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Developmental Characteristics of Rat Testicular Tissue and the Impact of Chronic Noise Stress Exposure in the Prenatal and Postnatal Periods

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Abstract

Recently claims have been raised that noise stress may exert adverse effects on the reproductive functions. The present study was undertaken to investigate first; developmental histological characteristics of rat testis at 3 critical age periods namely; full term fetus, pre-pubertal and adult stages. Second, the impact of chronic noise stress exposure on testicular tissue at these age periods.

Albino rats were used and assigned into 3 groups; pregnant rats (to obtain fetuses at day 20 of gestation), pre-pubertal and adult male rats. Each group was subdivided into control and experimental subgroups. Experimental subgroups were exposed to noise stress (100dB), 6 hours daily for 30 days for Pre-pubertal and adult groups. While, pregnant rats were exposed to noise stress from day 8-20 of gestation then rats were sacrificed, testicles of fetuses were extracted. At the end of experiment, all testicles were extracted from pre-pubertal and adult rats and processed for paraffin block embedding, light microscopic examination and immunohistochemical staining for apoptosis (TUNNEL assay). Histomorphometric measurements were done by image analysis. The current study demonstrated detailed histological, immunohistochemical and histomorphometric differences across developmental ages examined. The study also showed that noise stress induced several histopathological changes of testicular tissue, increased number of apoptotic germ cells, and decreased size and number of seminiferous tubules in prenatal and pre-pubertal groups. In Conclusion, the study demonstrated detailed histological features of early rat testicular development. Also, revealed that noise stress induced damage to rat testicular tissue at the three examined ages. Current findings support the notion that noise may adversely affect reproduction and fertility of exposed-males.

Recommendations: Noise as a form of stress is not well investigated. People should be aware of the hazards of exposure to high intensity sounds especially young age and pregnant females to ensure them a future healthy reproductive life.

Keywords: Rat; Fetus; Prepubertal; Adult; Testis; Chronic noise stress; Tunnel assay; Histomorphometry; Histology

Introduction

Stress is the sensation of strain or pressure. It is categorized into two types according to its duration. Acute stress is an abrupt, short-lasting (seconds to hours timescale), and isolated. Whereas chronic stress is recurring, persisting for several hours a day for weeks, months or longer [1]. Noise is the most prevalent insidious natural pollutant [2]. Living organisms are exposed daily to potentially hazardous noise levels coming from the environment [3].

The testis is the cardinal organ of reproduction and endocrinal functions in males. It is highly susceptible to damage due to its highly proliferative nature [4]. Claims have been raised that noise stress exposure could have an adverse effect on male reproductive organs. Several studies reported structural and functional affection of testicular tissue due to noise stress exposure in adult males [5-9]. However, adverse effects of chronic noise stress exposure on the testis in prenatal or pre-pubertal periods are deficient. Available studies only focused on the adverse pregnancy outcomes as a result of noise stress exposure during pregnancy. Increased risk of miscarriages, birth defects, pre-term birth, low-birth weight or intra-uterine growth retardation are the observations recorded [10].

Owing to the diffuse exposure to noise in modern life and rarity of histological studies on the effect of noise stress exposure in prenatal and pre-pubertal periods particularly on the testis.

The present research was conducted to investigate first; developmental histological characteristics of rat testis at 3 critical age periods namely; full term fetus, pre-pubertal and adult stages. Second, the impact of noise stress exposure on testicular tissues at these age periods.

The aim was also to reveal the most susceptible period of lifetime the testis is prone to injury. Histological, histomorphometric and immunohistochemical techniques were employed in this study.

Materials and Methods

Animals

Male albino rats were obtained and locally bred at the experimental unit-Medical Research Center, Faculty of Medicine, Ain Shams University. Rats used in the current study were of three age groups; pregnant rats from day 8-20 of gestation (to obtain fetuses at day 20 of gestation), pre-pubertal rats aged 3 weeks and about 150 g body

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weight (BW), and adult rats aged 3 months and weighing around 150-200 g BW. Rats were left in the animal house for one week before start of the experiment for acclimatization. Animals were maintained under pathogen free conditions with free access to food and water in a room maintained at a temperature of 21 ± 3°C. Animals were housed in standard laboratory conditions in a 12 h light: 12 h dark cycle.

The experimental design and procedures were approved by the Animal Care and Use Committee of Faculty of Medicine, Ain Shams University.

Induction of pregnancy

Adult cycling female albino rats weighing about 200 g were caged over- night with adult male fertile rat (two females/ male). In the next morning female rats that showed sperms in the vaginal plugs were considered to be in day 0 of pregnancy. Pregnant rats were divided into 2 subgroups; control and experimental. Only the experimental subgroup was exposed to noise from the 8^{th} - 20^{th} day of pregnancy. At day 20 of gestation all pregnant rats of control and experimental groups were sacrificed and fetuses were extracted from uteri of all rats.

Noise-stress induction procedure and duration

Rat experimental groups were exposed to noise using two loud speakers (15 watt) mounted 40 cm a part on the opposite sides of the cage and driven by a white noise generator (range 0-26 KHz) installed (suspended) 30 cm above the cage. The noise level was set at an intensity of 100 dB uniformly throughout the cage and monitored by a sound level meter (Radioshack Model 33-4050, China). The period of noise exposure was 6 hours daily from 8 am to 2 pm for all groups. Pregnant mothers were exposed to noise stress for 12 days (from day 8 – day 20 of gestation) while pre-bubertal and adult rats were exposed to noise for 4 weeks [8].

Pregnant mothers were not exposed to noise stress from day 1 of pregnancy to avoid abortion as reported previously [11].

Animal groups

Group I: full term fetal (pre-natal) groups (12 rats) which were subdivided into:

Ia (control): their pregnant mothers were not exposed to any stressors.

Ib (experimental): their pregnant mothers were exposed to noise stress from day 8 to day 20 of gestation.

Group II: Pre-pubertal groups (12 rats, 3 weeks old) which were subdivided into:

IIa (control): rats were not exposed to any stressors.

IIb (experimental): rats were exposed to noise stress for 4 weeks.

Group III: Adult groups (12 rats, 3 month old) which were subdivided into:

IIIa (control males): rats were not exposed to any stressors.

IIIb (experimental males): rats were exposed to noise stress for 4 weeks.

Tissue processing for light microscopy

At the end of the experiment, animals of all groups (fetuses, prepubertal and adult rats) were sacrificed using over dose of ether inhalation. Immediately, incision of the scrotal sac was done followed by extraction of the testes that were fixed in 10% neutral formalin in water. Tissue specimens were then processed for preparation of paraffin blocks and sectioned at 5-µm thick sections. Sections were stained with haematoxylin and eosin (Hx and E), periodic acid Schiff (PAS) and Masson's trichrome [12] then examined by the light microscope.

Light microscopic digital images were captured then transformed into 32-bit color images using a digital camera (FUJIX HC-2000; Fuji Photo Film, Tokyo, Japan) attached to a light microscope (VANOX AHBS3; Olympus, Tokyo, Japan).

Characterization of the histological findings was done as described previously.

Immunohistochemistry

Primary antibody: TUNEL Assay (Cat# A23210; APO-BrdUTM TUNEL Assay kit, Invitrogen).

Apoptotic fragmentation of DNA in histological sections was evaluated by TUNEL analysis (Terminal Deoxynucleotide Transferase dUTP Nick End Labeling). Standard protocol for paraffin sections was done as described before [13].

Staining technique

Immunohistochemical analysis was performed using streptavidin-biotin-peroxidase complex method (SAB). Paraffin sections were de-paraffinised in xylene and subjected to heat mediated antigen retrieval in sodium citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0). Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 20 minutes. To minimize non-specific reaction, sections initially were incubated with fetal calf serum for 30 min. at 37°C. Sections were then incubated with the primary antibody over night at 4°C and rinsed in Phosphate Buffer Saline (PBS). Then the secondary antibody was applied to the slides for 30 min. at 37°C. Finally sections were treated with diaminobenzidine-H_2O_2 mixture and counterstained with hematoxylin. Negative control staining was performed after omitting the primary antibody [14].

Image analysis and histomorphometry

Hx and E stained sections were used for assessment by image analyzer computer system (Leica Qwin 500, Cambridge, UK). Six readings in non-overlapping fields were obtained for each specimen of all groups examined.

The following histomorphometric parameters were assessed per cut section of the testis at a magnification of ×100 for all groups of the study:

1. Number of seminiferous tubules.

2. Surface area of the seminiferous tubules in μm^2.

3. Perimeter of the seminifersous tubules in µm.

4. Number of positive immune stained cells (Tunnel assay) at magnification of × 400.

Statistical analysis

Analysis of variance (one-way ANOVA) was used followed by post-hoc two-tailed Student's t test to compare between groups. Data were presented in the form of mean ± standard mean of error (SEM). The difference was significant at $P < 0.05$ and highly significant at $P < 0.001$.

Results

Light microscopic and immunohistochemical results

Group Ia: control 20 days old rat fetuses (control prenatal): Examination of Hx and E stained sections of this group revealed that, the testis was covered by a well-defined capsule formed of superficial fibrous coat, the tunica albuginea that consisted of thin compact bundles of collagen fibers. Deep to it was tunica vasculosa surrounding the parenchyma of the testis. Tunica vasculosa was composed of highly vascular loose areolar connective tissue with relatively large sized blood vessels. Testicular parenchyma was composed of several seminiferous follicles. The follicles were of variable shapes and sizes. Most of them appeared oval in shape and were arranged in groups separated by interstitial tissue (Figures 1,2).

The seminiferous follicles were surrounded by a well-defined basement membrane surrounded by spindle-shaped cells. The follicles were lined by two types of cells. The majority of them were the supporting cells which were peripherally arranged perpendicular to the basement membrane. They were small in size with deeply stained nuclei. The other type of cells was the gonocytes. These cells occupied a central position in the follicles. Apparently they were about two to five cells per follicle. They had pale stained cytoplasm. Their nuclei were pale stained with marginally arranged chromatin and a single nucleolus. The seminiferous follicles were separated by interstitial tissue consisting of connective tissue in which clumps of interstitial cells of Leydig were observed arranged in groups around blood vessels. They possessed deeply stained nuclei with one or two nucleoli (Figure 3).

PAS stained sections of this group showed strong intense positive reaction in the capsule especially tunica albuginea and in the basement membranes surrounding seminiferous follicles. Gonocytes appear darker than the peripherally arranged pale supporting cells (Figure 4).

Masson's trichrome stained sections showed dense deposition of collagen in the capsule and the basement membranes around most of the follicles (Figure 2b).

Stained sections of this group using TUNEL assay revealed negative immune reaction in the follicles and the interstitial tissue as well (Figure 5).

Group Ib: experimental 20 days old rat fetuses (pre-natal experimental): Examination of Hx and E stained sections of the testes of 20 days old albino rat fetuses whose mothers were exposed to noise from day 8 to day 20 of pregnancy, revealed that tunica albuginea was apparently thicker than control group. Tunica vasculosa showed more loose connective tissue compared to control group (Figure 6a and 6b). Testicular tissue consisted of clusters of irregular-shaped seminiferous follicles most of them was fused with each other (Figure 7).

The basement membrane with some supporting cells was sometimes detached from cells of seminiferous follicles. Supporting cells were frequently over crowded with degenerated hyperchromatic nuclei. Few supporting cells were seen sloughed into the lumen. The gonocytes also showed deeply stained hyperchromatic nuclei and vacuolated cytoplasm (Figure 8).

PAS stained sections showed faint reaction in capsule, basement membrane of follicles compared with control. Germ cells only appear dark coloured (Figure 9).

Masson's trichrome stained sections showed increased deposition of collagen fibers in tunica albuginea compared with control (Figure 6b).

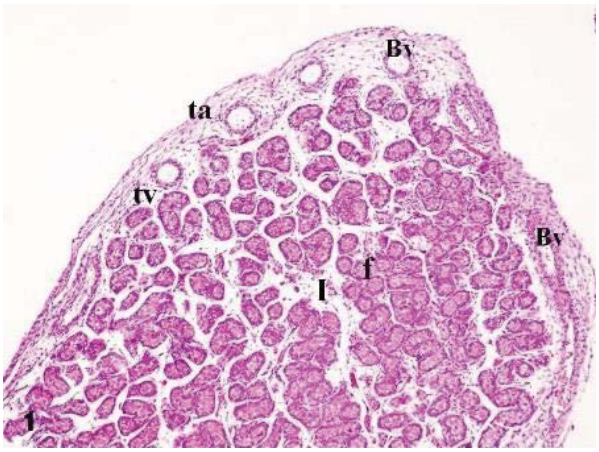


Figure 1: Photomicrograph of a section in testis of 20 days old rat fetus from control mother (group Ia) showing the capsule formed of tunica albuginea (ta) and the tunica vasculosa (tv), containing blood vessels (Bv), surrounding the testicular tissue. Most of the seminiferous follicles (f) are arranged in the groups separated by interstitial tissue (I). Hx & E x100

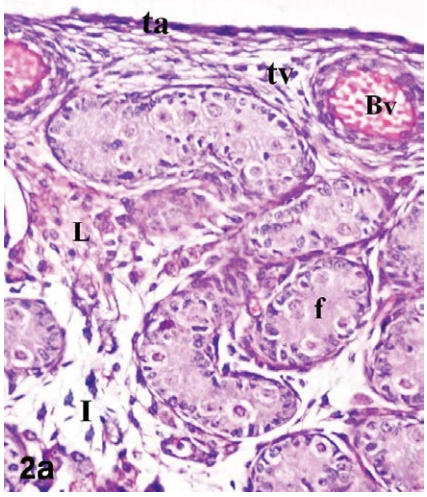
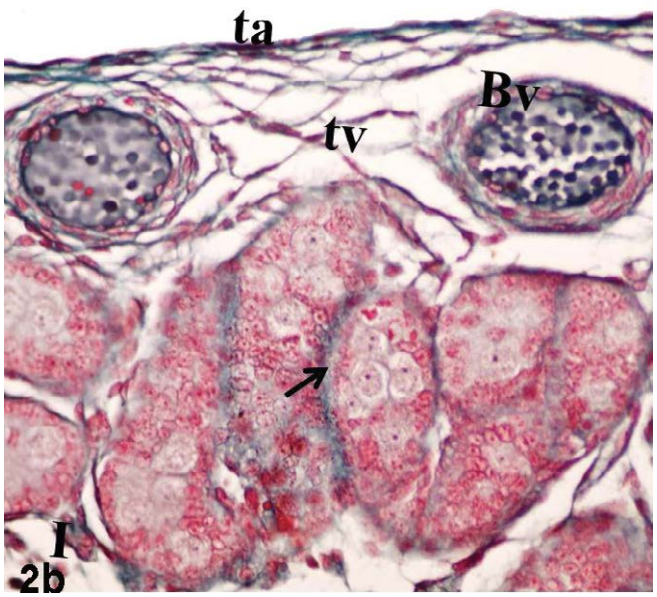


Figure 2a and b: a) Photomicrograph of section in the testis of 20 days old albino rat fetus from control mother (group Ia) showing the capsule formed of tunica albuginea (ta) and the tunica vasculosa (tv) surrounding the testicular parenchyma. The interstitial tissue (I) in between the seminiferous follicles (f) contain groups of interstitial cells of Leydig (L). Bv= blood vessel.

Stained sections of this group using TUNEL assay showed few scattered positive immune stained cells (apoptotic cells) within seminiferous follicles (Figure 10).

Group IIa: control 7 weeks old rats: (control pre-pubertal): Examination of Hx and E stained sections of testes of control rats aged 7 weeks showed that the testis was covered externally with a capsule formed of tunica albuginea, which appeared more compact and thicker than the prenatal group and tunica vasculosa, which was obviously diminished in thickness as age advanced and it was formed of loosely arranged connective tissue (Figures 11a and 11b).

Testicular tissue became formed of closely packed rounded seminiferous tubules separated by narrow interstitial tissue (Figure 11). Most of the tubules lumina were nearly empty while few tubules showed tails of spermatids in their lumina (Figure 12). The seminiferous tubules



b) Distribution of collagen fibers is seen. Basement membranes around the follicles (arrow). Hx&E; **b)** Masson's Trichrome X 400.

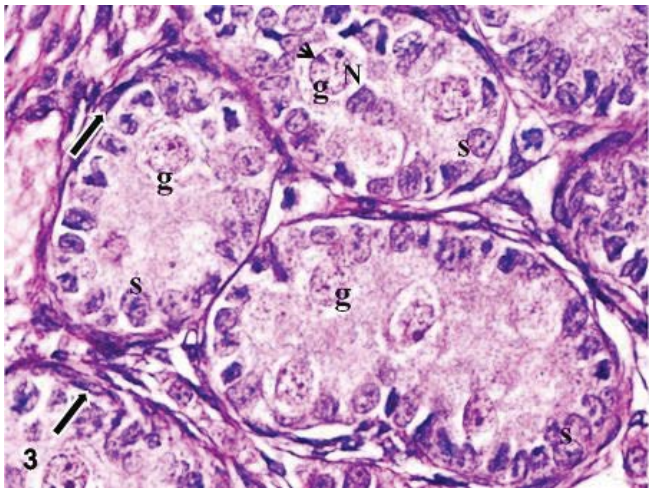


Figure 3: Photomicrograph of section in the testis of 20 days old albino rat fetus (group Ia) showing spindle-shaped cells (arrows) surrounding the basement membrane. The follicles are formed of two types of cells; the small sized peripherally arranged dark supporting cells (s) and the large centrally placed gonocytes (g) having pale cytoplasm and pale nucleus (N) with the presence of nucleolus (arrow head). Hx & E x1000

were lined by basement membrane surrounded by one layer of spindle-shaped cells. Stratified germinal epithelium resting on the basement membrane was formed of spermatogenic cells and Sertoli cells. The germinal epithelium was formed of several types of spermatogenic cells. These were the spermatogonia, the spermatocytes, the round immature spermatids and elongated spermatids in some tubules. Spermatogonia laid along the basement membrane with their longitudinal axis parallel to it. They were small in size with an oval deeply stained nucleus containing coarse clumps of marginal chromatin with single nucleolus. Different stages of spermatocytes were also detected. Most of them was large sized with large round centrally located nuclei with coarse punctate chromatin patches. Some of them had small nuclei with deeply stained chromatin (Figure 13). Round immature spermatids were present near the lumen of the tubule forming 2 to 3 rows. They were round or polygonal in shape. The cells contained scanty cytoplasm with pale stained nuclei and fine chromatin network. Few elongated mature spermatids were detected in this age group (Figure 14). Sertoli cells were hardly detected. They were pyramidal cells with basal triangular nuclei aligned perpendicular to the basement membrane of the tubules (Figures 12 and 13). The seminiferous tubules were separated by interstitial tissue formed of connective tissue and groups of interstitial cells of Leydig which were polygonal in shape with rounded eccentric nuclei and apparent nucleoli (Figure 15).

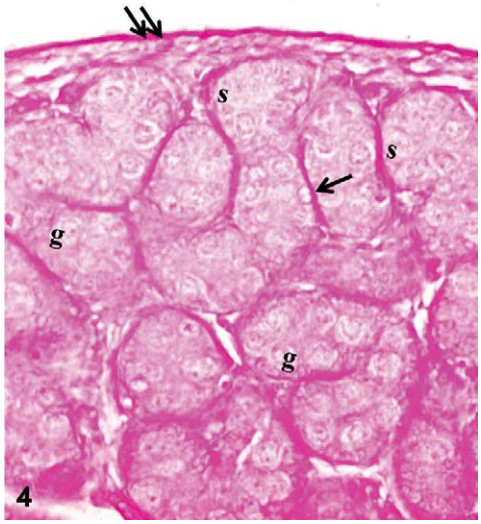


Figure 4: Photomicrograph of section in the testis of 20 days old albino rat fetus (group Ia) showing strong intense positive reaction in the capsule (two arrows) and in the basement membrane (arrow) surrounding the follicles. Gonocytes (g) can be easily distinguished from the supporting cells (s) which appear pale. PAS x400

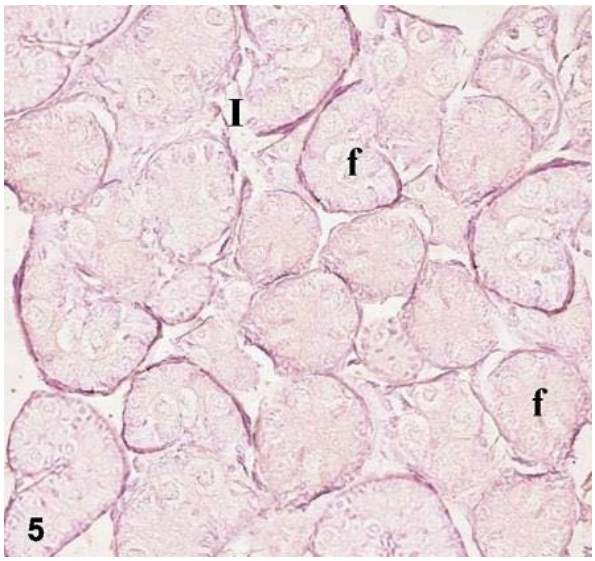


Figure 5: Photomicrograph of section in the testis of 20 days old albino rat fetus (group Ia) showing negative immune reaction in the seminiferous follicles (f) as well as the interstitial tissue (I). TUNEL Assay x400

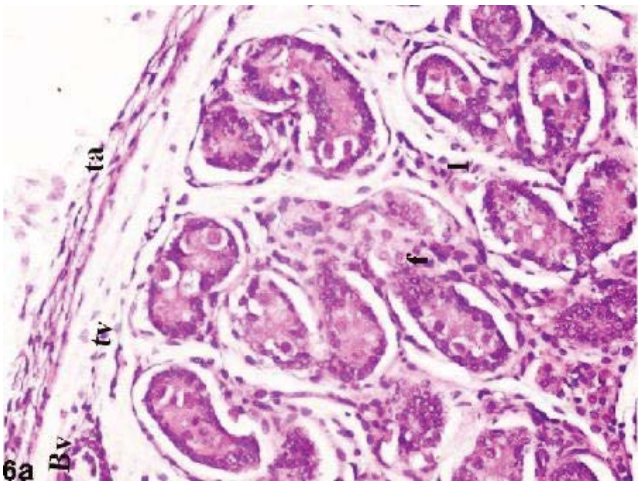
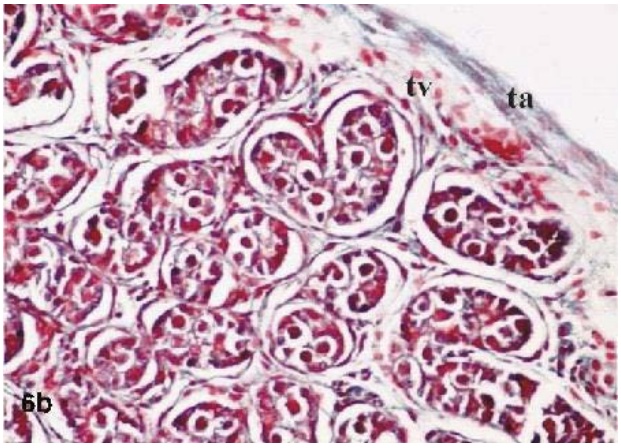


Figure 6a and b: a) Photomicrograph of section in the testis of 20 days old albino rat fetus (group Ib) showing thickened tunica albuginea (ta) which consists of collagen fibers with closely packed spindle-shaped fibroblasts and thinner tunica vasculosa (tv) which is formed of lightly stained loose connective tissue and blood vessels (Bv) compared with control. The seminiferous follicles (f) are arranged in clusters separated by interstitial tissue (I). b) collagen fibers in tunica albuginea (ta) & between follicles. **a)** Hx&E;



b) Masson's Trichrome X 400

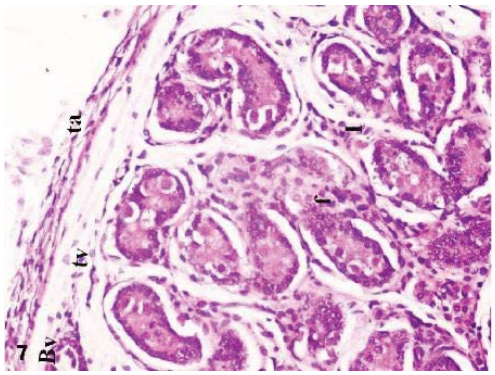


Figure 7: Photomicrograph of section in the testis of 20 days old albino rat fetus (group Ib) showing tunica albuginea (ta) and tunica vasculosa (tv). The seminiferous follicles (f) are irregular in outlines with occasional amalgamated ones. Interstitial tissue (I), blood vessels (Bv). Hx & E x400

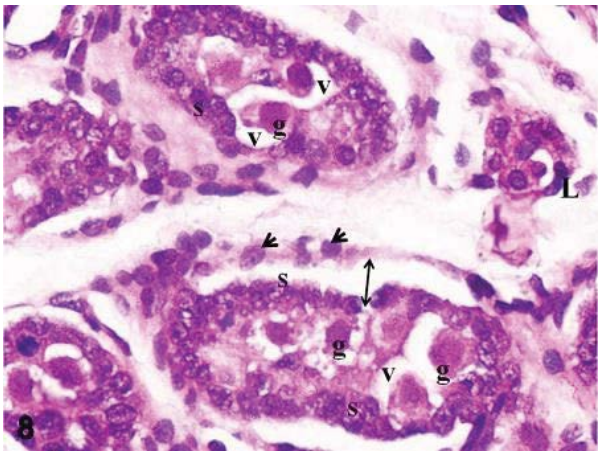


Figure 8: Photomicrograph of section in testis of 20 days old albino rat fetus (group Ib) showing cytoplasmic vacuolation (v) and dark nuclei of most of the gonocytes (g). Supporting cells (s) appear over crowded, some are degenerated with hyperchromatic nuclei. Separation of basement membrane (double head arrow) with some supporting cells (arrow heads) from the follicle is seen. Interstitial cells of Leydig (L) show indefinite cell boundries with cytoplasmic degeneration. Hx & E x1000

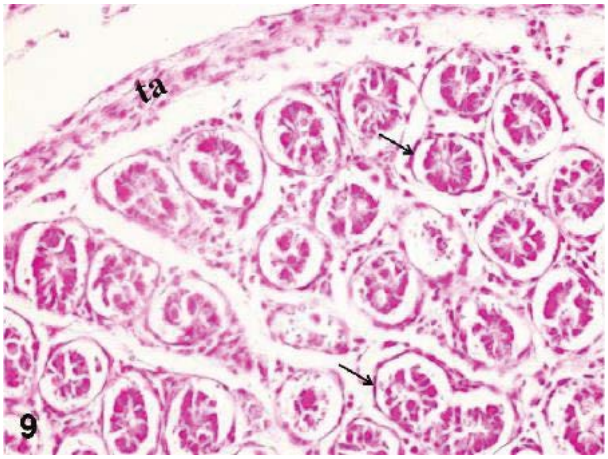


Figure 9: Photomicrograph of section in the testis of 20 days old albino rat fetus (group Ib) showing faint reaction of the capsule mainly tunica albuginea (ta), basement membrane of the follicles (arrows) only germ cells appear dark. PAS x400

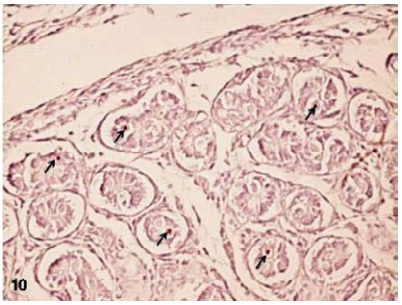


Figure 10: Photomicrograph of section in the testis of 20 days old albino rat fetus (group Ib) showing few scattered positive (apoptotic) immune stained cells (arrows) within the seminiferous follicles. TUNEL assay x400

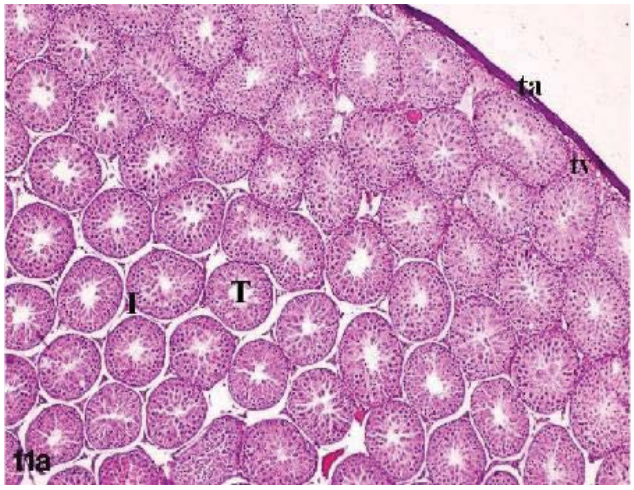
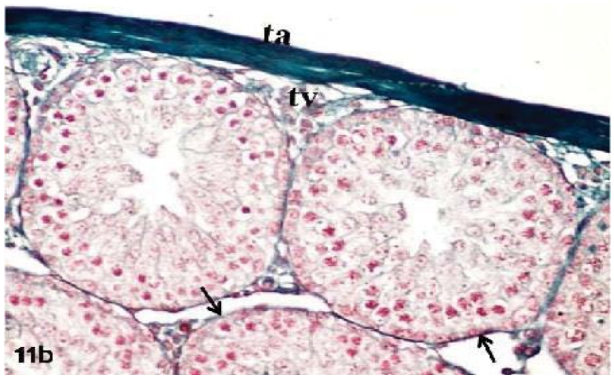


Figure 11 a and b: a) Photomicrograph of a section in testis of control albino rat aged 7 weeks (group IIa) showing capsule of thick dense tunica albuginea (ta) and thin tunica vasculosa (tv) surrounding testicular tissue. The seminiferous tubules (T) appear closely packed with the interstitial tissue (I) in between.



b) dense collagen deposition in tunica albuginea (ta) and mild in tunica vasculosa (tv). Minimal deposition of collagen fibers seen in basement membranes of tubules (arrows). **a)** Hx & E x100; **b)** Masson Trichrome x400

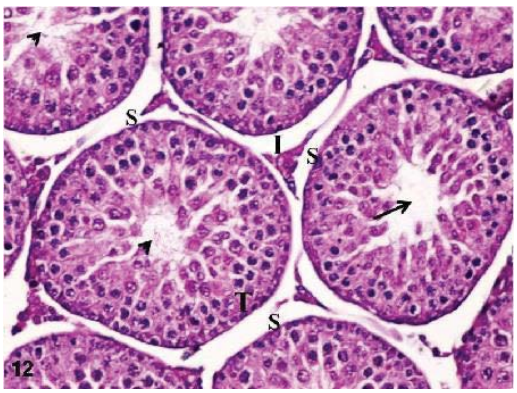


Figure 12: Photomicrograph of a section in testis of group IIa rats showing seminiferous tubules (T) with regular contour lined by stratified germinal epithelium and separated by interstitial tissue (I). Note the empty lumen of one tubule (arrow) and the tails of spermatids in the lumina of other tubules (arrow heads). S=Sertoli cells. Hx & E x400

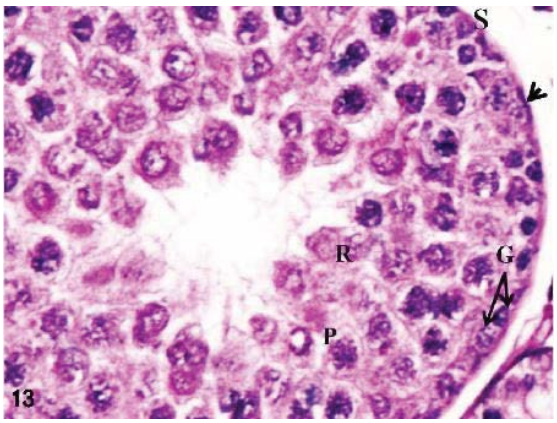


Figure 13: Photomicrograph of a section in testis of group IIa rats showing different stages of spermatogenic cells; spermatogonia (G), spermatocytes (P) and round spermatids (R). Spindle-shaped cells (arrow head) with dark flat nuclei are seen surrounding the basement membrane. S= Sertoli cell. Hx & E x1000

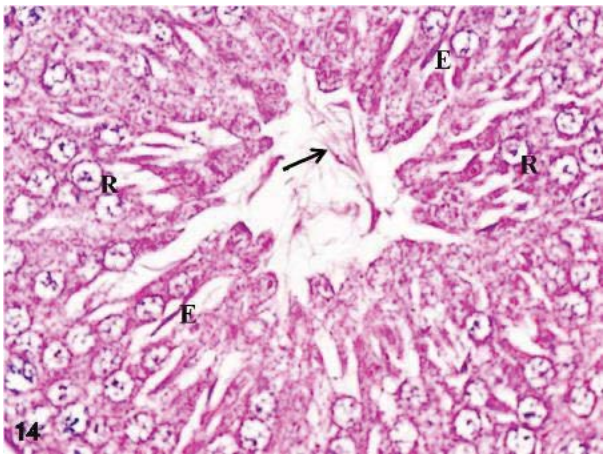


Figure 14: Photomicrograph of a section in the testis of group IIa showing round spermatids (R) and few elongated spermatids (E). Few tails of spermatids are observed in the lumen of the tubule (arrow). Hx & E x1000

PAS stained sections of this group showed strong positive reaction in the capsule and in the basement membranes of tubules. Few spermatocytes showed also moderate reaction.

Masson's trichrome stained sections showed dense deposition of collagen fibers in tunica albuginea and moderate deposition in tunica vasculosa of the capsule. Minimal deposition is seen in the basement membranes surrounding the follicles (Figure 11b).

Stained sections of this group using TUNEL assay revealed negative immune reaction in the tubule and the interstitial tissue (Figure 16).

Group IIb: experimental 7 weeks old rats: (experimental pre-pubertal): Examination of Hx and E stained sections of testes of albino rats aged 7 weeks, which were exposed to noise at 3 weeks of age for one month, showed localized thickening of tunica albuginea and congestion of blood vessels of tunica vasculosa of the capsule compared to the control group. Some areas showed wide separation of the tubules from the capsule. Peripherally located seminiferous tubules appeared more affected than centrally located ones. Most of the tubules showed rupture of their basement membranes or irregular ones (Figure 17a and 17b).

Most of seminiferous tubules revealed also loss of the normal arrangment of spermatogenic epithelium. Wide tubular lumen, sectorial loss of spermatogenic cells, vacuolated cytoplasm, faint nuclear staining, and fusion of cell membranes were among observations noted. Less frequently, Sertoli cells demonstrated cytoplasmic vacuolations or pale staining of their nuclei (Figure 18).

Leydig cells as well frequently showed pyknotic nuclei and were surrounded by congested blood vessels. Acidophilic hyaline material deposition in the interstitial tissue was also noticed (Figure 19). PAS stained sections revealed positive reaction in the capsule and basement membrane. Most germ cells showed faint reaction. Masson's trichrome stained sections revealed moderate deposition of collagen fibers in tunica albuginea.

Stained sections using TUNEL assay showed few scattered positive immune stained cells of the germinal epithelium inside the seminiferous tubules (Figure 20).

Group IIIa: control 4 months old rats: (control adult): Examination of Hx and E stained sections of testes of adult control rats aged 4 months showed that the testis was covered externally with tunica albuginea. Although tunica abuginea showed mild increase in thickness compared to the 7 weeks old group, tunica vasculosa didn't show visible difference (Figure 21a and b).

Testicular tissue was formed of many seminiferous tubules with interstitial tissue spaces in between. The basement membrane of the tubules was seen surrounded by spindle shaped cells. The seminiferous tubules were lined with spermatogenic cells and Sertoli cells. The tubules showed luminal whorly appearance due to the presence of mature elongated spermatids.

The seminiferous epithelium consisted of two types of cells, the spermatogenic cells and the supporting Sertoli cells. The spermatogenic cells included spermatogonia, spermatocytes, round spermatids and elongated spermatids, arranged in that order from the basal compartment to the adluminal compartment (Figure 22).

The spermatogonia were found in the basal compartment of the seminiferous tubules adjacent to the basement membrane. They were either small in size with oval deeply stained nucleus and single nucleolus or they were round with faint stained cytoplasm and nuclei having coarse clumps of marginally arranged chromatin. The spermatocytes were observed next to the spermatogonia and were characterized by their large size and their large rounded centrally located nuclei with condensed and dispersed punctate chromatin (Figure 22).

The round immature spermatids were detected in the adluminal compartment next to the spermatocytes. Their nuclei were smaller in size and appeared rounded in shape with pale fine granular chromatin. The elongated mature spermatids were identified by their elongated deeply stained nuclei (Figure 22). The tails of the elongated spermatids gave a whorly appearance to the lumen of seminiferous tubules (Figure 21).

Sertoli cells were resting on the basement membrane. Their nuclei were pyramidal indented in shape. They were more abundant than the 7 weeks old control group (Figure 22). Leydig cells were arranged in groups around interstitial blood vessels. The cells appeared polygonal or rounded in shape with granular acidophilic cytoplasm and single eccentric nucleus with apparent nucleolus (Figure 22).

PAS stained sections of this group showed strong positive reaction in tunica albuginea and basement membrane surrounding tubules.

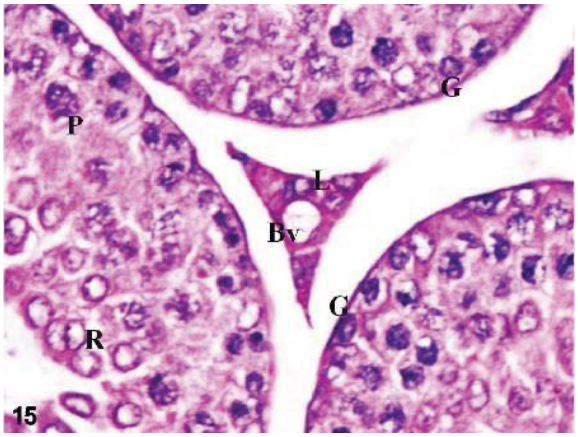


Figure 15: Photomicrograph of a section in testis of group IIa rats showing different stages of spermatogenic cells; spermatogonia (G) resting on basement membrane, spermatocytes (P) and round spermatids (R). Notice the arrangement of Leydig cells in groups (L) in interstitial tissue close to the blood vessel (Bv). Hx & E x1000

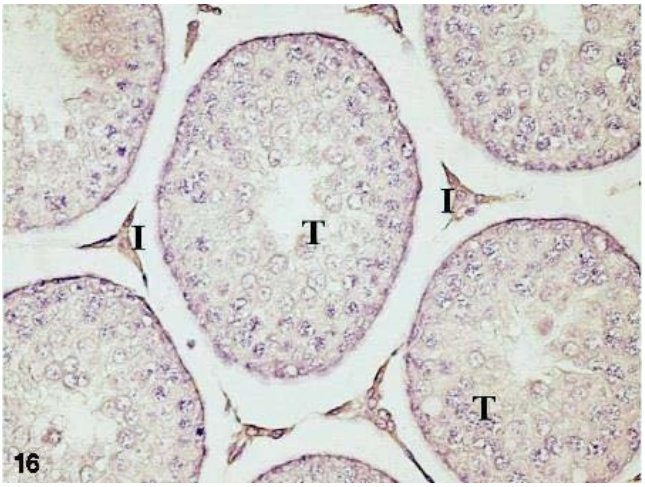


Figure 16: Photomicrograph of a section in testis of group IIa rats showing negative immune reaction in the seminiferous tubules (T) as well as interstitial tissue (I). TUNEL assay X 400

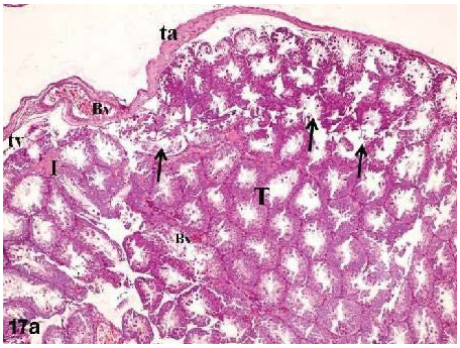
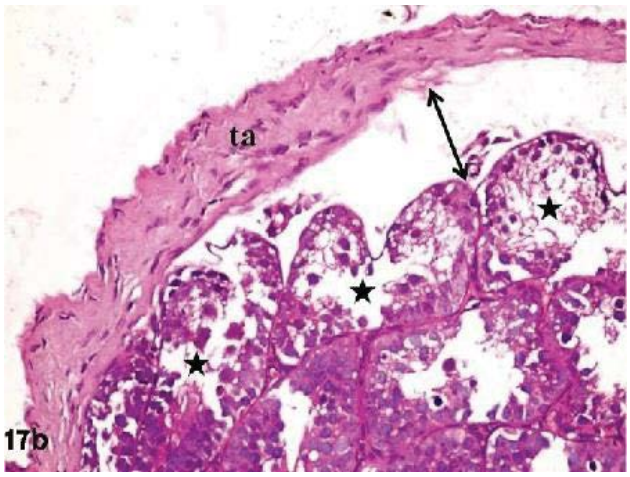


Figure 17a and b: a) Photomicrograph of a section in testis of albino rat aged 7 weeks exposed to noise (group IIb) showing localized thickness in tunica albuginea (ta) with congestion of blood vessels (Bv) of tunica vascuolsa (tv) and interstitial tissue (I). Seminiferous tubules (T) show marked distortion and irregular outlines especially peripherally (arrows).



b) Separation of capsule from underlying testicular tissue (double head arrow). Extensive affection of peripheral tubules (stars) with irregular basement membranes & distortion of spermatogenic cells. (Hx & E (**a)** x100; **b)** x 400)

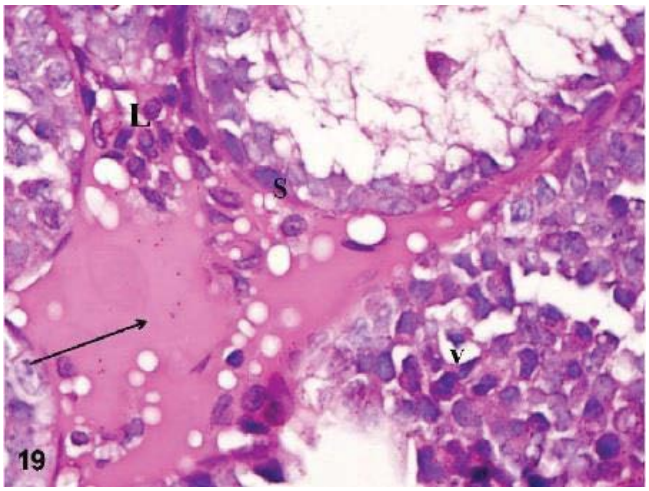


Figure 19: Photomicrograph of a section in testis of group IIb showing acidophilic hyaline material in the interstitial space (arrow) with interstitial cells of Leydig of variable shapes (L). Note that Sertoli cells more or less have normal appearance in contrast to other germ cells. V = vacuolations. Hx & E x1000

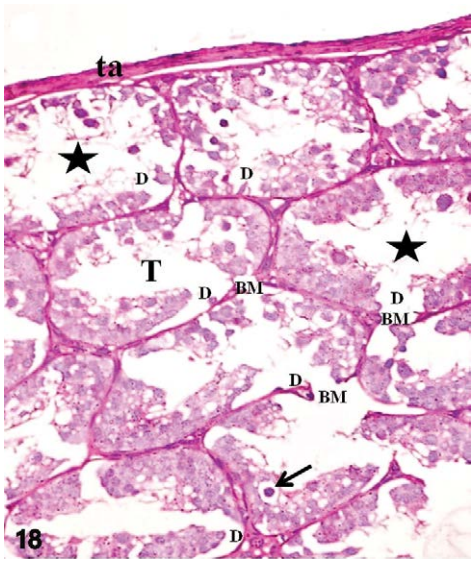


Figure 18: Photomicrograph of a section in testis of group IIb showing distortion of seminiferous tubules (T) with areas of ruptured basement membranes (BM) and reduction of interstitial tissue between them. Some tubules show focal areas of loss (D) of spermatogenic cells and degenerated cells with vacuolated cytoplasm and small dark nuclei (arrow). Wide tubular lumina (stars) are seen in most tubules. ta= tunica albuginea. Hx & E x400

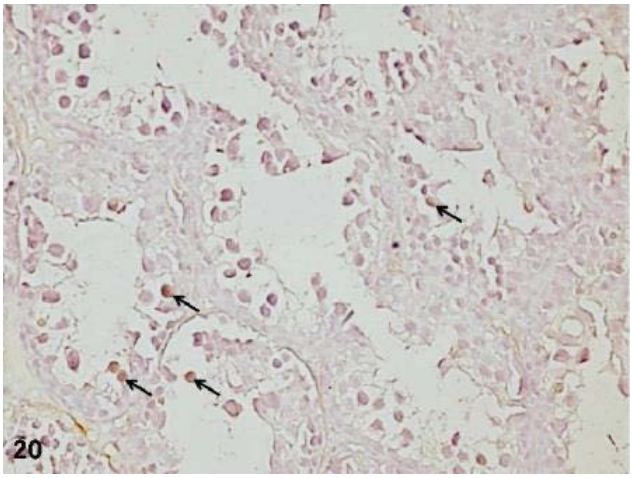


Figure 20: Photomicrograph of a section in testis of group IIb showing few scattered positive immune stained (apoptotic) cells within the seminiferous tubules (arrows). TUNEL assay x400

Most of spermatocytes and some elongated spermatids showed mild to moderate positive reaction. Masson's trichrome stained sections showed dense collagen deposition in the capsule and moderate deposition in the basement membrane (Figure 21b).

Stained sections of this group using TUNEL assay revealed few apoptotic cells localized at the basement membranes of the tubules and also in the interstitial tissue (Figure 23).

Group IIIb: experimental 4 months old rats: (experimental adult): Examination of Hx and E stained sections of testes of adult albino rats aged 4 months exposed to noise at 3 months of age for one month, revealed relative thinning of tunica albuginea in some areas and congestion of blood vessels in tunica vascuolsa compared to control group. The seminiferous tubules were markedly reduced in number and testicular parenchyma appeared loosely packed with tubules with extensive widening of the interstitial tissue spaces. The architecture of the seminiferous tubules was maintained but germinal epithelium showed numerous degenerative changes (Figure 24).

Most of seminiferous tubules showed variable degrees of affection. Localized detachment of spermatogenic cells from basement membrane, localized thickening of basement membrane or marked thinning of the germinal epithelium with extensive widening of their lumina were observed (Figure 25). Spermatogenic epithelium revealed variable degrees of affection. Areas of focal loss of germ cells, fusion of cell membranes of germ cells, cytoplasmic vacuolation with different degrees of nuclear affection, or complete disintegration of cells leaving ghost cells was seen. Also, Sertoli cells less frequently showed faint

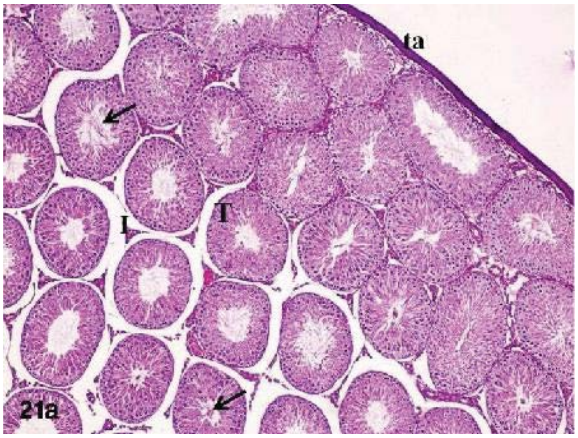
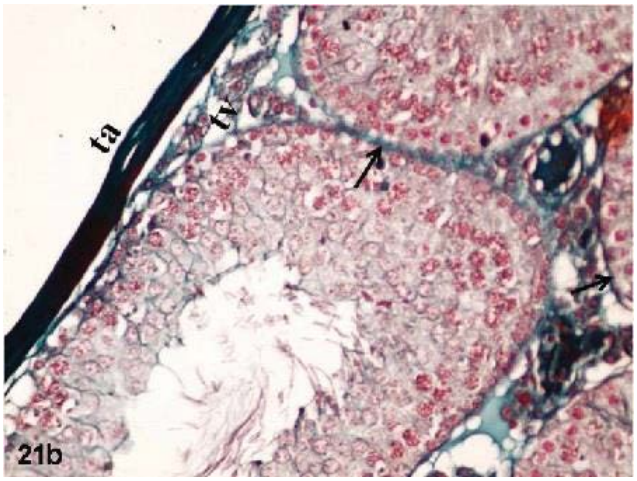


Figure 21a and b: a) Photomicrograph of section in testis of control adult albino rat aged 4 months (group IIIa) showing tunica albuginea (ta) surrounding the testicular tissue and seminiferous tubules (T) with the interstitial tissue (I) in between. Whorly appearance of mature elongated spermatids is observed in their lumina (arrows).



b) dense deposition of collagen fibers in tunica albuginea (ta) & moderate deposition in tunica vasculosa (tv) and basement membranes of seminiferous tubules (arrows).**a)** Hx & E x100; **b)** Masson's Trichrome x400

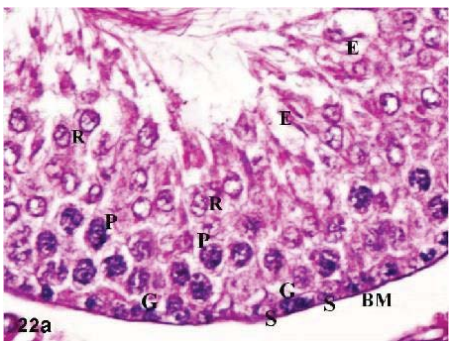
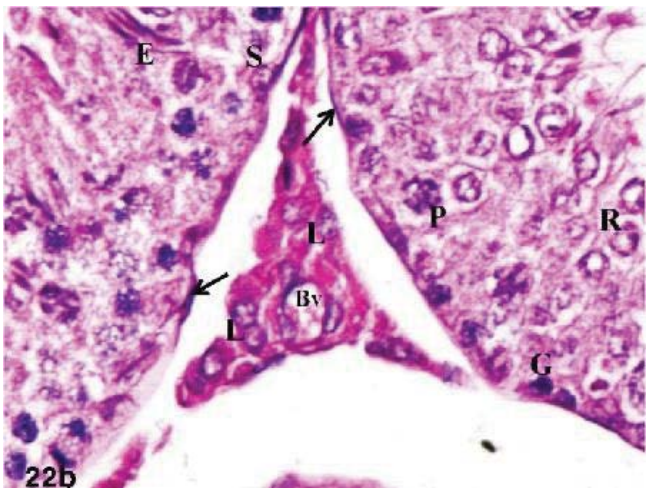


Figure 22a and b: a) Photomicrograph of section in testis of group IIIa showing different types of spermatogenic cells; spermatogonia (G) resting on basement membrane (BM), spermatocytes (P), round spermatids (R) and elongated spermatids (E). Note Sertoli cells (S) with triangular basal nucleus aligned perpendicular to the basement membrane.



b) notice leydig cells polygonal or rounded in shape with granular acidophilic cytoplasm and single eccentric nucleus with apparent nucleolus. **a&b** Hx & E x1000

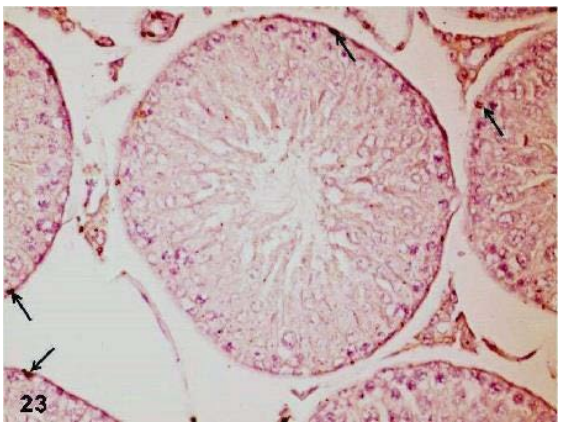


Figure 23: Photomicrograph of a section the testis of group IIIa showing few apoptotic cells localized at basement membrane (arrows) & interstitial tissue. TUNEL Assay x400

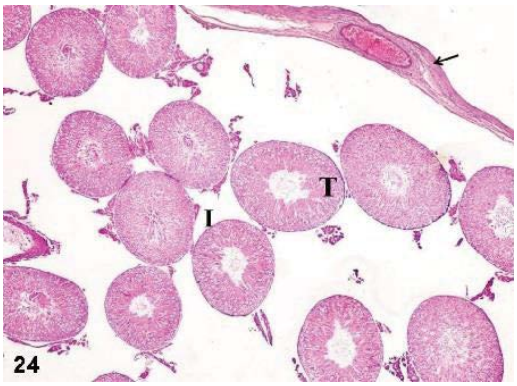


Figure 24: Photomicrograph of section in testis of adult albino rat aged 4 months exposed to noise (group IIIb) showing irregular thickness of capsule (arrow). Marked decrease in number of seminiferous tubules (T) with extensive widening of the interstitial tissue spaces (I) is observed. Hx & E x100

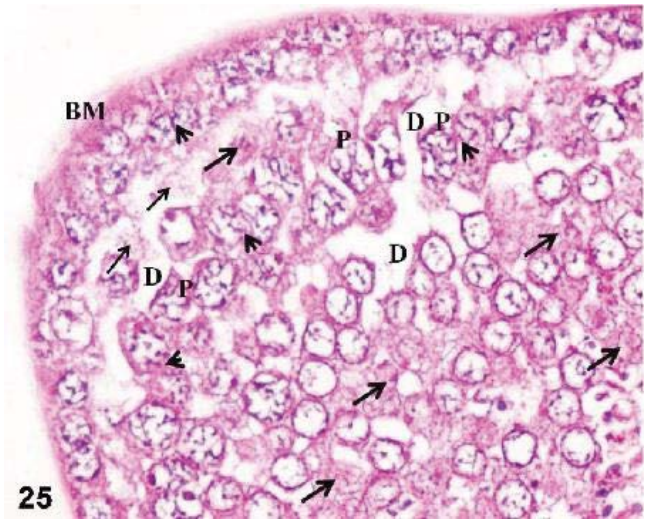


Figure 25: Photomicrograph of section in the testis of group IIIb showing a seminiferous tubule with localized thickening of the basement membrane (BM). Most of the germ cells show fusion of their cell membranes (arrow heads). Different shapes of the spermatocytes (P) are observed. Focal areas of depletion (D) are seen in the regions of spermatocytes and round spermatids. Complete nuclear degeneration of few spermatocytes and round spermatids is noticed giving them ghost cell appearance (arrows). Hx & E x1000

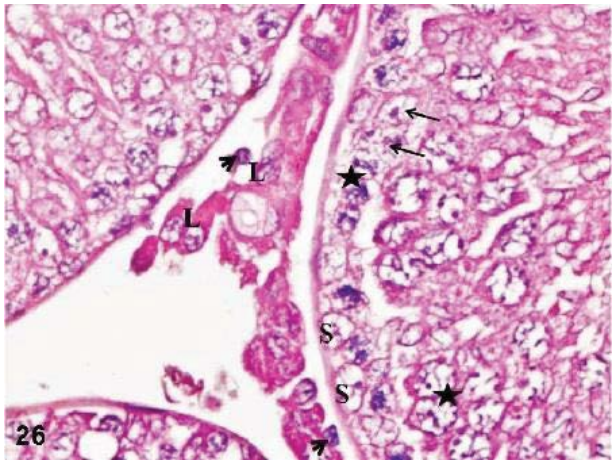


Figure 26: Photomicrograph of section in testis of group IIIb showing germ cells with vacuolated cytoplasm and small dark degenerated nuclei (arrows) and Sertoli cells (S) with faint stained disintegrated nuclei. Note fusion of cell membranes of germ cells (stars). The interstitial cells of Leydig (L) have faint staining of their nuclei and dark cytoplasm while few of them have small dark nuclei (arrow heads). Hx & E x1000

stained disentegrated nuclei (Figure 26). Interstitial cells of Leydig showed faint stained or pyknotic nuclei and dark cytoplasm (Figure 26).

PAS stained sections of this group revealed moderate positive reaction in tunica albuginea and basement membrane. Although most of germ cells showed negative reaction, elongated spermatids showed positive reaction. Masson's trichrome stained sections of this group showed mild collagen deposition in the capsule with apparent decrease in thickness compared to control.

Stained sections of this group using TUNEL assay revealed moderate number of dark positive stained cells distributed among spermatogenic epithelium (Figure 27).

Histomorphometric Results

Number of apoptotic cells (Tunnel positive stained cells) per cut section of testis

Control groups: prenatal and prepubertal groups showed negative immune staining for Tunnel assay. However, adult control groups had mean number of (5 ± 0.2).

Experimental groups: mean number of apoptotic cells was (5 ± 0.3) in prenatal group and (13 ± 0.5) in prepubertal group. In adult experimental group it was (26 ± 0.4). There was highly statistical significant increase in the mean number of positive cells between prenatal, prepubertal and adult experimental groups ($P < 0.0000002$) and between adult control versus adult experimental groups ($P < 0.000003$).

(Bar chart 1) illustrates the mean number of apoptotic cells (Tunnel positive stained cells) in seminiferous tubules counted per cut section of the testis in the three ages of the current study in control and experimental groups.

Number of seminiferous tubules per cut section of testis

Control groups (developmental changes): Mean number of tubules per cut section of the testis was (111 ± 9.69) in control prenatal group while it was (51.8 ± 3.48) in control 7 weeks old group and it was (19.8 ± 3.57) in control adult group. There was highly statistical significant decrease in the mean number of tubules between prenatal and 7 weeks old groups ($P < 0.0002$) and between prenatal and adult groups as well as between 7 weeks old group and adult group ($P < 0.0001$) (Bar chart 2).

Experimental groups: In group I, 20 days old rat fetuses (prenatal group), mean number of seminiferous follicles was (111 ± 9.68) in control group while it was (91.5 ± 6.78) in noise exposed group. There was no statistical difference in the mean number of the seminiferous follicles per cut section of the testicular tissue in the noise exposed group Ib ($P < 0.13$) compared to its control group Ia.

In group II, 7 weeks old rats, mean number of seminiferous tubules was (51.8 ± 3.47) in control group while it was (38.5 ± 4.5) in noise exposed group. There was statistical significant decrease in the mean

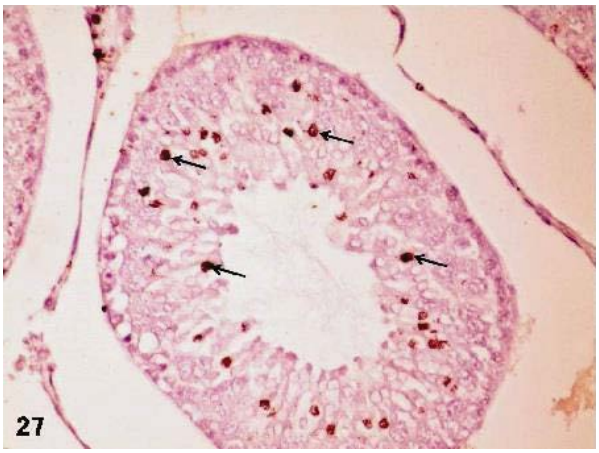
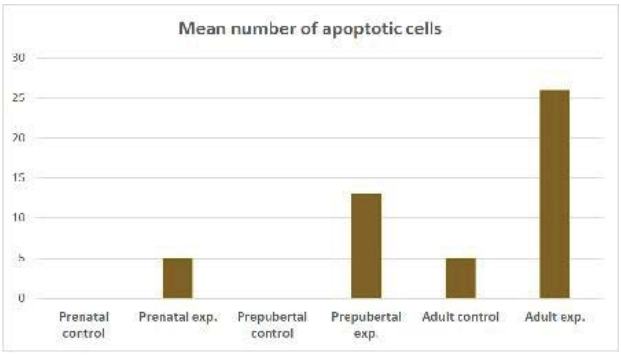
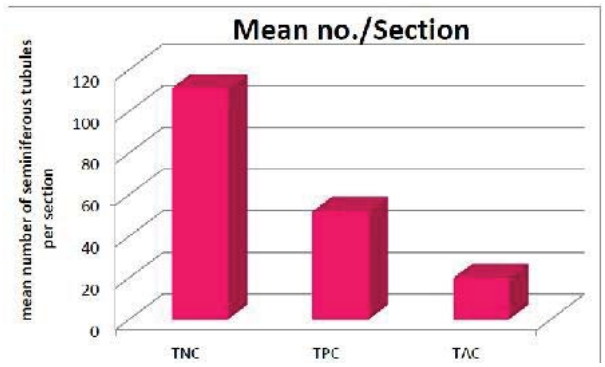


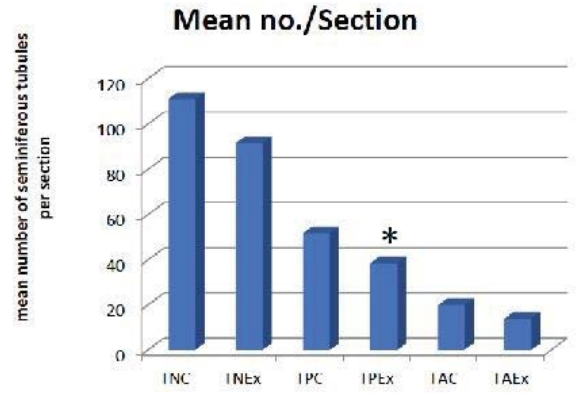
Figure 27: Photomicrograph of section in testis of group IIIb showing moderate number of dark positive stained apoptotic cells distributed among different spermatogenic cell layers (arrows). TUNEL assay x400



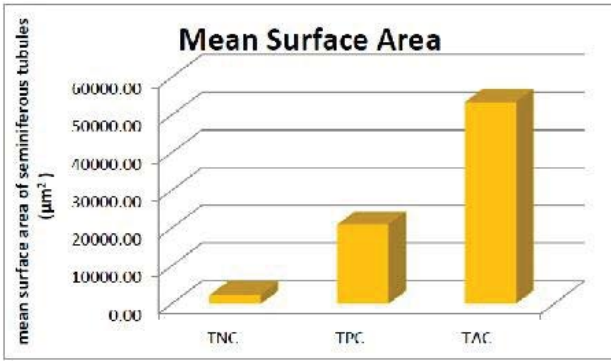
Bar Chart 1: illustrates the mean number of apoptotic cells (Tunnel positive stained cells) in seminiferous tubules counted per cut section of the testis in the three ages of the current study in control and experimental groups.



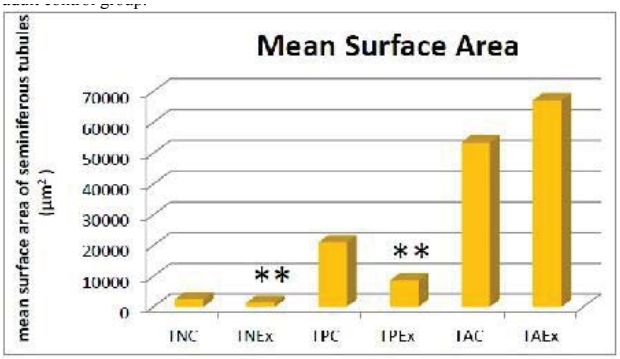
Bar Chart 2: Representing the mean number of seminiferous tubules in three age control groups in the current study. There is highly statistical significant decrease in the mean number of tubules between the three age groups. TNC= testis prenatal control group, TPC= testis 7 weeks old control group, TAC= testis adult control group.



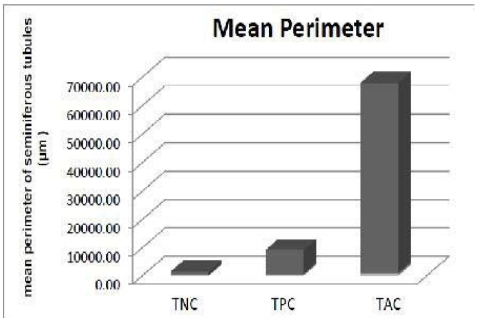
Bar Chart 3: Illustrates the mean number of seminiferous tubules counted per cut section of the testis in the three ages of the current study in control and experimental groups. Statistical significant decrease is noticed in group II whereas no significant difference is observed in group I and group III. TNC= testis prenatal control group, TNEx= testis prenatal experimental group, TPC= testis 7 weeks old control group, TPEx= testis 7 weeks old experimental group, TAC= testis adult control group, TAEx= testis adult experimental group (star= significant difference).



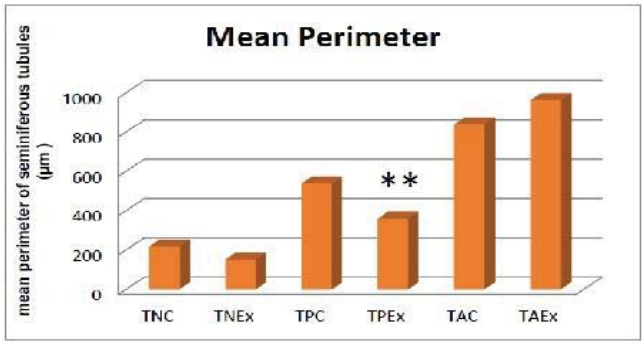
Bar chart 4: Representing the mean surface area of seminiferous tubules in three age control groups in the current study. There is highly statistical significant increase in the mean surface area measurements between prenatal and 7 weeks old groups as well as between prenatal and adult groups. There is significant increase between 7 weeks old group and adult group. TNC= testis prenatal control group, TPC= testis 7 weeks old control group, TAC= testis adult control group.



Bar chart 5: Illustrates the mean surface area of seminiferous tubules of the testis in the three ages of the current study in control and experimental groups. Highly statistical significant decrease is noticed in group I and group II whereas no significant difference is observed in group III. TNC= testis prenatal control group, TNEx= testis prenatal experimental group, TPC= testis 7 weeks old control group, TPEx= testis 7 weeks old experimental group, TAC= testis adult control group, TAEx= testis adult experimental group (two stars= highly significant difference).



Bar chart 6: Representing the mean perimeter of seminiferous tubules in three age control groups in the current study. There is highly statistical significant increase in the mean perimeter measurements between prenatal and 7 weeks old groups as well as between prenatal and adult groups. There is significant increase between 7 weeks old group and adult group. TNC= testis prenatal control group, TPC= testis 7 weeks old control group, TAC= testis adult control group.



Bar chart 7: Illustrates the mean surface area of seminiferous tubules of the testis in the three ages of the current study in control and experimental groups. Highly statistical significant decrease is noticed in group I and group II whereas no significant difference is observed in group III .TNC= testis prenatal control group, TNEx= testis prenatal experimental group, TPC= testis 7 weeks old control group, TPEx= testis 7 weeks old experimental group, TAC= testis adult control group, TAEx= testis adult experimental group (two stars= highly significant difference).

number of the seminiferous tubules per cut section of the testicular tissue in the noise exposed group IIb ($P < 0.04$) compared to its control group IIa.

In group III, 4 months old rats (adult group), mean number of seminiferous tubules was (19.8 ± 3.57) in control group while it was (14 ± 1.4) in noise exposed group. There was no statistical difference in the mean number of the seminiferous tubules per cut section of the testicular tissue in the noise exposed group IIIb ($P < 0.16$) compared to its control group IIIa.

(Bar chart 3) illustrates the mean number of seminiferous tubules counted per cut section of the testis in the three ages of the current study in control and experimental groups.

Surface area of seminiferous tubules measurements

Control groups (developmental changes): Mean surface area measurement of seminiferous tubules of the testis was (2372.57 ± 80.2) in control prenatal group while it was (20956.36 ± 1209.2) in control 7 weeks old group and it was (53490.34 ± 11079.69) in control adult group. There was highly statistical significant increase in the mean surface area measurements between prenatal and 7 weeks old groups as well as between prenatal and adult groups ($P < 0.0001$). However, there was significant increase between 7 weeks old group and adult group ($P < 0.015$) (Bar chart 4).

Experimental groups: In group I, 20 days old rat fetuses (prenatal group), mean surface area of seminiferous follicles was (2372.57 ± 80.2) in control group while it was (1373.5 ± 49.9) in noise exposed group. There was highly statistical significant decrease in the mean surface area of the seminiferous follicles in the noise exposed group Ib ($P < 0.001$) compared to its control group Ia.

In group II, 7 weeks old rats, mean surface area of seminiferous tubules was (20956.36 ± 1209.2) in control group while it was (8592.78 ± 281.86) in noise exposed group. There was highly statistical significant decrease in the mean surface area of the seminiferous tubules in the noise exposed group IIb ($P < 0.0017$) compared to its control group IIa.

In group III, 4 months old rats (adult group), mean surface area of seminiferous tubules was (53490.34 ± 11079.69) in control group while it was (67076.87 ± 3716.04) in noise exposed group. There was no statistical difference in the mean surface area of the seminiferous tubules in the noise exposed group IIIb ($P < 0.27$) compared to its control group IIIa.

(Bar chart 5) illustrates the mean surface area calculation of the testis in the three ages of the current study in control and experimental groups.

Perimeter of seminiferous tubules measurements

Control groups (developmental changes): Mean perimeter measurement of seminiferous tubules of the testis was (216.86 ± 6.78) in control prenatal group while it was (538.38 ± 23.2) in control 7 weeks old group and it was (838.5 ± 97.47) in control adult group. There was highly statistical significant increase in the mean perimeter measurements between prenatal and 7 week's old groups as well as between prenatal and adult groups ($P < 0.0001$). However, there was significant increase between 7 weeks old group and adult group ($P < 0.013$) (Bar chart 6).

Experimental groups: In group I, 20 days old rat fetuses (prenatal group), mean perimeter of seminiferous follicles was (216.85 ± 6.7) in control group while it was (151.86 ± 3.53) in noise exposed group. There was highly statistical significant decrease in the mean perimeter of the seminiferous follicles in the noise exposed group Ib ($P < 0.001$) compared to its control group Ia.

In group II, 7 weeks old rats, mean perimeter of seminiferous tubules was (538.38 ± 23.18) in control group while it was (360.14 ± 7.43) in noise exposed group. There was highly statistical significant decrease in the mean perimeter of the seminiferous tubules in the noise exposed group IIb ($P < 0.001$) compared to its control group IIa.

In group III, 4 months old rats (adult group), mean perimeter of seminiferous tubules was (838.45 ± 97.47) in control group while it was (961.8 ± 38.72) in noise exposed group. There was no statistical difference in the mean perimeter of the seminiferous tubules in the noise exposed group IIIb ($P < 0.27$) compared to its control group IIIa.

(Bar chart 7) illustrates the mean perimeter calculations of the testis in the three ages of the current study in control and experimental groups.

Discussion

The present study explored and compared first; the developmental structural characteristics of rat testicular tissue at three age periods; full term fetal, pre-pubertal and adult. Second, the effect of chronic noise stress exposure on testicular tissue was investigated at these critical age groups. Offsprings of pregnant rats exposed to chronic noise were examined at full term fetus stage. The aim of the study was also to reveal the most susceptible period of lifetime during which the testis is prone to injury. Light microscopic, immunohistochemistry for apoptosis and histomorphometric techniques were employed in the current study.

Rat reproductive system had been chosen as it is very similar to human one [15]. The rat has been considered as an ideal animal model for such studies [16]. The noise stress model employed in the current study was previously used by [8]. In rat, the implantation window lasts for about 24 h on day 4–5 of fertilization. Thus, to avoid abortion regarding the prenatal group, the present study started on the 8th day of pregnancy to ensure the completion of implantation [11].

In the present developmental study, the three successive age control groups showed histological differences that were confirmed by histomorphometric measurements. The tunica albuginea increased in

thickness in contrast to tunica vascuolsa which diminished in thickness as age advanced. Size of the seminiferous tubules increased with age.

Regarding the germinal epithelium, the testes of full term rat fetuses were composed only of two types of cells; the gonocytes and the supporting cells. The gonocytes were few in number and large in size whereas the supporting cells were smaller and much more numerous resting against the basement membrane. Reviewing the literature, studies describing the histology of this age group are seldom.

In the 7 weeks old rats, the testis was formed of seminiferous tubules which were larger than the prenatal group. Most of them acquired narrow lumina hence they changed into tubules rather than follicles. The germinal epithelium was formed of several cell types; spermatogonia, spermatocytes, round immature spermatids. At this age, early or round spermatids started their transformation into elongated or mature ones. Few elongated mature spermatids were detected in this age group. Moreover, the immature supporting cells became the Sertoli cells. The lumina appeared within the follicles at age earlier than 7 weeks hence they were called seminiferous tubules. Current results were in agreement with the observations made by [17].

Regarding 4 months old rats, seminiferous tubules increased in size. The germinal epithelium showed the same sequence of cells as the previous group, but elongated mature sperms were more frequently encountered giving the characteristic whorly appearance in the lumina. Sertoli cells were more detected in the adult group compared to the 7 weeks old rats. The interstitial cells of Leydig increased in number as age advanced. Hooker (1970) reported that they were numerous in the cat and pig testis, fewer in the human testis and scarce in birds [18]. The current study observations were in accordance with that performed by [19-21].

In the current study testes of 20 days old rat fetuses, whose mothers were exposed to noise during pregnancy, revealed obvious distortion of the normal histological structure of the testis. Both supporting cells and gonocytes that constituted the testicular parenchyma were highly affected. The supporting cells were detached from the basement membrane in most of the seminiferous follicles. The cells were closely packed up to adherence between cell membranes of neighboring supporting cells. Few supporting cells were sloughed towards the lumen or demonstrated nuclear affection. The gonocytes, which are the precursors of the germinal epithelium, also showed degenerative changes in the form of nuclear pyknosis and cytoplasmic vacuolation. Reviewing the literature, there is a gap of knowledge regarding the immediate effects of prenatal stress exposure on the full term fetal testicular histology. One study described the late effects of prenatal noise stress on the offsprings at adult stage. Other studies reported late effects of other forms of stress exposure during prenatal life.

[7] Examined the effects of prenatal noise stress exposure on the offspring of rats at adulthood. Dilatation of the tubular lumen and disorganized epithelium and sloughing of germ cells was observed. Chehreie et al. (2013) applied water deprivation stress on pregnant rats and examined the effect on their offsprings after reaching puberty. They found decrease in offspring's testes weight and damage of testicular histology.

Chehreie et al. (2013) showed that restraint stress to pregnant rodent mothers induced low birth weight in newborn pups that remained small up to adulthood [22]. Others examined the effect of exogenous administration of cortisol on the offspring of pregnant rats [24]. The author reported reduced fetal growth in rats. It was reported that exposure of pregnant animals to stressful conditions (capture, noise, immobilization, introduction of a strange male, crowding, etc.) often resulted in a smaller litter size, embryo resorption, structural malformations, growth retardation, lower birth weight of the puppies, and even a shift in the sex ratio [25,26].

According to [27] the application of stressful stimuli during pregnancy in rats affected male offsprings hypothalamic pituitary gonadal (HPG) axis. Stress reduces the frequency and amplitude of gonadotropin releasing hormones (GnRH) and luteinizing hormone (LH) pulses, that decreased the gonadotropic function [28]. The hypothalamic-pituitary- testicular axis of the rat is already functional in fetuses during late gestation and in newborns [29]. In male rats there is a surge in plasma testosterone during 18 and 19 gestation days. Testosterone as an inhibitor of HPA axis modulates animal behavior. It has been demonstrated that prenatal stress reduces the level of testosterone in male rats and guinea pigs [30] these study results were explained in view of central nervous system development during fetal life. The third trimester of gestation is the period of rapid brain myelination and occurrence of fetal brain growth spurt therefore it is a critical period of time for exposure to prenatal stress [31] Additionally, epidemiological studies in humans have demonstrated that prenatal exposure to stress resulted in permanent modification of hypothalamic-pituitary-adrenal (HPA) function in the form of stress-related behaviors in the offspring [32].

In the present work, testes of 7 weeks old rats exposed to noise at 3 weeks of age for one month duration, showed obvious histological alterations to testicular structure. Mature sperms were not detected in the examined sections. No similar studies were found in the literature focusing on the effect of chronic noise stress on prepubertal rat testes.

The only relevant study was that of [17] who found that administration of exogenous dexamethazone to prepubertal rats for 2 weeks resulted in marked histological affection of testicular tissue. It was suggested that chronic stress elevates cortisol levels in blood. Chronically elevated cortisol levels affected the HPG axis. Moreover, cortisol acts directly on Sertoli cells and/or on germ cells and induces retardation of testicular development [33].

In the present work, testes of adult group exposed to chronic noise revealed marked alterations in normal testicular architecture as stated in the results. In agreement with previous reports [8,19]. In the current study, Sertoli cells as well were frequently affected as stated by [35]. Sertoli cell affection can cause severe damage to spermatogenesis being the nurse cells for germinal epithelium [35,36].

In the present study, immunohistochemical staining for apoptotic cells using TUNEL assay in control consecutive ages revealed scarce number of positive stained cells. It is recognized that during normal germ cell development, physiological germ cell apoptosis occurs to maintain the quality of the germ cell [4].

immunohistochemical staining for apoptosis in the three experimental groups showed increased number of apoptotic cells in the seminiferous tubules compared to their corresponding controls. Late effects of various forms of stress were studied by numerous authors [22] stated that water deprivation in pregnant rats increased number of TUNEL positive cells and germ cell apoptosis of the offsprings when reached puberty [9] found that immobilization stress to pregnant rats resulted in increased apoptotic cells of the offsprings at adulthood. The authors hypothesized that the increase in testicular apoptosis was the result of decline of gonadotropins and subsequently testosterone hormonal level that influenced the testicular cellular viability [37]

found that testosterone replacement at birth in offspring males exposed to prenatal stress was able to reverse changes in sexual behavior. Numerous studies also found increase in number of apoptotic cells in spermatogenic epithelium after exposure of adult rats to chronic stress [38-41] suggested that high levels of cortisol, the stress hormone, in the blood is the reason for germ cell apoptosis as glucocorticoids control mitosis and apoptosis induction of the germinal epithelium. Interestingly, apoptosis was seen to occur in a developmental stage-specific manner. Spermatocytes and spermatids were the most prone to damage. It is believed that germ cells are more vulnerable to injury as they have high mitotic activity [4].

In the current study, morphometric analysis of the three parameters examined revealed highly statistically significant difference between control and noise exposed groups of full term fetuses and pre-pubertal rats but not adult rats. Actual decrease in the mean perimeter, mean surface area and mean number of seminiferous tubules was obtained in noise exposed groups. No similar studies were reported but few studies described late observations of prenatal stress. A study investigated late effects of prenatal noise stress [7]. Weight, length and width of the testes and height of germinal epithelium were remarkably decreased. Other group found reduction in spermatid count in rats subjected to immobilization stress during prepubertal period and left to be assessed when reached adulthood [41].

Conclusion

The current study demonstrated the histological characteristics, histomorphometry of number, surface area, and perimeter of seminiferous tubules, immunohistochemistry for apoptotic cells of the rat testis at three sequential developmental stages; full term fetus, pre-pubertal, and adult periods. The study also revealed that exposure to chronic noise stress resulted in; first structural affection of testicular tissue in the three age groups. Second, increased germ cell apoptosis most pronounced in adult rats. Thirdly, significant decrease in morphometric parameters in prenatal and pre-pubertal groups. Current results imply that chronic noise stress exposure particularly in pre-natal and pre-pubertal stages (as tubular size reduced) might hamper male reproductive capacity in future life. Further studies are needed to explore whether these effects are reversible or not.

Conflict of Interest

There is no conflict of interest to declare.

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