control).

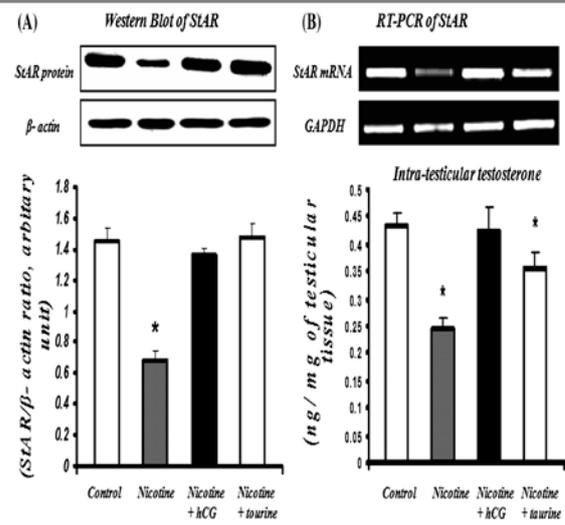


FIG. 3.

(A) Western blot analysis of testicular StAR expressions and (B) RT-PCR analysis of testicular mRNA expressions of StAR genes along with corresponding bar diagrammatic representation of ITT concentrations (nanogram per milligram of testicular tissue) following after repeated ip injections of normal saline (1 ml/rat/day as control) or nicotine at the dose of 0.6 mg/kg body weight/day (nicotine) along with hCG at the dose of 5 IU/kg body weight/day (nicotine + hCG) or taurine at the dose of 50 mg/kg body weight/day (taurine + nicotine) for 12 weeks (about the duration of six spermatogenesis cycle) in mature male rats. Total protein was extracted from testis and 100 μg of protein lysate was used for Western blot analysis. The relative intensity of each band was quantified by densitometry using the β-actin as an internal control. The corresponding histogram showing means ± SEM, of relative arbitrary units of the bands for four immunoblots, were conducted with separate experiments in each group and expressed as arbitrary units ("StAR:β-actin ratio"). For RT-PCR analysis, total RNA isolated from testis was reverse transcribed, and the cDNA obtained was subjected to PCR for StAR with a standard gene GAPDH. The PCR products were separated by electrophoresis on 2% agarose gels containing ethidium bromide visualized by UV-light by densitometric scanning using gel doc scanner (\*p < 0.05 as compared with respective control).

## 5. CONCLUSION

From the present study, it may be suggested that nicotine impairs testicular structure and functions. It appears, therefore, that nicotine treatment produces degenerative changes in the germ cells and inhibits the androgen production acting primarily at the level of hypothalamicopituitary axis to inhibit the release of gonadotropins. The other way can be that the nicotine is directly modulating the extent of testicular lipid peroxidation through free radicals generation, which may interfere in the testicular spermatogenesis, steroidogenesis, and StAR gene expression. However, not only nicotine but also many other compounds (i.e., hydrocarbons, aldehydes, ketones, etc.) present in cigarette smoke may also be responsible for the testicular dysfunction and that needs further investigation. Finally, this study recommends further studies on the compound present in cigarette smoke in order to understand the harmful effects of smoking on testicular functions and in order to extrapolate the results of the present study to human chronic moderate smokers; further study in this area is an imperative necessity.

## 6. REFERENCES

1. Alvarez JG, Storey BT. Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. *Biol. Reprod.* 1983;29:548-555. Abstract
2. Angelova M, Nedkova V, Bojinova AS, Milanova VN, Stoyanova V (2012) Smoking and thiocyanates in school children. *Trakia Journal of Sciences* 10: 52-58.
3. Arabi M . Nicotinic infertility: assessing DNA and plasma membrane integrity of human spermatozoa. *Andrologia* 2004;36:305-310.
4. Armitage P, Berry G, Matthews JNS (2000) *Statistical methods in medical research*:4th edition. Oxford; Malden, MA: Blackwell Sciencepp: 760-783.
5. Aruldas MM, Subramanian S, Sekar P, Vengatesh G, Chandrahasan G, Govindarajulu P, Akbarsh a MA. Chronic chromium exposure-induced changes in testicular histoarchitecture are associated with oxidative Stress: study in a non-human primate (*Macaca adiata* Geoffroy. *Human Reprod.* 2005;20:2801-2813.
6. Asiyah HA, Syazana NS, Hashida NH, DurriyyahSharifah HA, Kamaruddin MY (2011) Effects of nicotine and Gelam honey on testis parameters and sperm qualities of juvenile rats. *Scientific Research and Essays* 26: 5471-5474.
7. Aydos K, Güven MC, Can B, Ergün A (2001) Nicotine toxicity to the ultrastructure of the testis in rats. *BJU Int* 88:622-626.
8. Aydos K, Güven MC, Can B, Ergün A. Nicotine toxicity to the ultra-structure of the testis in rats. *BJU Int.* 2001;88:622-626.
9. Bandopadhyay G, Sinha S, Chattopadhyay BD, Chakraborty A (2008) Role of cucumin against nicotine induced genotoxicity on rat liver under restricted dietary protein. *EurJ Pharmacol*588:151-157.
10. Bandopadhyay G, Sinha S, Chattopadhyay BD, Chakraborty A. Role of cucumin against nicotine induced genotoxicity on rat liver under restricted dietary protein. *Eur. J. Pharmacol.* 2008;588:151-157.
11. Barrow PC, Barbellion S, Stadler J (2011) Preclinical evaluation of juvenile toxicity. *Methods MolBiol* 691:17-35.
12. Basta PV, Basham KB, Ross WP, Brust ME, Navarro HA (2000) Gestational nicotine exposure alone or in combination with ethanol down-modulates offspring immune function. *Int J Immunopharmacol* 22: 159-169.