

The Effect of Prenatal Exposure to Nicotine versus Thiocyanate on Pituitary-Testicular Axis of Juvenile Albino Rats: A Comparative Histological and Electron Microscopic Study

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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 work aims at clarifying whether the prenatal exposure to either nicotine or thiocyanate could be the responsible factor in induction of the possible effect of smoking on spermatogenesis and the cytoarchitecture of the testis and hence influencing the male fertility later on. Twenty pregnant rats were randomly divided into four groups from gestational day 4. Groups Ia and b served as control. Group II animals were injected with nicotine (6 mg/kg/day) subcutaneously as a single dose. Group III Animals were treated with oral potassium thiocyanate (25 mg/rat/day) as a single dose through gastric gavages. Five male litters from each group (one/each pregnant rat) were sacrificed at the age of 1 month. Pituitary and testicular tissue samples were subjected to histological and electron microscopic study. The counts of different cells of seminiferous tubules were statistically analyzed for significance. Results revealed apoptotic changes at the subcellular level of the pituitary gonadotrophs and different spermatogenic and Sertoli cells of the testis. The number of Sertoli and different spermatogenic cells- rather than spermatogonia- showed a very highly significant decrease in the nicotine treated group in comparison to the control. The dense cells revealed a very highly significant increase in the nicotine treated group. In conclusion, the results of the present study were more consistent with a suggested dual effect of nicotine on the pituitary-testicular axis rather than a purely direct effect on the testis. On the other hand, thiocyanate induced subcellular effect on the testicular histology with minimal affection of gonadotrophs.

Keywords: Nicotine; Thiocyanate; Prenatal; Testis; Gonadotrophs

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 [1]. 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 [2]. Maternal cigarette smoking during pregnancy has multiple deleterious effects on the offspring, which persist into adulthood [3].

The male reproductive system is known to be highly sensitive to many chemicals [4]. Growing evidence indicates that over 4000 chemical compounds are usually concentrated and condensed into tobacco mixture [5]. Nicotine has always been identified as the most powerful toxin of cigarette smoke [6]. Thiocyanate, which develops in the vapour phase of tobacco smoke, exerts adverse cardiovascular effects with consequent increased damage caused by nicotine and carbon monoxide. In addition, it reacts at different steps of body metabolism [7]. Structural changes in DNA are observed among active and passive smokers at puberty [8]. Smoking has multiple effects on hormones, some of which are associated with important clinical implications [9].

According to investigators, knowledge of the exact chemical in tobacco which intensely affects the male fertility is still contradictory. The present work aims at clarifying whether the prenatal exposure to either nicotine or thiocyanate could be the responsible factor in induction of the possible effect of smoking on spermatogenesis and the cytoarchitecture of the testis and hence influencing the male fertility.

Materials and Methods

Test drugs

1. Nicotine solution (Sigma-Aldrich chemical company, St.Louis,

USA) freshly diluted with sterile saline solution.

2. Potassium thiocyanate (Sigma-Aldrich chemical company, St.Louis, USA) in 4 mg tablets freshly dissolved in distilled water.

Animals

A total number of acclimatized 20 pregnant female albino rats aged four months (average weight 150-200 gm) were used in the present study. They were randomly selected from mixed breeding cages after detection of the post-coital vaginal mucous plug, indicator of day one of pregnancy. Rats were obtained and housed separately in labeled cages in the animal house in the faculty of Medicine, Assiut University. They were kept under normal day and light cycles and appropriate temperature, fed rat chow and water *ad libitum*.

Experimental design

The experiment protocol has been approved by the institutional ethics committee of Assiut University before the start of the experiment. The pregnant rats were randomly divided into four groups from gestational day 4. Rats of each group were treated as follows until day 20 of gestation:

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Group Ia (n=5): served as control for nicotine-injected group. Rats were injected with 0.5 ml sterile saline solution subcutaneously at the same time where group II rats were injected with the drug.

Group Ib (n=5): Served as control for thiocyanate-treated group. Animals were given distilled water orally at the same time where group III rats were given the drug.

Group II (n=5): Animals were injected with nicotine (6 mg/kg/day) subcutaneously as a single dose [10].

Group III (n=5): Animals were treated with oral potassium thiocyanate (25 mg/rat/day) as a single dose through gastric gavages [11].

Five male litters from each group (one/each pregnant rat) were sacrificed at the age of 1 month. Rats were anesthetized with ether, thorax opened, heart exposed and the appropriate fixative was perfused. After perfusion, right testes and the pituitary glands were dissected free for processing.

Electron microscopic study

Immediately, specimens of the pituitary glands and the testes were fixed in 5% cold glutaraldehyde for at least 24 hours then washed in 3-4 changes of cacodylate buffer (pH 7.2) for 20 minutes each and post fixed in cold osmium tetroxide for 2 hours. They were washed in four changes of cacodylate buffer for 20 minutes each. Dehydration was done by using ascending grades of alcohol (30,50 and 70%) each for 2 hours and then 90%, 100% two changes 30 minutes each. Embedding was done in Epon 812 using gelatin capsules for polymerization. The embedded samples were kept in incubator at 35°C for one day, at 45°C for another day and for three days at 60°C.

Semithin sections (0.5-1 microns) were cut by using LKB ultramicrotome, stained with toluidine blue [12] then examined and photographed with light microscope.

Ultrathin sections (50-80 nm) from selected areas of the trimmed blocks were made and collected on copper grids. The ultra-thin sections were contrasted with uranyl acetate for 10 minutes then lead citrate for 5 minutes [13]. Finally, sections were examined and photographed by transmission electron microscopy (Jeol, E.M.-100 CX11; Japanese Electron Optic Laboratory, Tokyo, Japan) and photographed at 80 kV. Electron micrographs were taken using a series of magnifications ranging from 3600 to 7200X for each specimen. The ultra thin processing, examination and photography has been performed in the service laboratory, National Research Center, Cairo.

Morphometric study

Morphometric measurements of digitalized images from toluidine blue stained sections of the testicular tissue were performed. A "Leica QWin 500" image analyzer computer system (England) and a Zeiss light microscope (Germany) using a magnification of X1000 were used in this process. Quantitative morphometric study included counting of Sertoli and different spermatogenic cells (spermatogonia, primary spermatocytes, spermatids and dense cells) in ten complete sections of seminiferous tubules per subject.

Statistical analysis

Data were presented as means $\pm$ standard deviation (SD). Comparison between the two groups was carried out by student's T test. The Statistical Package for the Social Sciences (SPSS for windows Version 22; SPSS Inc., Chicago, Illinois, USA) was used. Results were considered significant when probability (p) was ≤ 0.05 , highly significant when (p) ≤ 0.01 and very highly significant when p ≤ 0.001 [14].

Results

Pituitary gland

Microscopic examination of the control groups, I a&b demonstrated the same architecture and cellularity of the pituitary gland with evident blood sinusoids. The ultra-thin sections examination revealed that gonadotrophs had rounded euchromatic nuclei. Their cytoplasm contained the characteristic rounded secretory granules of variable size and electron density (Figure 1). The cytoplasm also exhibited Golgi body, numerous mitochondria and rough endoplasmic reticulum (Figure 2).

Nicotine treated group (GII) revealed some gonadotrophs with distorted nuclei associated with indefinite nuclear outline. Clumps of heterochromatin were peripherally arranged associated with empty vacuoles in the nucleoplasm. The cytoplasm contained destructed mitochondria and dilated rough endoplasmic reticulum with evident whorly arrangement (Figure 3).

Thiocyanate treated group (GIII) showed gonadotrophs with oval euchromatic nuclei and intact nuclear envelope. The cytoplasm exhibited dilated destructed mitochondria, dilated rER, ribosomes and the characteristic granules (Figure 4).

Testis

Histological examination: Control groups Seminiferous tubules had regular basal membrane and well distinct myoid cells. Normal spermatogenic series formed of 4-6 layers at different stages of spermatogenesis (spermatogonia, primary spermatocytes with characteristic curled chromatin and rounded spermatids) were observed. Pyramidal Sertoli cells were resting on the basement membrane and exhibited prominent nucleoli. There was complete absence of sperms in the non-canalized seminiferous tubules (Figure 5).

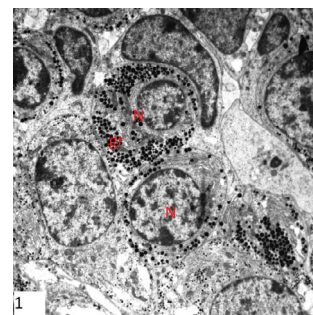


Figure 1: An electron photomicrograph of a section in the pituitary gland of group I showing a group of gonadotrophs. The nuclei are rounded euchromatic (N) and the cytoplasm contains rounded secretory granules of variable size and electron density (gr) (X3600).

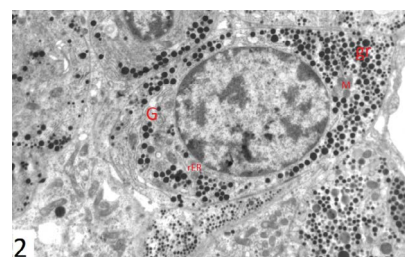


Figure 2: An electron photomicrograph of a section in a gonadotroph of group I showing rounded euchromatic nucleus with double layer nuclear envelope. The cytoplasm contains Golgi body (G), numerous mitochondria (M), rough endoplasmic reticulum (rER) and characteristic secretory granules (gr) (X7200).

In nicotine-treated group (GII) many seminiferous tubules suffered from reduction and disorganization in the spermatogenic layers with complete absence of spermatid. The spermatogonia, Sertoli and some of the primary spermatocytes seemed similar to control. Dense cells with pyknotic nuclei were obviously and frequently noted in some tubules in addition to intercellular vacuoles. Still the basement membrane showed intact trilaminar structure (Figure 6).

Thiocyanate treated group (GIII) showed seminiferous tubules that exhibited vacuoles of different sizes in between and surrounding the spermatogenic cells. The cells appeared disorganized and dissociated from one another. Moreover, large multinucleated cells were occasionally noted. There was complete absence of spermatids except for a degenerated one seen inside this multinucleated cell. On the other hand, some of the spermatogonia, primary spermatocytes, and Sertoli cells seemed to be more or less similar to the control. Other cells exhibited pyknotic nuclei and densely stained cytoplasm (Figure 7).

Electron microscopic examination

Control groups (GIa and b): Sertoli cells were clearly resting on the basal membrane with its characteristic tri-laminar appearance and few myoid cells. Sertoli cells had folded nucleus with well distinct nucleolus. The cytoplasm exhibited many mitochondria and ribosomes. Spermatogonia type B were resting on the basement membrane and showed rounded euchromatic nuclei with peripheral clumps of heterochromatin. Primary spermatocytes had rounded euchromatic nuclei with mitochondria and ribosomes in their cytoplasm. Spermatids had rounded nuclei characterized by the presence of small rounded clump of heterochromatin (B body) in addition to small peripherally arranged mitochondria (Figure 8).

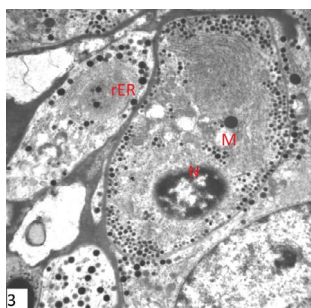


Figure 3: An electron photomicrograph of a section in the gonadotrophs of group II showing distorted nucleus with indefinite nuclear outline (N). Clumps of electron dense heterochromatin were peripherally arranged with loss of the nuclear sap. The cytoplasm contained destructed mitochondria (M). The rough endoplasmic reticulum is dilated and whorly arranged (rER) (X3600).

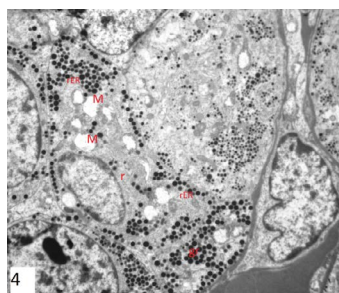


Figure 4: An electron photomicrograph of a section in the pituitary gland of group III showing oval euchromatic nucleus with intact nuclear envelope. The cytoplasm exhibits dilated destructed mitochondria (M), dilated rough endoplasmic reticulum (rER) and ribosomes (r). The characteristic secretory granules (gr) are still detected (X3600).

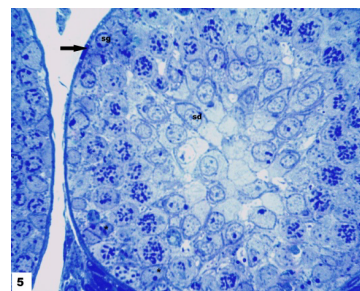


Figure 5: A photomicrograph of a semithin section in seminiferous tubule of group I showing regular basal membrane and well distinct myoid cell (†). Normal spermatogenic cells formed of 4-6 layers at different stages of spermatogenesis, Spermatogonia (Sg), primary spermatocytes (Sc) with curled chromatin and spermatids (Sd). Pyramidal Sertoli cells exhibit prominent nucleolus resting on the basement membrane (*). Note the absence of sperms in the non-canalized seminiferous tubule (Toluidine blue, X1000).

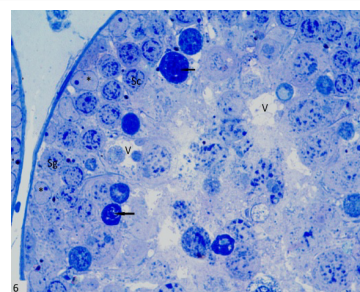


Figure 6: A photomicrograph of a semithin section in seminiferous tubule of group II revealing intact basement membrane with reduction and disorganization in the spermatogenic layers with complete absence of spermatids. The spermatogonia (Sg), Sertoli (*) and some of the primary spermatocytes (Sc) seemed similar to control. Dense cells with pyknotic nuclei are obviously noted in the tubule (†). Note the presence of vacuoles (v) in between the cells (Toluidine blue, X1000).

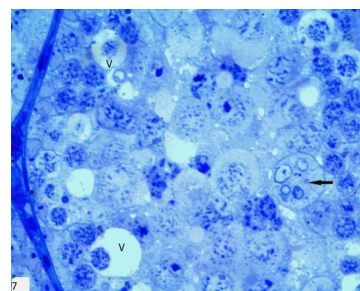


Figure 7: A photomicrograph of a section in seminiferous tubule of group III revealing vacuoles of different sizes in between and surrounding the spermatogenic cells (v). The spermatogenic cells show variable nuclear staining affinity. The cells appear disorganized and dissociated from each other. Note the presence of large cell containing multiple nuclei (†) and absence of spermatids except for one degenerated spermatid is seen inside this multinucleated cell (Toluidine blue, X1000).

It was rather unexpected but interesting to find accidentally a late spermatid starting spermiogenesis at this age. The spermatid exhibited rounded nucleus with acrosomal cap and acrosomal vesicle, well distinct flagellum in addition to many out pouching and shedded cytoplasmic plebs (Figure 9). Interstitial cells of Leydig had rounded euchromatic nuclei and their cytoplasm appeared full of lipid droplets, well developed smooth endoplasmic reticulum and few lysosomes (Figure 10).

Nicotine treated group (GII): Sertoli cells had indented nuclei with

indistinct nuclear envelope and vacuolated cytoplasm. Spermatogonia had oval nuclei with irregular nuclear envelope (Figure 11). Some primary spermatocytes looked like the control. Damaged cells with irregular nuclei were detected. The nuclei contained amalgamated clumps of heterochromatin. The cytoplasm appeared granular, electron dense and devoid of cell organelles (Figures 12 and 13). Interstitial cells of Leydig showed variable degree of affection. Some cells were completely ruptured and the remaining cytoplasm contained dilated smooth endoplasmic reticulum and lysosomes. Other cells exhibited oval irregular nuclei with peripheral clumps of heterochromatin.

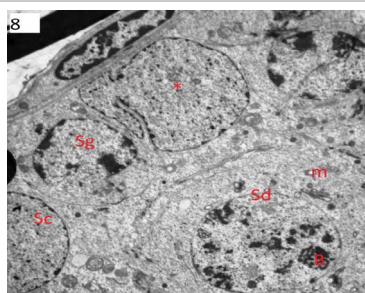


Figure 8: An electron photomicrograph of a section in a seminiferous tubule of group I demonstrating Sertoli cell with folded nucleus (*), Spermatogonia type B (Sg), part of primary spermatocyte (Sc) and early spermatid (Sd). The spermatid has rounded euchromatic nucleus containing small rounded clump of heterochromatin (B-body). The cytoplasm exhibits small peripherally arranged mitochondria (m) (X3600).

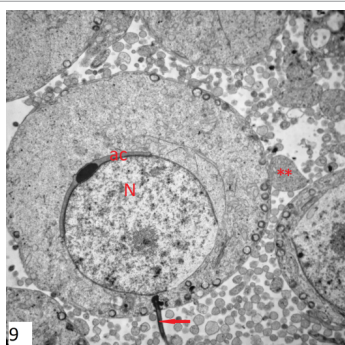


Figure 9: An electron photomicrograph of a section in the late spermatid of group I showing rounded nucleus (N). The spermiogenesis is manifested by the presence of well developed acrosomal cap and vesicle (ac), flagellum (↑) and many shed cytoplasmic blebs (**) (X3600).

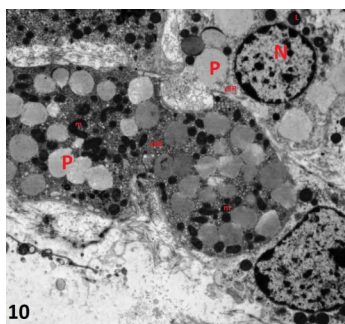


Figure 10: An electron photomicrograph of a section in the interstitial cells of Leydig of group I showing rounded euchromatic nucleus (N). The cytoplasm is rich in smooth endoplasmic reticulum (sER), mitochondria (m) and some lysosomes (L). Note the presence of numerous lipid droplets occupying most of the cytoplasm (P) (X4800).

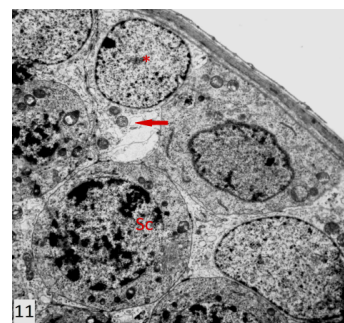


Figure 11: An electron photomicrograph of a section in the testis of group II showing a primary spermatocyte (Sc) very similar to that of the control. Sertoli cell (*) has indented nucleus with indistinct double layer nuclear envelope. The cell has relative electron dense vacuolated cytoplasm (↑) (X3600).

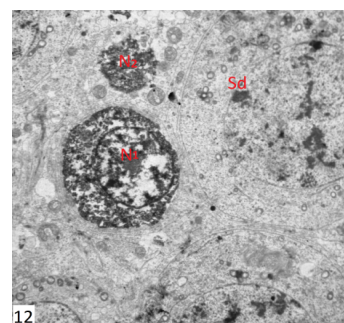


Figure 12: An electron photomicrograph of a section in the testis of group II showing normal spermatid (Sd). There are two cells which had electron dense nuclei with amalgamation and segmentation of the chromatin clumps (N1 & N2). The cytoplasm of N1 is electron dense and fragmented with loss of the organelles. While the other cell N2 exhibits vacuolated cytoplasm with numerous mitochondria (X3600).

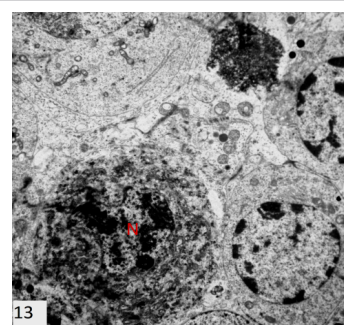


Figure 13: An electron photomicrograph of a section in the testis of group II showing damaged cell with hyperchromatic irregular nucleus (N) is surrounded by degenerated electron dense cytoplasm (X3600).

The cytoplasm was electron dense contained numerous smooth endoplasmic reticulum, mitochondria and numerous secondary lysosomes but lacked lipid droplets (Figure 14).

Thiocyanate treated group (GIII): Sertoli cells had irregular nuclei and the cytoplasm exhibited mitochondria with destructed cristae. Tight junctions were still detected in spite of the presence of intercellular vacuoles (Figure 15).

Primary spermatocytes had rounded euchromatic nuclei with destructed mitochondria in their cytoplasm. Damaged cells with complete nuclear lysis could be noted frequently (Figure 16).

Spermatids had irregular distorted nuclei associated with loss of the nuclear envelope. The cytoplasm exhibited characteristic small numerous mitochondria (Figure 17). Leydig's cells had irregular euchromatic nuclei with plenty of smooth endoplasmic reticulum, destructed mitochondria, few lipid droplets (Figure 18).

Morphometric results and statistical analysis: The percentage of the counted numbers of different cell types of the control and the two experimental groups are represented in Figure 19.

Statistical analysis of the number of Sertoli and different spermatogenic cells-rather than spermatogonia- showed a very highly significant decrease in the nicotine treated group in comparison to the

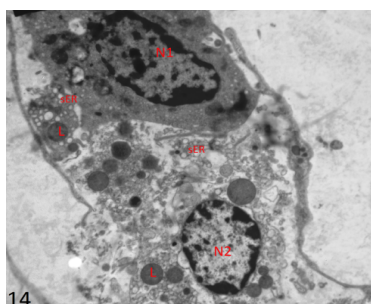


Figure 14: An electron photomicrograph of a section in the interstitial tissue of the testis of group II showing two Leydig's cells. One (N1) has irregular oval nucleus and electron dense cytoplasm containing numerous smooth endoplasmic reticulum (sER) and secondary lysosomes (L). There is absence of lipid droplets. N2 was ruptured with dilated smooth endoplasmic reticulum (sER) and lysosomes (L) in the cytoplasm (X4800).

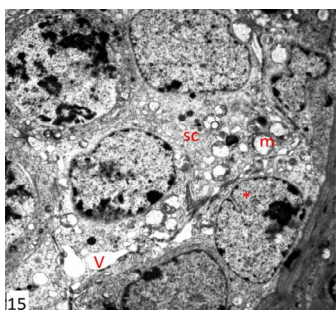


Figure 15: An electron photomicrograph of a section in the testis of group III showing Sertoli cell with irregular nucleus (*). The cytoplasm exhibits mitochondria with destructed cristae (m). Primary spermatocyte has euchromatic nucleus (Sc). Note the presence of vacuoles (v) among the cells (X3600).

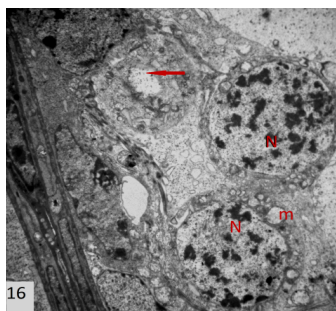


Figure 16: An electron photomicrograph of a section in the testis of group III showing two primary spermatocytes have rounded euchromatic nuclei (N) and destructed mitochondria (m) in the cytoplasm. Note the presence of a damaged cell with complete nuclear lysis (↑) (X3600).

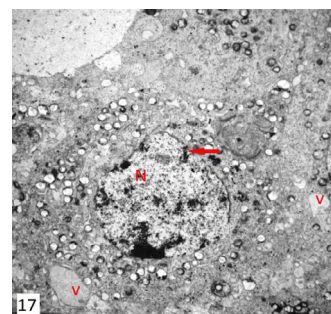


Figure 17: An electron photomicrograph of a section in spermatid of group III showing irregular nucleus with loss of the nuclear envelope (N). Note the presence of irregular cell outline (↑) and intercellular vacuoles (v) (X3600).

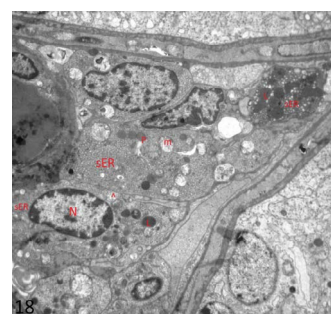


Figure 18: An electron photomicrograph of a section in the interstitial tissue of testis of group III showing Leydig's cells. One cell has irregular euchromatic nucleus (N). Its cytoplasm exhibits smooth endoplasmic reticulum (sER), destructed mitochondria (m), few lipid droplets (P) and lysosomes (L). Note the presence of mitochondria with tubular cristae (^) (X4800).

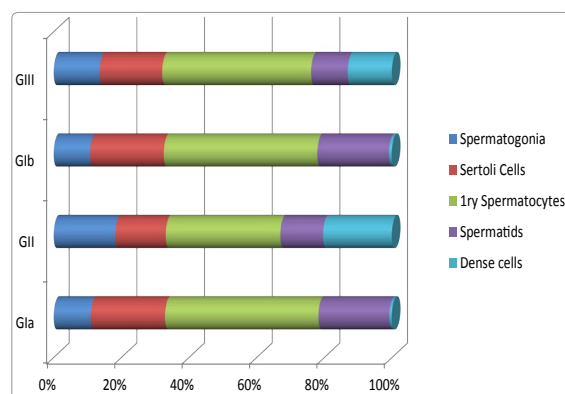


Figure 19: The percentage of cell counts of each cell type in all groups.

control. However spermatogonia demonstrated a slight increase in their number which was statistically non-significant. On the other hand the dense cells revealed a very highly significant increase in the nicotine treated group (Table 1).

In case of thiocyanate treated group all the counted cells, except the dense cells, showed a decrease in comparison to its control. This decrease was non-significant only in case of spermatogonia but of very high significance in the rest. The dense cell count revealed a very highly significant increase as well (Table 2).

When the nicotine and thiocyanate groups were compared together it was clear that the number of spermatogonia was significantly higher

Values Type of cells	Cell count (Mean ± SD)		P Value
	Group Ia (Control)	GROUP II (Nicotine)	
Spermatogonia	4.56 ± 2.555	5.75 ± 3.274	0.333
Sertoli cells	10.00 ± 3.240	4.42 ± 1.213	0.0001***
Primary spermatocytes	19.78 ± 7.190	10.00 ± 4.273	0.0001***
Spermatids	8.89 ± 6.412	3.79 ± 2.934	0.003***
Dense cells	0.33 ± .5	6.67 ± 4.440	0.0001***

***Very highly significant when $p \leq 0.001$

Table 1: Statistical comparison between the mean ± standard deviation of different cell counts of the nicotine treated group and its control.

Values Type of cell	Cell count (Mean ± SD)		P Value
	Group Ib (Control)	GROUP III (thiocyanate)	
Spermatogonia	4.67 ± 1.803	3.63 ± 3.270	0.386
Sertolicells	11.44 ± 2.603	4.95 ± 2.272	0.0001***
Primary spermatocytes	19.22 ± 6.457	11.11 ± 3.665	0.0001***
Spermatids	11.44 ± 4.667	2.89 ± 3.680	0.0001***
Dense cells	0.78 ± .667	3.79 ± 2.016	0.001***

***very highly significant when $p \leq 0.001$

Table 2: Statistical comparison between the mean±standard deviation of different cell counts of the thiocyanate treated group and its control.

Values Type of cells	Cell count (Mean ± SD)		P Value
	Group II (Nicotine)	GROUP III (Thiocyanate)	
Spermatogonia	5.75 ± 3.274	3.63 ± 3.270	0.041*
Sertoli cells	4.42 ± 1.213	4.95 ± 2.272	0.331
Primary spermatocytes	10.00 ± 4.273	11.11 ± 3.665	0.376
Spermatids	3.79 ± 2.934	2.89 ± 3.680	0.379
Dense cells	6.67 ± 4.440	3.79 ± 2.016	0.012*

**Significance was considered $P \leq 0.05$

Table 3: Statistical comparison between the mean±standard deviation of different cell counts of the nicotine- versus thiocyanate treated groups.

in the nicotine treated group. The rest of the cell counts didn't reveal any significant changes between the two experimental groups. However, the increase in dense cell count recorded a significantly higher value in nicotine treated group as well (Table 3).

Discussion

Despite remarkable advances in the treatment of severe male factor infertility, significant numbers of men cannot be effectively treated because of deficiencies in our understanding of testicular failure. Therefore, the mechanisms responsible for abnormal spermatogenesis should be clarified in order to develop and improve treatment options [15].

In mammals, the functional maturation of male reproductive organs is under strict regulation by hormones of the pituitary–gonadal axis in a species-specific mode. With stages regulated by testosterone, it is quite logical that any affection to this axis brings about a constitutional delay in the spermatogenic process [16].

Rats were chosen as the experimental animal in this research for their well-defined reproductive systems and the fact that compounds which could cause infertility in human males were also found to be active in rats [17]. Five to six developmental time periods, indicated in post natal days (PNDs), are recognized in the male rat and were referenced when characterizing microscopic changes. These stages include the neonatal (PND 0–7), infantile (PND 8–20), juvenile (PND 21–32), peri-pubertal (PND 33–55), and late pubertal (PND

56–70) periods [18,19]. These periods loosely correlate with similar developmental stages in humans [20,21].

Examination of the pituitary sections in the current work revealed changes in both nicotine- and thiocyanate-treated groups with more evident affection in the former. The gonadotroph of the nicotine treated group showed distorted nucleus associated with loss of the nuclear envelope. There are empty vacuoles in the nucleoplasm. The cytoplasm contained destructed mitochondria. The rough endoplasmic reticulum was dilated and whorly arranged. The thiocyanate treated group examination revealed much less affection. This could be attributed to apoptosis or programmed cell death. Apoptosis is morphologically characterized by disruption of the cell skeleton, cell shrinkage and nuclear condensation [15].

The apoptotic changes may result partially from the deleterious toxic effects of nicotine through increased production of reactive oxygen species (ROS) [22,23]. The free radicals produced would lead to cellular injury. The structure and fluidity of cell membrane would be altered when membrane phospholipids and lipid peroxidation were disintegrated, marked by the release of unsaturated fatty acid from membrane phospholipids [24].

Although nicotine readily crosses the placenta, there is no evidence that it is metabolized by the placenta. It is therefore likely that the blood concentrations of nicotine reached in the fetus are similar to those in the mother. Furthermore, enzymatic protection mechanisms of the fetus are not well developed [8]. Metabolism of nicotine in the fetal liver is slow and a longer half-life of nicotine in the fetus can be expected. This is confirmed by the higher concentrations of nicotine in fetal tissue compared to maternal blood levels [25].

Similarly, there is higher level of thiocyanate in the amniotic fluid which accumulates in the rat embryo. This may be due to the pH gradient in the maternal plasma, which is more acidic in relation to the pH of the fetus [26].

The adverse effect of nicotine on the pituitary hormones is still unclear. It is not settled yet whether nicotine affects the pulsatile gonadotropin secretion or not [27]. However, recently, it has been established that nicotine inhibits pulsatile LH secretion in human males but not in human females [28]. In this respect the current results may add subcellular explanation to the low level of plasma LH and FSH concentration following nicotine exposure.

The available literature did not provide clear evidences regarding the direct effect of thiocyanate on the hypothalamic-pituitary axis. This turned on the curiosity about the possible effect of thiocyanate on the testis. It was justified that administration of thiocyanate to pregnant rats promoted toxic effects in the fetuses in many organs rather than reproductive organs [29].

In addition, rapidly dividing cells are more vulnerable to the effects of foreign substances such as nicotine [30]. Now it is more interesting to show our finding on examining testicular sections.

The results of the control testis run parallel to histological finding in the Juvenile Period in rats: PND 21 to PND 32. The juvenile period is characterized by maintenance of the first wave of spermatogenesis to round spermatids. The presence of numerous large pachytene spermatocytes and the formation of secondary spermatocytes are characteristics of this stage [31].

The interesting finding of very early spermiogenesis and the appearance of acrosomal vesicle and cap and flagellar formation in the late spermatid in the control of the current study was unexpected at

this age. But, this was supported by the studies of Igwebuike and his group in 2011 in addition to Ramos et al. in 2012 [32,33]. Both teams demonstrated sperms during their experiments in different testicular sections at same age. Still it was postulated earlier that the start of canalization and lumen formation in the seminiferous tubules starts on day 15 and at day 31 some of the tubules [34].

In mammalian species, the adult Leydig cells -the only source of testosterone- are absent at birth. Th. Adult Leydig cells are the dominant cell type of the Leydig cell lineage from Postnatal Days 26–28 onward. The total number and the size of lipid droplets are more in Leydig's cells of the juvenile rat than that in the adult rat [35]. This clearly explains the plenty of lipid droplet in the Leydig cells found in control group of the present study.

The result of the current work of nicotine treated group revealed that the seminiferous tubules exhibited vacuoles of different sizes. Cells appeared disorganized and dissociated from each other and exhibited pyknotic nuclei and densely stained cytoplasm. These findings are in agreement with a previous study which reported that male Wistar rats treated with varied doses of nicotine for 30 days also showed a reduction in germ cells and spermatids of their seminiferous tubules [17]. On the other hand, some temporary effects on testes histopathology of rats aged 7 weeks when exposed to nicotine were also reported [24].

Abnormalities in the present Sertoli cells differentiation, polymorphic mitochondria, residual bodies, irregular cristae and an electron-dense matrix on exposure to nicotine are similar to those after experimental ischaemia, suggesting that vascular lesions may be important in nicotine toxicity [36].

Our result as regard the Leydig cells with few lipid droplets are in agreement with reduction in the number of these cytoplasmic lipid droplets occurred in nicotine-exposed newborn rats [37].

Indeed, it has been shown that ROS inhibits steroidogenesis by interfering with cholesterol transport to mitochondria and/or catalytic function of P450 enzymes. Moreover, ROS also inhibits steroidogenesis during cholesterol transfer by suppression of the steroidogenic acute regulatory (StAR) protein expression in Leydig cells. However, the data concerning the effects of cigarette smoking and nicotine on testosterone levels are conflicting [38].

Moreover, it was reported that smoking has been associated with testicular degeneration, disorganization of the cytoarchitecture and decreased serum testosterone levels [39]. Arrest of spermatogenesis may probably occur as a consequence of reduction in serum testosterone. It has been shown to be essential for the completion of meiotic division during spermatogenesis [4].

The examination of the thiocyanate treated group revealed Sertoli cells had irregular nucleus. The cytoplasm exhibited mitochondria with destructed cristae. Tight junctions were detected in spite of the presence of intercellular vacuoles. Damaged cells with complete nuclear lysis could be noted as well. The cytoplasm showed mitochondria with destructed cristae and large vacuoles. Some nuclei appeared distorted associated with loss of the nuclear envelope. Leydig's cells had irregular nuclei and their cytoplasm exhibited plenty of smooth endoplasmic reticulum, destructed mitochondria, few lipid droplets and lysosomes.

Multinucleated cells observed in the thiocyanate treated group could be explained as a normal change. Multinucleates due to formations of young spermatids present in sexually immature normal rat testes. Multinucleation apparently results from a failure of intercellular bridges to break down in the early phases of spermiogenesis [40].

To our knowledge there is a great shortage in data explaining the effect of thiocyanate on the juvenile testis and testicular hormones. Still it was proved that Low-level exposure to thiocyanate is positively associated with thyroid stimulating hormone TSH and negatively associated with free T4 in first trimester of pregnancy [41]. Furthermore, thiocyanate can cross the placenta in the rat and produces alterations directly in the fetal thyroid gland [42]. In addition, thiocyanate inhibits NA/I symporter potentially reducing iodine transport and blocking the production of thyroid hormone [43].

It was proved by Kumar and his group in 2014 that the presence of thyroid hormone receptors on germ cells suggests a probable role of thyroid hormones in sustaining different population of germ cells [44].

In 2008, Sahoo et al. [45] reported that altered thyroid function during early stages of development adversely affect testicular growth, physiology, and antioxidant defense status. This compromised testicular antioxidant status might have contributed to poor growth and development by affecting the spermatogenesis and steroidogenesis in rats before puberty. Furthermore, this was recently endorsed by a similar study that showed specifically reduced germ cell number, complete absence of round spermatids, decreased seminiferous tubule diameter, and decreased testosterone level [46]. It has been reported that hypothyroidism prolongs the proliferative phase of Sertoli cells and inhibits their differentiation, leading to a higher number of Sertoli cells [47].

Thus, it could be hypothesized that the lesions observed in the offspring from the experimental groups were induced by an indirect toxic effect of thiocyanate. However, in contrast to this some authors considered this possibility is unlikely. As among all the parameters analyzed in reproductive toxicity, none showed significant differences between the control and experimental groups receiving thiocyanate [26].

Still the statistical analysis of the morphometric results revealed significant decrease in the number of the spermatogenic cells in the treated groups apart from spermatogonia. It is to be recalled that the stem spermatogonia, maintain their numbers by self-renewal while some differentiate. This differentiation may be blocked in different ways. Undifferentiated spermatogonia proliferate but their numbers remain relatively constant because of apoptosis [48]. The constant number or even sometimes increased number of spermatogonia may be noted in cases of hypogonadism [49].

Because each Sertoli cell supports a limited number of germ cells in a species-specific manner, it is currently accepted that the number of Sertoli cells established during testis development determines the rate of sperm production in sexually mature animals [50]. In addition, reports declared that spermatogenic efficiency is usually positively correlated with the number of germ cells supported by each Sertoli cell [51]. This rings an important alarm that the prepubertal insult to the number of Sertoli cells may cause permanent effect on the fertility of the individuals.

The number of spermatogonial generations, the rate of germ cell loss during spermatogenesis, the number of Sertoli cells per gram of testis and the size of Sertoli cells are also important parameters for the determination of spermatogenic efficiency [52].

Conclusion

The results of the present study were more consistent with a suggested dual effect of nicotine on the pituitary-testicular axis rather than a purely direct effect on the testis. On the other hand, thiocyanate induced subcellular effect on the testicular histology with minimal affection of gonadotrophs. It is recommended to design further studies

to investigate the possible effect of thiocyanate on the pituitary-thyroid-testicular crosstalk.

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