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RESEARCH ARTICLE

Light and electron microscopic studies of the effect of alprazolam on the testicular tissue of mice

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Abstract

Alprazolam (8-chloro-1-methyl-6-phenyl-4H-s-triazolo, 4,3-á) is a benzodiazepine derivative used in the treatment of panic attacks, generalized anxiety, and depression. The present study aimed to assess the reproductive efficiency of male mice after exposure to different doses of alprazolam (ALP). This was done by estimation of serum testosterone hormone, sperm morphology, histopathological and ultrastructural investigations of the testicular tissue. Sixty male mice were divided into four experimental groups (15 mice/group); the control and three experimental mice groups. The control mice were given no treatment. The experimental mice were received daily oral by gavage three different dose levels of ALP (0.5, 1.5 and 4.5 mg/kg bw) for three months. The obtained results revealed a decrease in the body weight gain, absolute and relative testes and epididymal weights in experimental-treated group. Significant findings were detected in higher dose treatment. Also, there was dose-dependent decrease of serum testosterone hormone of ALP-treated mice, being significant at high dose level. Following investigating sperm anomalies, experimental groups exhibited significant increase. The prominent sperm deformities were as such the detached head, the amorphous head and the highly folded tail. The applied drug treatment possessed testicular damage including atrophy of seminiferous tubules (ST) and reduction of spermatogenic cells. Furthermore, detachment and sloughing of spermatogenic epithelium were a marked increase of inactive ST with degenerated spermatids, spermatozoa and severe Leydig cells. Ultrastructurally, the cytoplasm of spermatocytes showed marked vacuolation and the nuclei appeared with convoluted nuclear envelope and disintegration of their nuclear chromatin. The mitochondria in secondary spermatocytes contained wide intercristae apaces and they formed dense masses of intermitochondrial cement. Accumumulation of myelin figures were observed in the cytoplasm of these cells. Spermatids had deformed nuclei and irregularly-shaped acrosomes. In conclusion, it was found that treatment with alprazolam produced pronounced male reproductive toxicities in testes and epididymis associated with increased incidence of sperm shape abnormalities and a decrease of testosterone hormone. Also, ALP caused degeneration in seminiferous tubules, depletion of germinal epithelium, intercellular disassociation of germ cells, along with disruption in germ cell arrangement, and an increase in Leydig cells damage.

INTRODUCTION

Alprazolam (Zolam®) (benzodiazepine) is used in the treatment of generalized anxiety, sedative, anticonvulsant, panic attack with or without agoraphobia, and skeletal muscle relaxant **(Kessler et al., 1994; Anderson et al., 2000; Rathod, 2001; Isbister et al., 2004).** Alprazolam is biotransformed by hepatic microsomal oxidation and conjugation to hydroxylated metabolites, 4-hydroxy alprazolam (4-OHALP) and α-hydroxy alprazolam (α-OHALP). Approximately 80% of alprazolam is excreted by the kidney as unchanged drug **(Allison and Pratt, 2003; Verster and Volkerts, 2004).** Disturbances of reproductive and sexual health are common in people with epilepsy. The testicular function can be affected in men with epilepsy. Psychosocial complications associated with epilepsy can also affect reproductive health and sexuality.

Dana-Haeri et al. (1982) concluded that long-term antineoplastic drugs use could lead to the testicular failure, inability to respond to raised levels of LH, falling testosterone and eventually impaired spermatogenesis. Many investigators have been shown that the epileptic discharges have been shown to be associated with abnormal bioavailable serum testosterone and gonadotropin concentrations, altered LH response to GnRH stimulation, and increased serum prolactin concentrations **(Herzog et al., 1986; Bauer et al., 1992)**. **Montouris and Morris (2005)** found that some antiepileptic drugs (AEDs) could alter concentrations of sex steroid hormones.

Despite extensive medical and therapeutic use of alprazolam, little information related to its possible influence on the fertility and the reproductive organs were available. So, it was thought to be of particular interest to determine the effect of this commonly used drug on the male reproductive organs including testis and epididymis.

2. Materials and Methods

2. 1. Chemicals

Alprazolam (Zolam®) is produced by Amoun Pharmaceutical Company, Egypt. The applied doses of alprazolam (0.25, 1 or 2 mg/kg bw) were dissolved in saline solution (0.9 % NaCl).

2. 2. Experimental animals

Sexually mature albino male mice, weighing approximately 30 ± 3 g each, 3 months old were obtained from the Medical Research Institute, Alexandria University, Egypt. Animals were maintained at the animal care facility under controlled temperature (23 ± 2°C) and 12-hr light/dark cycle. Free access of standard diet and water were available ad libitum. Mice were acclimatized to the laboratory environment for two weeks prior to the starting of the experiment.

2. 3. Experimental design

Sixty male mice were randomly assigned into four groups (15 mice/ each) according to their approximately equal mean body weight. Control (saline – treated mice) and experimental mice received either daily therapeutic doses of 0.5, 1.5 and 4.5 mg/kg bw, for three months. The applied dose-treatment was dissolved in 0.5 ml saline solution.

2. 4. Monitoring of the signs of toxicity

All mice of the experiment were carefully examined daily throughout the experimental period in order to depict any apparent behavioural changes and/or signs of toxicity.

2. 5. Body weights and reproductive organs weights

Body weights of all experimental mice were recorded weekly during the period of treatment. Means of the body weights and body weight gains were estimated. At the end of the experiment, both control and experimental groups were sacrificed and dissected. The testes and epididymes were excised out quickly, weighed and the absolute and the relative weights were calculated according to **Matousek (1969)** I.W. = organ weight (g)/100×body weight (g).

2. 6. Estimation of serum testosterone hormone

At the end of experiment, blood was collected in non-heparinized tubes from control and ALP-treated mice. Serum was obtained by centrifugation of the blood samples at 4500 rpm for 15 min, and kept at a refrigerated at −20°C. Testosterone levels were assayed by microtiter plate enzymimmunoassay (EIA) method, using commercial Kit, Calbiotech, Spring Velley, CA, USA **(Chen et al., 1991).**

2. 7. Detection of sperm morphology

To evaluate epididymal sperm deformities, the left epididymis was removed from the adhering connective tissue. The samples were then minced with small scissors in a Petri dish containing 2 ml of warm saline and left for 30 min at 37 ◦C for sperm release **(Oliveira et al., 2009).** Aliquots of spermatozoa were smeared on clean, grease-free microscope slides. Smears were air-dried, then fixed in methanol. After fixation, the samples were stained with 1% aqueous eosin-Y solution for 10 minutes, washed with distilled water, dehydrated through ascending series of ethyl-alcohol, cleared in xylol and mounted in Canada Balsam **(Narayana et al., 2005)**. Spermatozoa from each sample were evaluated, using a light microscope with an oil immersion objective lens (1000× magnification) and classified as follows: normal, hookless head, amorphous head, compacted head, highly folded tail and coiled tail (**Burruel et al., 2000).**

2. 8. Histopathological studies

Testis of both control and alprazolam-treated mouse of the different experimental group was removed and quickly fixed in 10% formalin for 24 h, and processed through the conventional paraffin embedding technique **(Bancroft and Gamble, 2002),** sectioned at 5 µm thick and stained with haematoxylin and eosin (H & E).

2. 9. Transmission electron microscope

Very small slices of the testes of the control and ALP-treated mice were taken out quickly and immediately fixed in 2 % 4F1G, rinsed in 0.1M phosphate buffer, pH = 7.4 at 4˚C for around 1 h, then rinsed in 0.1 M phosphate buffer (pH 7.4). This was followed by post-fixation using 2% buffered OsO4 (osmium tetroxide) for 1-2 hr at 4˚C. Then the specimens were washed with phosphate buffer for several times for 30 min, dehydrated in ascending grades of ethanol concentration, and embedded in epoxy resin. Ultrathin sections (50-60 nm) were cut with a diamond knife on LKB ultra-microtome. Samples were collected in naked copper mesh-grids and stained with 2% aqueous uranyl acetate for 30 minutes and lead citrate for 20 min **(Robards and Wilson, 1993).** These sections were examined and photographed on a Joel, 100 cx transmission electron microscope.

2. 10. Statistical analysis

Data were expressed as mean values ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) to assess significant differences among treatment groups. For each significant effect of treatment, the post hoc Tukey's test was used for comparisons. The criterion for statistical significance was set at $P < 0.05$. All statistical analyses were performed using SPSS statistical version 8 software package (SPSS Inc., USA).

3. Results

The findings of the present results revealed no detected increase in mortality rate in experimentl treated mice, depending on dose level of treatments, and compared with the control (Table 1).

3. 1. Body and the reproductive organs weights

At the end of experiment, the results revealed that the body weights and the weight gains of mice administered with 0.5 & 1.5 mg/kg bw ALP were insignificantly decreased versus the control. However, these values were significantly decreased in mice administered with 4.5 mg/kg bw, comparing to the control (Table 1). Statistically, there were no significant decreases in testes and epididymal weights of mice treated with 0.5 & 1.5 mg/kg bw ALP, comparing to the control. However, these values were significantly decreased in mice administered with 4.5 mg/kg bw ALP.

3. 2. Testosterone level

Depending on the dose level of alprazolam, the present results revealed gradual decreases in the levels of serum testosterone in mice administered with 0.5, 1.5 and 4.5 mg/kg bw ALP, comparing to the control **(Fig. 1).**

3. 3. Sperm morphology

As shown in **Fig. 2,** it is evident that mice administered with 4.5 mg/kg bw ALP resulted in profound altered sperm morphology. Unlike the control mice, in which 19.71% of the epididymal spermatozoa exhibited normal morphology **(Fig.2a),** 0.5, 1.5 & 4.5 mg/kg bw ALP-treated mice showed 21.9, 41.0 % and 67.54 % abnormal spermatozoa, respectively. These abnormalities included: amorphous head, hookless head, calyculated head, doublet heads, compact head tail with a cytoplasmic droplet, irregular tail and coiled tail **(Figs. 2 e-h).** The notable abnormality in 4.5 mg/kg bw ALP-treated sperms was the appearance of predominant deformities such as the detached and amorphous head with the highly folded tail.

3. 4. The histopathological results

At the light microscopic level, the testis of control mice revealed that it is covered by a collagenous layer of dense connective tissue capsule containing fibroblasts and a layer of loose connective tissue, containing many blood vessels. Each testis is composed of many seminiferous tubules (ST), separated by thin sheath of connective tissue. These tubules are highly convoluted and roughly circular structures with a relatively narrow lumen **(Fig. 3).** Each tubule is surrounded myoid cells that exhibit smooth muscle characteristics **(Fig. 4).** Each ST contains regular arrangement of spermatogenic cells: spermatogonia; primary and secondary spermatocytes; spermatids, and spermatozoa (**Figs. 4 & 5**). Few number of Sertoli cells are observed resting on the basement membrane of the tubules at fairly regular intervals. Each of these cells had a large pale, indented nucleus and a densely-stained cytoplasm. Many metamorphosed spermatids and spermatozoa are observed near the lumen. Further, the STs are closely packed with each other leaving triangular spaces which occupied by groups of large polyhedral interstitial Leydig cells and blood vessels **(Fig. 5).** At the ultrastructural level, the primary spermatocytes are large spherical cells, having centrally located nuclei with finely granular dark nucleoplasm and clumps of irregular chromatin **(Fig.**

17). The cytoplasm contains abundant mitochondria, Golgi body, fairly little rough endoplasmic reticulum (rER) and free scanty ribosomes may be observed **(Fig. 17).**

The secondary spermatocytes are rarely observed among the germinal epithelium. They are spherical in shape and their nuclei are almost ovoid in shape and covered by the acrosomal vesicles which are spread to cover the anterior half of the condensing nucleus **(Figs. 18 & 19)**. Different forms of spermatids are detected. The early one is rounded in shape, having large spherical and centrally located nuclei, containing few chromatin clumps. The mitochondria in these cells are distributed in the cytoplasm with wide intercristae spaces. Also, there were flattened Golgi saccules opposite a depression in one pole of the nucleus **(Fig. 19)**.

The late stages of spermatids are elongated, having different degrees of nuclear elongation and have a developing acrosomal cap. Spermatozoa have variable position in relation to the surface of the supporting Sertoli cells. The flagellum is emerged at the lower region below the nucleus, and a cylindrical bundle of microtubules limits the nucleus laterally **(Fig. 20)**. The interstitial Leydig cells appear as oval or polyhedral in shape, containing large slightly irregular nuclei and have one or two visible nucleoli at the periphery. The cytoplasm shows varying degrees of vacuolization, and contains many mitochondria with dense matrix and inconspicuous cristae, and few small dense lysosomal particles and abundant smooth endoplasmic reticulum **(Fig. 21)**.

In ALP-treated mice, many histopathological changes were observed, depending on dose level of treatment. At low dose level, most seminiferous tubules were closely similar to the normal one, where they showed orderly arranged layers of spermatogenic stages **(Figs. 6 & 7)**. However, few tubules revealed a slightly loosened organization of spermatogenic epithelium represented by intratubular degeneration (Fig. 8). The interstitial tissue was abundant and contained many Leydig cells which were distributed evenly in cords **(Figs. 6 & 7).**

Ultrastructurally, few cytoplasmic alterations were detected in the primary and secondary spermatocytes. Some primary spermatocytes possessed clumping of chromatin with signs of pyknosis **(Fig. 22)**. The secondary spermatocytes were almost spherical in shape and covered by the acrosomal vesicle **(Fig. 23)**. Many of the spermatids become smaller in size and showed vacuolated cytoplasm. Spermatozoa were irregularly arranged in haphazard fashion in relation to the surface of the supporting Sertoli cells **(Fig. 24)**. The interstitial Leydig cells were large in size, polyhedral in shape and containing irregularly-shaped nuclei with peripherally-placed nucleoli. The cytoplasm contained many mitochondria with conspicuous cristae. Also, few small dense lysosomal particles and short abundant smooth endoplasmic reticulum were observed **(Fig. 25)**. These changes were severe and more pronounced in testes of mice administered with 1.5 and 4.5 mg/kg bw ALP. Most seminiferous tubules were separated from each other, leaving wide intratubular spaces, had irregular outlines and greatly depleted germ cells **(Figs. 8-10)**. Furthermore, detachment and sloughing of spermatogenic epithelium were apparently seen in many of these ST **(Fig. 11)**. In other tubules, there were loss of elongated spermatids and spermatozoa. In addition, severe degeneration in Leydig cells was observed in the loosely packed interstitial tissues among ST **(Figs. 10 & 11)**.

Ultrastructurally, the primary spermatocytes were moderately affected, where marked vacuolation in the cytoplasm was observed **(Fig. 26)**. The secondary spermatocytes were the most affected stage, where the mitochondria in these cells were numerous and contained wide intercristae spaces. Also, they formed dense masses with inter-mitochondrial cement. Accumulation of myelin-like inclusions was observed in cytoplasm of these cells **(Fig. 27)**.

Different stages of spermatozoa formation with abnormal formation of flagellum and wide cytoplasmic regions were seen. Among the commonest head anomalies were the deformed head shape, and the abnormal chromatin condensation. The heads of most late spermatids were broken and detached from the middle piece **(Figs. 27 & 28)**. They were still embedded in Sertoli cell after sloughing of their middle pieces into the lumina. The interstitial Leydig cells acquired many cytoplasmic processes; having few organelles, contained few and small rounded-shaped mitochondria, and their nuclei were apparently pyknotic **(Fig. 29)**.

Sections of testes of mice treated with 4.5 mg/kg bw ALP displayed more advanced stages of injury and marked degree of vacuolar degenerative changes in the spermatogenic epithelium, most ST were separated from each other, leaving wide intertubular spaces. In some ST, most spermatogenic cells were pyknotic **(Fig. 13)**. The spermatogenic epithelium had a slightly loosened organization represented by intratubular vacuolation **(Fig. 14)**. Many of these tubules showed low height of germinal epithelium, and induced incomplete spermatogenesis. The lumens of these tubules were severely devoid of spermatozoa **(Figs. 14 & 15)**, and there was a disruption in the continuous sheath of myoid cells. The tunica albuginea was much thicker and contained vacuolized areas. Severe degeneration of Leydig cells was observed in the interstitial tissues among ST **(Fig. 16)**.

One of the most important ultrastructural alterations observed in sections of testes of mice administered with 4.5 mg/kg bw ALP was that the intercellular spaces of spermatogenic stages became abnormally wide. The

primary spermatocytes showed marked vacuolation in their cytoplasm **(Fig. 30)**, and the secondary spermatocytes were the most affected stages where the mitochondria in these cells were numerous; contained wide intercristae spaces and they formed dense masses of inter-mitochondrial cement. Accumulation of myelin-like inclusion in cytoplasm of these cells was observed **(Fig. 31)**. Spermatids have a tendency to show more severe ultrastructural changes, where they had deformed nuclei, and irregularly-shaped acrosomes **(Fig. 32)**. In other spermatids, the acrosomes were stretched out unusually, and different stages of spermatozoa formation with abnormal formation of flagellum and wide cytoplasmic region were detected **(Figs. 33 & 34)**.

The Sertoli cells maintained little morphological characteristics as an indented nucleus and evident nucleolar associated chromatin. Also, it had marked decrease in cytoplasmic area and very thin cytoplasmic extensions. The cytoplasm showed marked loss of cytoplasmic organelles and large lysosomes-like vacuoles were observed **(Fig. 31)**. Concerning the interstitial Leydig cells, their nuclei were apparently pyknotic, having prominent nuclear irregularity and contained small patches of chromatin in the electron lucent nucleoplasm. The cytoplasm contained few mitochondria, and other organelles were not clearly observed **(Fig. 35)**.

Table 1. Body and organs weights of male mice after oral administration with differentdoses of alprazolam for three months.

Parameters	Doses of alprazolam (mg/kg bw/day)			
	Control	0.5	1.5	4.5
Number of male mice	15	15	15	15
Number of dead mice	0	1	3	7
Rate of mortality (%)	0	6.6	26.3	46.6
Initial body weight (g)	32.9 ±4.01	33.8 ± 3.25	33.5± 2.45	32.2 ± 2.98
Final body weight (g)	34.3 ± 2.98 ^{a}	32.1 ± 3.01^{ab}	28.1 ± 2.41^{b}	17.3 ± 1.98^{c}
Weight gain	12.4 ± 1.69 ^{a}	12.3 ± 1.28 ^{a}	11.7 ± 1.36 ^{a}	4.1 ± 0.52^{b}
Absolute organs weights (g)				
Paired testes	0.25 ± 0.031 ^{a}	0.25 ± 0.031 ^{a}	0.22 ± 0.016 ^{a}	0.12 ± 0.010^{b}
Paired pididymides	0.07 ± 0.003 ^{a}	0.07 ± 0.013 ^{a}	0.06 ± 0.018^{ab}	0.05 ± 0.074^{b}
Relative organs weights				
Paired Testes	0.55 ± 0.036 ^{a}	0.45 ± 0.033 ^{a}	0.49 ±0.025 ^{a}	0.33 ± 0.013^{b}
Paired pididymides	0.07 ± 0.003 ^{a}	0.07 ± 0.013 ^{a}	0.06 ± 0.018^{ab}	0.05 ± 0.074^{b}

Data are presented as mean ± SD, (*n* = 15/ group)

The same small letters show no significant differences from the control,

The different small letters indicate that there were significant differences at value of $p \leq 0.05$.

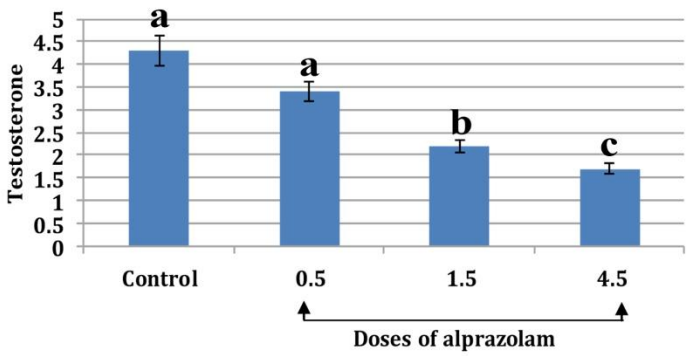


Fig. 1. Serum testosterone levels in the control mice and mice administered with different doses of alprazolam, for three months.

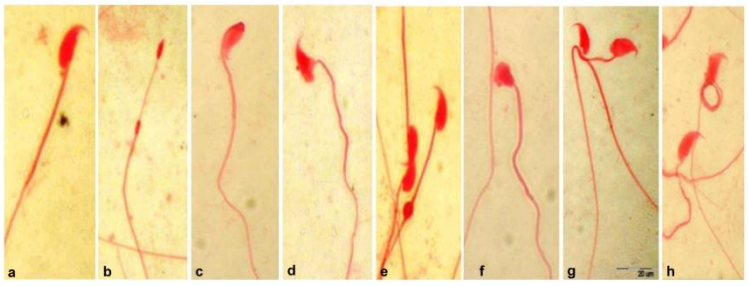
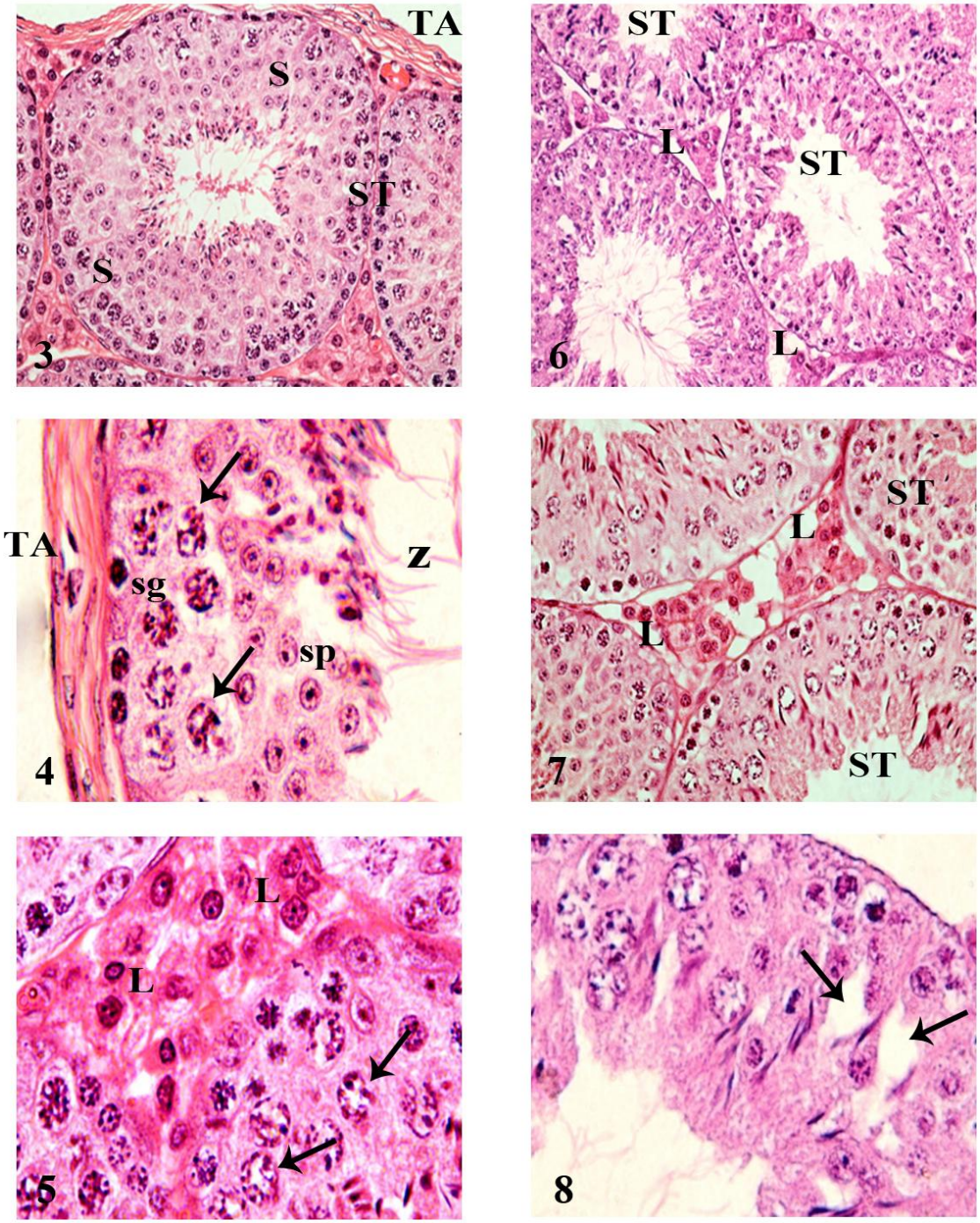
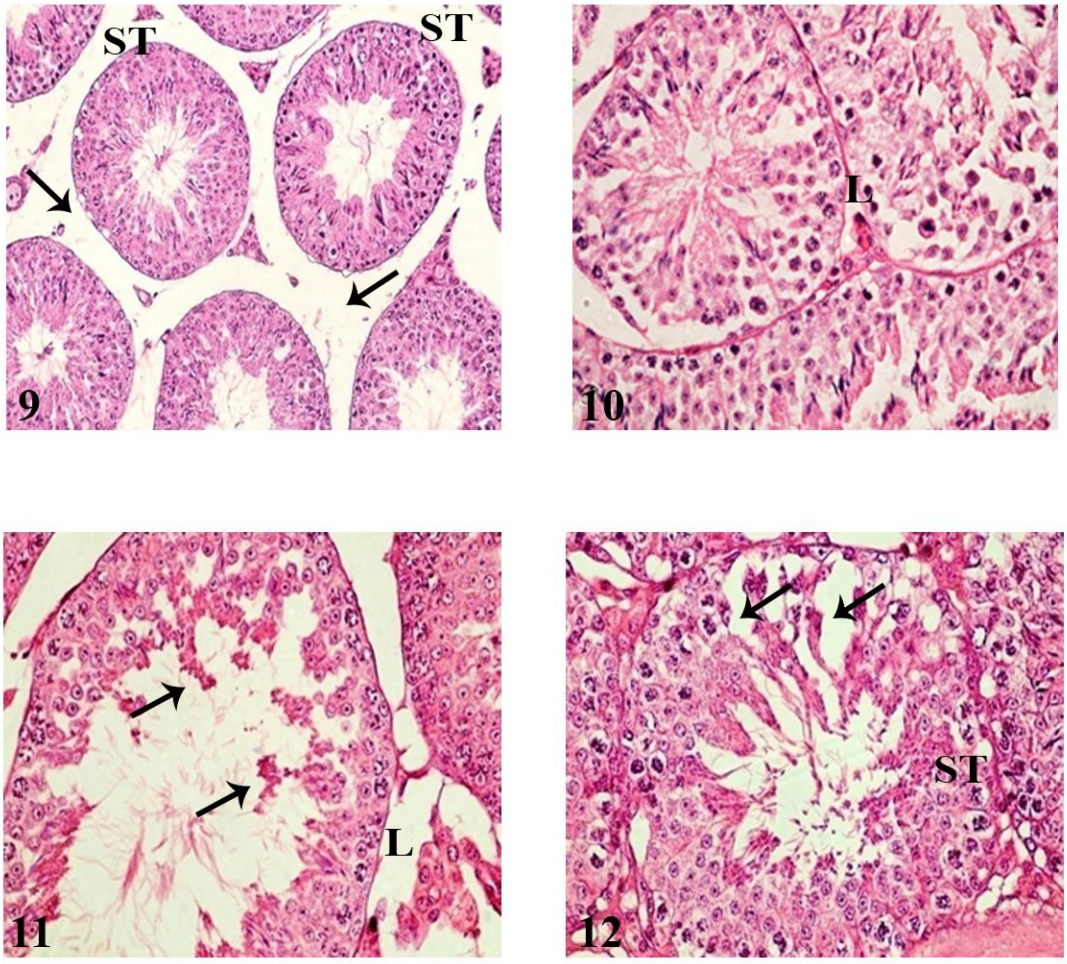


Fig. 2. Photomicrographs of the epididymal sperm shapes, stained with eosin, showing: normal sperm: (a); amorphous head (b); hookless head (c); calyculate head (d); doublet heads and tail with a cytoplasmic droplet (e); compact head (f); irregular tail (g); coiled tail (h). (Burruel *et al*., 2000).

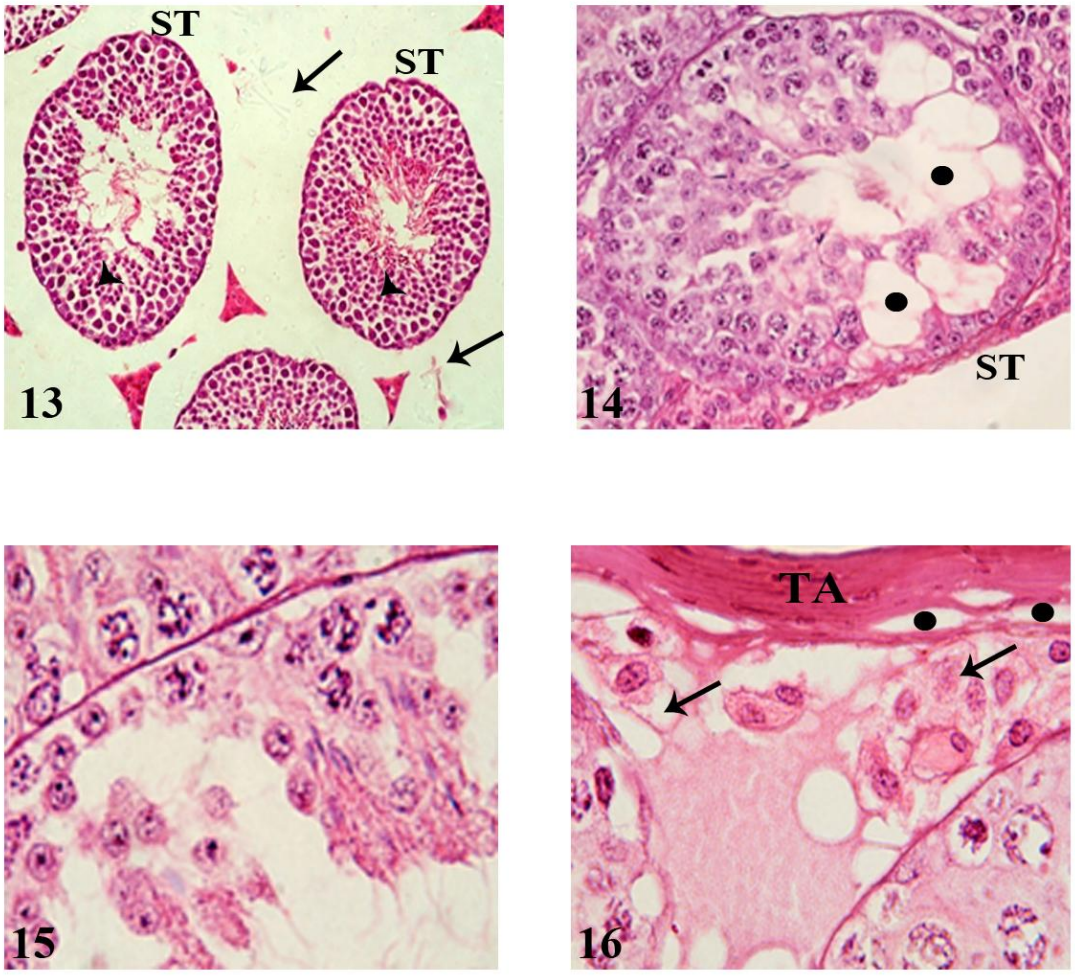


Figs. 3 - 5: Light micrographs of testis of control mouse showing: (3): The normal structure of seminiferous tubules (ST), each one is lined with spermatogenic cells and sertoli cells (S), tunica albugenea (TA); (4): Tunica albugenea (TA) contains myoid cells (m), dark nuclei of spermatogonia (sg), primary spermatocytes (arrows), spermatids (sp), spermatozoa (Z); (5): interstitial Leydig cells (L), X 630.

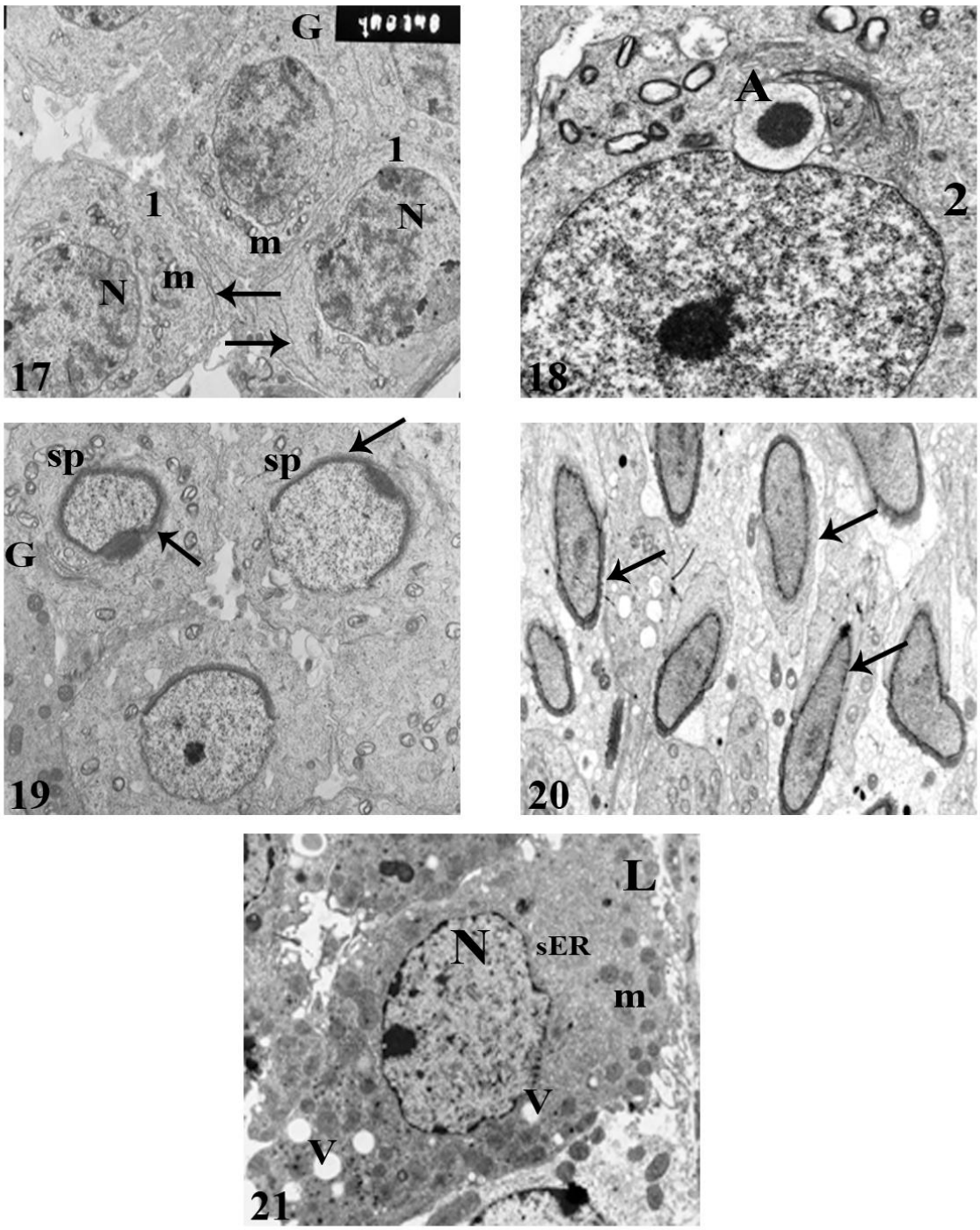
Figs. 6 - 8: Light micrographs of testis of mice treated with 0.5 mg/kg bw/d alprazolam, showing: (6): well organized ST with an orderly arranged spermatogenic stages; Leydig cells (L); (7): well organized ST with an orderly arranged spermatogenic stages; Leydig cells (L); (8): intratubular degeneration in spermatogenic epithelium (arrow), X 630.



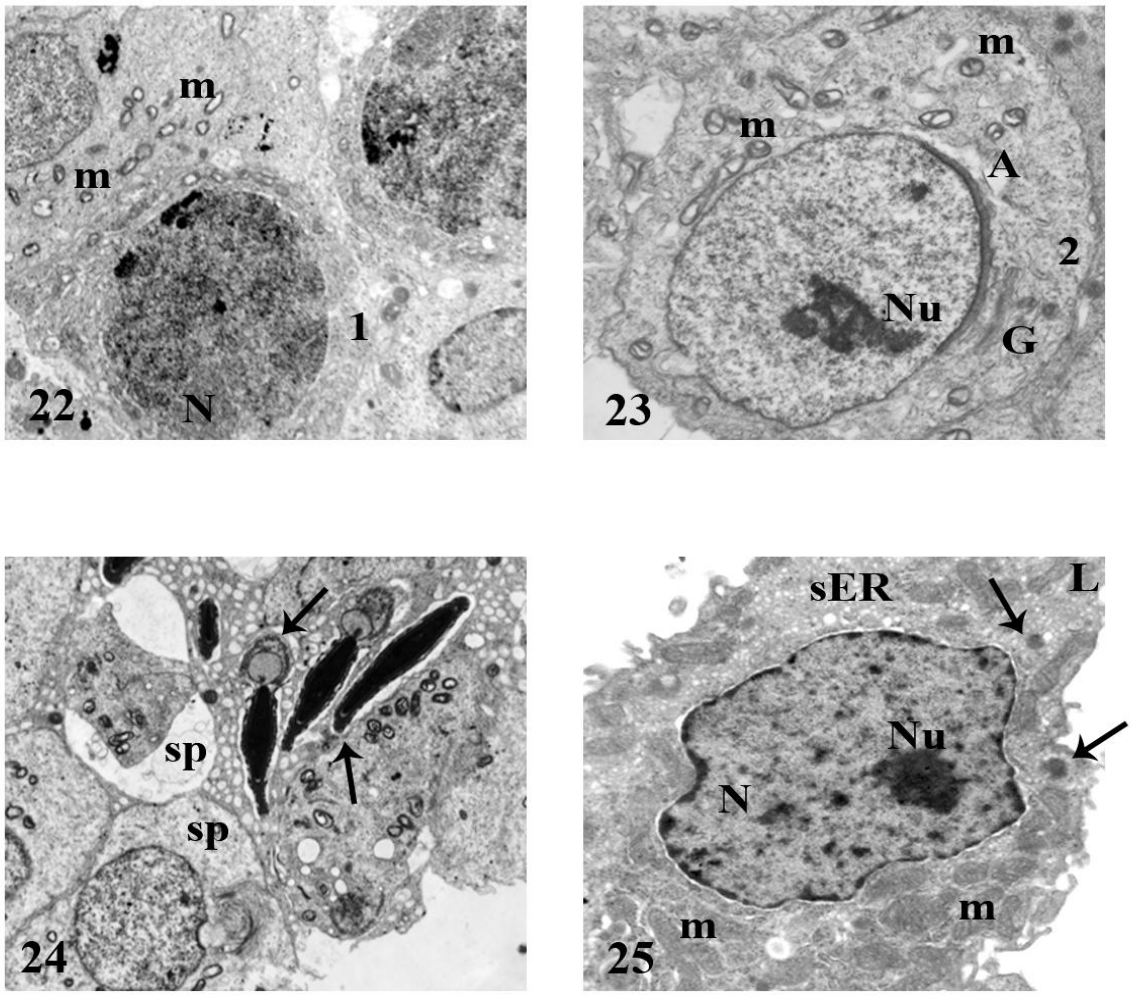
Figs. 9 - 12: Light micrographs of testes of mice treated with 1.5 mg/kg bw/d alprazolam, showing: (9): wide intertubular spaces (arrows) of ST; (10): loss of spermatogenic stages, loss of elongated spermatids, degenerated Leydig cells (L); (11): loss of spermatogenic stages, loss of elongated spermatids, detachment and sloughing epithelium (arrows), degenerated Leydig cells (L); (12): vacuolation in the intratubular ST (arrows), X 630.



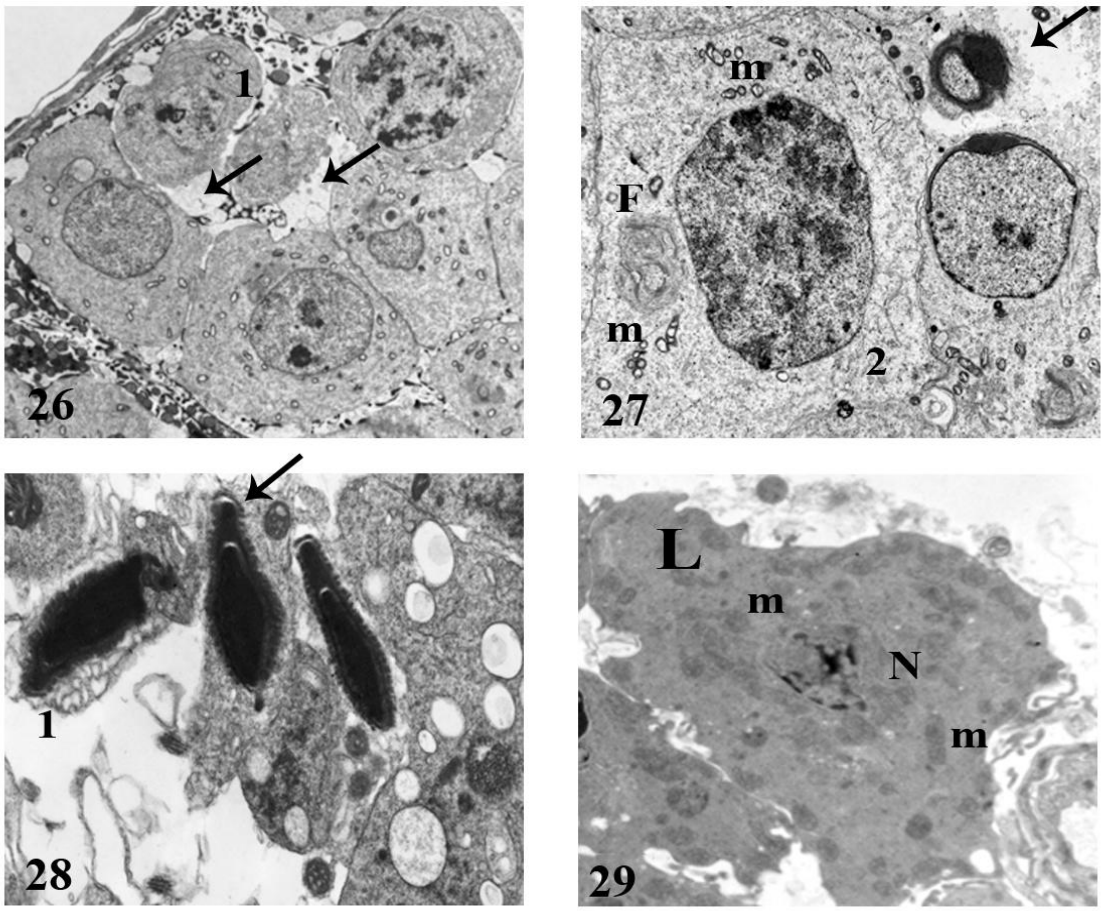
Figs. 13-16: Light micrographs of testis of mice treated with 4.5 mg/kg bw/d alprazolam, showing: (13): Seminiferous tubules (ST) are separated by wide intertubular spaces (arrows), pyknotic spermatogenic cells (arrowheads); (14): intratubular vacuolation (●) of ST; devoid of spermatozoa; (15): degenerated spermatogenic epithelium with incomplete spermatogenesis, devoid of spermatozoa; (16): thick TA containing vacuolized area (●), severe degeneration of Leydig cells (arrows), X 630.



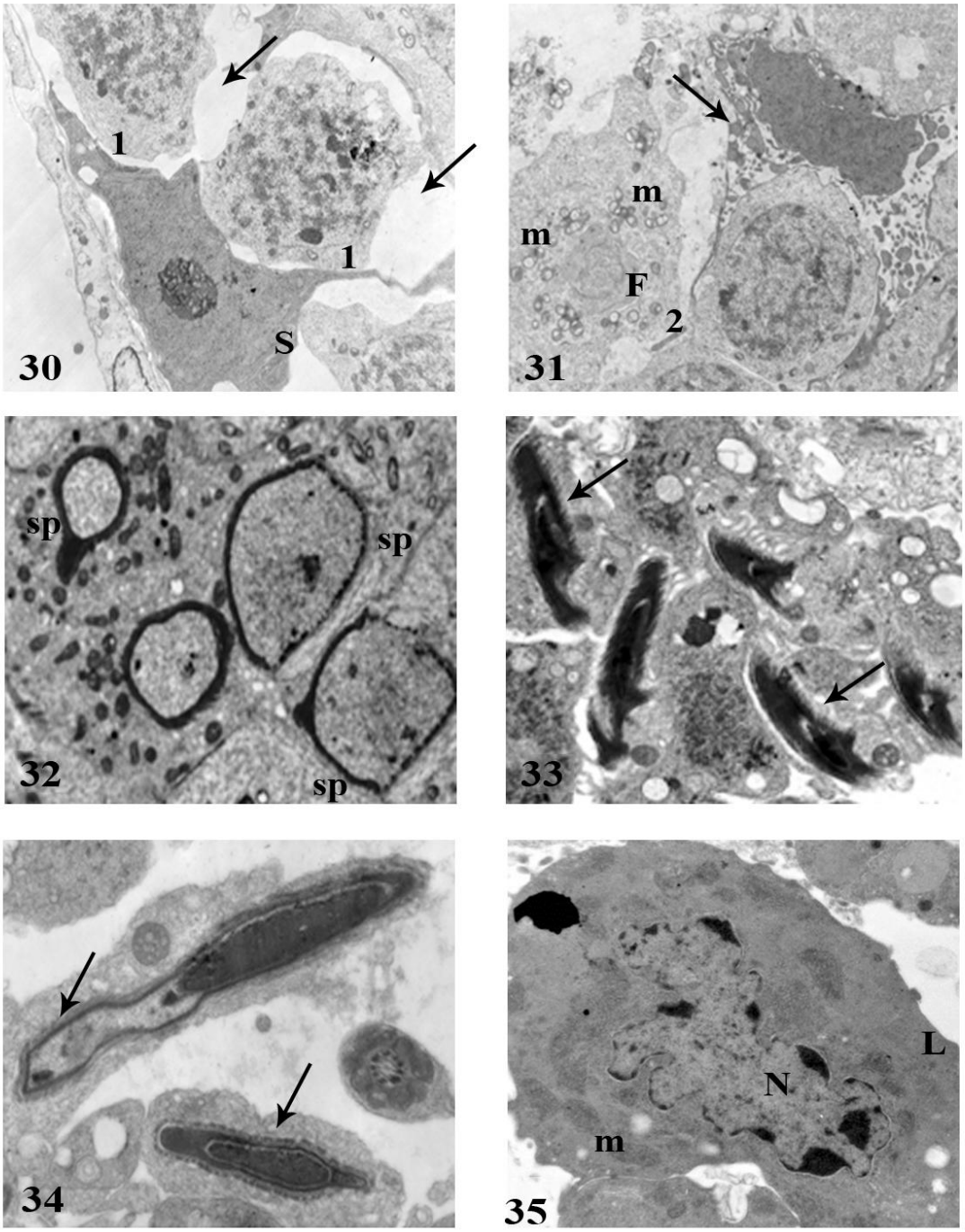
Figs. 17-21: Electron micrographs of testis of control mouse showing: (17): large spherical nuclei (N) of the primary spermatocytes (1), ovoid-shaped mitochondria (m), Golgi body (G), rough endoplasmic reticulum (arrows); (18): secondary spermatocyte (2) is covered by acrosomal vesicle (A); (19): spermatids (SP) are covered anteriorly by the acrosomal vesicles (arrows) and contain flattened Golgi areas (G); (20): late elongated spermatids (arrows) forming spermatozoa with flagellum; (21): slightly irregular nucleus (N) of Leydig cell (L), the cytoplasm contains many ovoid-shaped mitochondria (m), few dense lysosomal (arrows) particles, small vacuoles, smooth ER (sER), X 5000.



Figs. 22-25: Electron micrographs of testis of mice treated with 0.5 mg/kg bw/d alprazolam, showing: (22): the primary spermatocytes (1) have large spherical nuclei (N), mitochondria (m); (23): secondary spermatocyte (2) is covered with acrosomal vesicle (A), nucleolus (Nu), in the cytoplasm the mitochondria (m) contain wide intercristae spaces, Golgi area; (24): spermatids (SP) have vacuolated cytoplasm, elongated spermatids (arrows); (25): irregularly-shaped nucleus (N) of Leydig cells (L) containing a peripherally-placed nucleolus (Nu), the cytoplasm contains many mitochondria (m), few dense lysosomal particles (arrows), smooth ER (sER), X 5000.



Figs. 26 - 29: Electron micrographs of testis of mice treated with 1.5 mg/kg bw/d alprazolam, showing: (26): marked vacuolation (arrow) in the cytoplasm of the primary spermatocytes (1); (27): the mitochondria (m) in secondary spermatocyte (2) contain wide intercristae spaces, and form intermitochondrial cement, myelin figure (F) deformed head shape (arrows); (28): the heads of late spermatids (arrows) are broken and detached; (29): pyknotic nucleus (N) of the interstitial Leydig cell (L), the cytoplasm contains few organelles and small rounded-shaped mitochondria (m), X 5000.



Figs. 30 - 35: Electron micrographs of testis of mice treated with 4.5 mg/kg bw/d alprazolam, showing: (30): marked vacuolation (arrows) in the cytoplasm of the primary spermatocytes (1); Sertoli cell (S) contains marked decrease in cytoplasmic area and very thin cytoplasmic extensions; (31): the mitochondria (m) in secondary spermatocyte (2) contain wide intercristae spaces, and form intermitochondrial cement; myelin figure (F), Sertoli cell (S) contains large phagolysosomes; (32): deformation in the nuclei of most spermatids (SP) and the acrosomes are stretched out unusually; (33): abnormal head formation of spermatozoa (arrows); (35): abnormal formation of spermatozoa, pyknotic nucleus (N) of the interstitial Leydig cell (L), the cytoplasm contains few mitochondria (m), X 5000.

4. Discussion

The present study on the reproductive toxic effect of alprazolam (ALP) was undertaken in view of its widespread use as an anxiolytic antisedative drug. Hence, in the current study, evaluation of the reproductive endpoint was recorded in male mice administered orally by gavage with 0.5, 1.5 and 4.5 mg/kg bw ALP, for three months.

In toxicological studies, absolute and relative organs weights are important criteria for evaluation of organ toxicity **(Crissman et al., 2004).** The current results revealed significant decreases in the body weights, weight gain, absolute and relative testes and epididymal weights in mice treated with 4.5 mg/kg bw ALP in comparison with the other experimental groups and the control. These results are supported with the study of **Udoh and Kehinde (1999)** who suggested that the decrease in the testicular weight was accompanied by necrotic changes. In this respect, we suggest that, the reduction of the relative testes weight may be attributed to the parenchymal atrophy in seminiferous tubules after oral treatment with the high dose level of ALP.

Further, the present results showed significant decrease in testosterone levels in serum of all ALP-treated mice, which explained ALP exerted its suppressive effects on the testicular function and lead to infertility of mice. The decrease in testosterone levels, body weight, relative testes and epididymis weights observed in the present results confirmed earlier results of **Grote et al. (2004)** in rats and **Sarpa et al. (2007)** in mice.

It is known that normal spermatogenesis depends on the level of testosterone gonadotropic hormones of LH and FSH, where LH stimulates testosterone production **(MacLachlan et al., 2002; Spaliviero et al., 2004).** In the adult, testosterone is responsible for the establishment of secondary sexual characteristics, epididymal sperm maturation, and the promotion of spermatogenesis **(Orth, 1993).** Spermatogenesis and fertility are critically dependent upon the maintenance of adequate levels of testosterone **(Walsh et al., 2000).** The histological examination of testes of control mice showed normal cellular arrangement in ST along with plenty of spermatid steps correlating with the sufficient serum testosterone level.

The testicular tissue of mice revealed many histopathological changes in the structure of testes of ALP-treated mice, which were of dose dependent. These changes were summarized in: most STs were shrunken and had irregular outlines and greatly depleted germ cells; detachment and sloughing of spermatogenic epithelium; loss of elongated spermatids, spermatozoa, and severe degeneration in Leydig cells. The loss in germinal epithelium might be the cause of decrease in the number of spermatocytes and spermatids, which would eventually, resulted in the decrease of spermatozoa. In addition, there was pronounced testicular histopathology evidenced by thickening of basement membrane in some seminiferous tubules and the appearance of a wavy outline. **Richardson et al. (1998)** stated that the basement membrane plays an important role in maintaining the structural and functional integrity of tissues and that any structural changes in this membrane are associated with severe functional impairment of the testis.

Ultrastructurally, the results showed that the ST were surrounded by two collagenous fibres and elongated myoid cells between them. These myiod cells were believed by **Goyal and Williams (1987)** to be responsible for the rhythmic shallow contraction of ST. **Leeson et al. (1988)** explained that the neighboring myoid cells exhibit junctional complexes that retard, but do not entirely prevent, the passage of macromolecules from the interstitial space to the seminiferous epithelium.

In the testicular tissue of ALP-treated mice, the primary spermatocytes showed marked vacuolation in their cytoplasm. These vacuoles could be attributed to shrinking and appearance of degeneration in germ cells. **Creasy (2005)** explained the presence of vacuoles within the seminiferous epithelium as a common early response to a variety of toxicants. They added that these empty spaces may be phagocytic vacuoles remaining after digestion of necrotic germ cells. **Ramzan and Qureshi (2011)** presumed that cellular vacuolization is due to a lowered plasma testosterone concentration.

Sertoli cells function in that they may mediate most, if not all, hormonal stimuli regulating spermatogenesis, and when disturbed caused epithelial disorganization and subsequent tubular atrophy **(Lin and Jones, 1993; Bedwal et al., 1994)**. At any differentiation step (stage), germ cells contact and associate with Sertoli cells. These cellular interactions between the germ cells and the Sertoli cells are complex **(Toyama et al., 2001)**. In the present study, the Sertoli cells showed an accumulation of lysosome-like structure in the testicular tissue of mice administered with high dose of ALP, and the tight junctions of them seem to be weakened that lead to separation of spermatogenic cells. Many investigators (**Bizarro et al., 2003; Fiorini et al., 2004; Morales et al., 2004)** had reported that the main morphologic responses of Sertoli cells were the vacuolization, alteration of intercellular junctions, and the accumulation of lysosome-like structures with polymorphous interiors.

The decrease in testosterone levels was accompanied by significant increase in sperm shape abnormalities. The present results revealed that many spermatozoa had several abnormalities in both heads and tails. These abnormalities were as such the detached head, the amorphous head and the highly folded tail. High incidence of the

coiled or curved flagella was detected. This may be due to the injury effect of ALP and destruction of germ cells. **Takihara et al. (1987)** had reported that the reduction in the number of spermatogenic elements and spermatozoa leads to reduction in the weight of testes.

Further, **De Lamirande and Gagnon (1992)** explained that the rapid loss of intracellular ATP might lead to damage in sperm flagellum and increase of morphological defects of sperms (amorphous, hookless, bicephalic, coiled, or abnormal tails) with deleterious effects on sperms capacity and acrosome reaction. **Narayana et al. (2005)** believed that sperm abnormalities indicated points of mutation in germ cells, which it could be attributed to the chromosomal variations. Anyhow, defects in the axonemal cytoskeleton should be considered, since they have been correlated with loss of motility and fertilization potential **(Hancock and de Krester, 1992; Chemes and Raue, 2003).**

Tasdemir et al. (1997) had reported that defects in the sperm head morphology could reflect abnormalities in spermatogenesis. Also, **Lister and McLean (1997)** had postulated that these morphological abnormalities in heads of sperms resulted from mutations in the testicular DNA, which in turn disrupts the process of differentiation of spermatozoa.

Many investigators had reported that the induction of faulty head differentiation that developed in spermatids is a result of alterations in the pattern of chromatin condensation and/or the development of the acrosome (**Barth and Oko, 1989; Zamboni, 1992; Pinart et al., 1998; Predes et al., 2011)**.

Isojarvi et al. (1993) suggested that the partial epilepsy might affect sperm concentration, morphology and motility; and that generalized epilepsy may affect sperm morphology and motility. Sperm morphology is another important aspect in assessing sperm quality as well as a key index to evaluate the reproductive toxicity and mutagenicity of exogenous chemicals **(Wang et al., 2006).** These data were clearly demonstrated that treating male mice with ALP resulted in profound altered epididymal sperm morphology. The increase in the total sperm shape abnormalities were more frequent in mice treated with high doses of ALP (1.5 & 4.5 mg/kg bw) compared with those treated with the low dose (0.5 mg/kg bw). This was accompanied by significant decrease in serum testosterone levels.

Ultrastructural examination of the testes revealed more detailed structural alterations represented by the disarrangement and vacuolization of mitochondria in the middle piece, and fibrous sheath deficiency in the principal piece. However, there was no evidence of tail folding recognized during ultrastructural inspection, which indicates that ALP sperm folding is not an intrinsic spermatozoa defect, but rather a morphological aberration of an epididymal origin.

Furthermore, the remarkable increase in the frequency of spermatozoa with an amorphous head is confirmed with the current ultrastructural observations of numerous late spermatids with head deformities. **Wyrobek and Bruce (1978)** and **Letz (1990**) had reported that head abnormalities could be due to hormonal alterations which affect spermatogenesis.

Leydig cells are of great importance for the progression of the spermatogenic process **(Bustos-Obregon and Croxatto, 2003),** and the reduction of the hormone levels indicated alterations in structure and function of these cells. In high dose treatment with ALP, the population of Leydig cells had dispersed, and their nucleoli had disappeared suggesting an atrophy of them **(Saxena et al., 1987). Dutta and Meijer (2003)** explained that the decrease in the number of Leydig cells could be the cause of testosterone shortage. In conclusion, the results showed that ALP possess a deleterious effect on the testis and the epididymal spermatozoa, and adversely influence the male reproductive fertility of albino mice. Although these results provide no definitive explanation of the mechanism of action of ALP, they offer additional insights into the ultrastructural events related to its testicular toxicity.

References

Allison C, Pratt J A. 2003. Neuroadaptive processes in GABAergic and glutamatergic systems in benzodiazepine dependence. Pharmacol. Ther., 98 (2): 171-195.

Anderson I M, Nutt D J, Deakin F W. 2000. Evidence-based guideline for treating depressive disorders with antidepressants: A revision of the 1993 British Association for Psychopharmacology guidelines. J. Psychopharmacol., 14: 3 – 20.

Bancroft J D, Gamble M. 2002. Theory and Practice of Histological Techniques. 5th ed. Harcourt publisher limited, London. 153 p.

Barth A D, Oko R J. 1989. Abnormal morphology of bovine spermatozoa. Ames, IA: Iowa State Univ. Press; 130 – 266.

Bauer J, Stefan H, Schrell U. et al. **1992.** Serum prolactin concentrations and epilepsy: a study which compares healthy subjects with a group of patients in presurgical evaluation and circadian variations with those related to seizures. Eur. Arch. Psychiatry Clin. Neurosci., 241: 365 – 371.

Bedwal R S, Edwards M S, Katoch M, Bahuguna A, Dewan R. 1994. Histological and biochemical changes in testes of zinc deficient Bal B/C strain mice, Indian J. Experim. Biol., 32 (4): 243 – 247.

Bizarro P, Acevedo S, Niño-Cabrera G. et al. 2003. Ultrastructural modifications in the mitochondrion of mouse Sertoli cells after inhalation of lead, cadmium or lead-cadmium mixture. Reprod. Toxicol., 17: 561 – 566.

Burruel R V, Raabe O G, Overstreet J W, Wilson B W, Wiley L M. 2000. Paternal effects from methamidophos administration in mice. Toxio. Appl. Phamacol., 165: 148 - 157.

Bustos-Obregon E, Croxatto H. 2003. Gametogenesis, A. Alfredo Pérez Sánchez, Editor, Ginecologia, Editorial Mediterraneo, Chile, 60 – 75 p.

Chemes H E, Raue V Y. 2003. Sperm pathology: a step beyond descriptive morphology. Origin characterization and fertility potential of abnormal sperm phenotypes in infertile men. Hum. Reprod. Update, 9: 405 – 428.

Chen A, Bookstein JJ, Medrum D R. 1991. Diagnosis of a testosteronescreting adrenal adenoma by selective venous catheterization. Fertile Steril., 55: 1202 - 1203.

Creasy D M. 2005. The male reproductive system, In: Turton J, Hooson J, editors. Target Organ Pathology: A Basic Text. London, Bristol: Taylor & Francis, 334 – 365 p.

Crissman, J. W.; Goodman, D. G.; Hildebrandt, P. K.; Maronpot, R. R.; Prater, D. A.; Riley, J. H. et al. (2004). Best practice guideline: toxicologic histopathology. Toxicol. Pathol., 32: 126 – 131.

Dana-Haeri J, Oxley J, Richens A. 1982. Reduction of free testosterone by antiepileptic drugs. Br. Med. J. (Clin Res Ed). 284: 85 – 86.

De Lamirande E, Gagnon C. 1992. Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. J. Androl., 13: 379 – 386.

Dutta H M, Meijer H J. 2003. Sublethal effects of diazinon on thestructure of the testis of bluegill, Lepomis macrochirus: a microscopic analysis. Enviromental pollution, 125: 355 - 360.

Fiorini C, Tilloy-Ellul A, Chevalier S. et al. 2004. Sertoli cell junctional proteins as early targets for different classes of reproductive toxicants. Reprod. Toxicol., 18: 413 – 421.

Goyal HO, Williams CS. 1987. The rete testis of the goat, a morphological study. Acta Anat., 130: 151 – 157.

Grote, K, Stahlschmidt B, Talsness C, Gericke C. Appel K, Chahoud I. 2004. Effects of organotin compounds on pubertal male rats. Toxicol., 202: 145–158.

Hancock A D, de Krester D M. 1992. The axonemal ultrastructure of spermatozoa from men with asthenospermia. Fertil. Steril., 57: 661 – 664.

Herzog A G, Seibell M M, Schomer D L, Vaitukaitis J L, Geschwind N. 1986. Reproductive endocrine disorders in men with partial seizures of temporal lobe origin. Arch. Neurol., 43: 347 – 350.

Isbister G K, OaeRegan L, Sibbritt D. et al. 2004. Alprazolam is relatively more toxic than other benzodiazepines in overdose British Journal Clinical Pharmacology, 58 (1): 88 - 95.

Isojarvi J I T, Laatikainen T J, Pakarinen A J, Juntunen K T S, Myllyla V V. 1993. Polycystic ovaries and hyperandrogenism in women taking valproate for epilepsy. N. Engl. J. Med., 329: 1383 – 1388.

Kessler R C, McGonagle K A, Zhao S. et al. 1994. Lifetime and 12-month prevalence of DSM- III-R psychiatric disorders in the United State: results from the National co-morbidity survey. Arch. Gen. Psychiatry, 51: 8 - 19.

Leeson T S, Leeson C R, Paparo A A. 1988. A text/ Atlas of Histology. 9th ed. W.B. Saunders Company, Philadelphia, London and Toronto.

Letz G. 1990. Male Reproductive Toxicology. In: Ladou J, editor. Occupational Medicine. connecticut: Appleton and Lange, 288 – 296 p.

Lin M, Jones R C. 1993. Spermatogenesis and spermiation in the Japanese quail. J. Anat., 183: 525 - 535.

Lister C F, McLean A E. 1997. Inhibition of DNA synthesis by paracetamol in different tissues of the rat in vivo. Toxicology, 116: 49 – 57.

MacLachlan R I, Meachem S J, Stanton P G, deKretser D M, Pratis K. Robertson D M. 2002. Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkey, and man. Rec. Prog. Hormon Res., 57: 149 – 179.

Matousek J. 1969. Effect on spermatogenesis in guinea pigs, rabbits and sheep after their immunization with sexual fluid of bulls., J. Report Fert., 19: 63 – 72.

Montouris G D, Morris G L. 2005. Reproductive and sexual dysfunction in men with epilepsy Epilepsy & Behavior, 7; S7 –S14.

Morales E, Horn R, Pastor L M. et al. 2004. Involution of seminiferous tubules in aged hamsters: an ultrastructural, immunohistochemical and quantitative morphologic study. Histol Histopathol., 19: 445 – 455.

Narayana K, Prashanthi N, Nayanatara A, Kumar H H, Abhilash K, Bairy K L. 2005. Effects of methyl parathion (o,o-dimethyl o-4-nitrophenyl phosphorothioate) on rat sperm morphology and sperm count, but not fertility, are associated with decreased ascorbic acid level in the testis. Mutat. Res., 588: 28 – 34.

Nibedita N, Bhar R B, Mukherjee A. et al. 2005. Structural alteration of spermatozoa in the person's employed in lead acid battery factory. Indian Journal of Physiology and Pharmacology, 49: 153 - 162.

Oliveira H, Span M, Santosc C, Pereira M. 2009. Adverse effects of cadmium exposure on mouse sperm. Reprod. Toxicol., 28: 550 – 555.

Orth J M. 1993. Cell and Molecular Biology of the Testis (eds. Desjardins,C, Ewing). University Press, New York: 3 - 43.

Pinart E, Camps R, Briz M D, Bonet S, Egozcue J. 1998. Unilateral spontaneous abdominal cryptorchidism: structural and ultrastructural study of sperm morphology. Anim. Reprod. Sci., 49: 247 – 268.

Predes F S, Monteiro J C, Matta S L P, Garcia M C. Dolder H. 2011. Testicular histomorphometry and ultrastructure of rats treated with cadmium and Ginkgo biloba. Biol. Trace Elem. Res., 140: 330 – 341.

Ramzan F, Qureshi I Z, 2011. Intraperitoneal kisspeptin-10 administration induces dose-dependent degenerative changes in maturing rat testes. Life Sci., 88: 246 – 256.

Rathod N R. 2001. Alprozolam poisoning. Indian J. Med. Sci., 55: 218 – 221.

Richardson L L, Kleinman H K, Dym M. 1998. Altered basement membrane synthesis in the testis after tissue injury. J Androl., 19: 145 – 155.

Robards A W, Wilson A J. 1993. Procedures In Electron Microscopy. Wiley, Chichester, United Kingdom.

Tasdemir M, Tavukcuoglu S, Kahraman S, Biberoglu, K. 1997. Effect of abnormal sperm head morphology on the outcome of intracytoplasmic sperm injection in humans. Hum. Reprod., 12: 1214 – 1217.

Sarpa M, De-Carvalho R R, Delgado I F, Paumgartten F J R. 2007. Developmental toxicity of triphenyltin hydroxide in mice. Regul. Toxicol. Pharmacol., 46: 235 – 242.

Saxena D K, Srivastara R S, Lal B, Chndra S V. 1987. The effects of lead exposure on the testis of growing rats. Exp. Pathol., 31: 240 – 252.

Spaliviero J A, Jimenez M, Allan C M, Handelsman D J. 2004. Luteinizing hormone receptor-mediated effects on initiation of spermatogenesis in gonadotropin-deficient (hpg) mice are replicated by testosterone. Biol. Reprod., 70: 32 – 38.

Takihara H, Cossentino M J, Sakotoku J, Cockett ATK. 1987. Significance of testicular size measurements in andrology. II. Correlation of testicular size with testicular function. J. Urology, 137: 416 - 419.

Toyama Y, Hosoi I, Ichikawa S. et al. **2001.** Estradiol 3-benzoate affects spermatogenesis in the adult mouse. Mol Cell Endocrinol., 178: 161 – 168.

Udoh P, Kehinde A. 1999. Studies on antifertility effect of pawpaw seeds (Carica Papaya) on the gonads of male albino rats. Phytotherapy Resource, 13 (3): 226 – 228.

Verster J C, Volkerts E R. 2004. Clinical Pharmacology, Clinical Efficacy, and Behavioral Toxicity of Alprazolam: A Review of the Literature CNS Drug Reviews, 10 (1): 45 – 76.

Walsh L P, McCormick C, Martin C, Stocco D M. 2000. "Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression". Environmental Health Perspectives, 108 (8): 769 – 776.

Wang H, Meng Z, Chang F. 2006. The toxic effects of in vivo derivatives of sulfur dioxide on sperms in male mice. J.Application and Environmental, 40: 249 - 255.

Wyrobek A J, Bruce W R. 1978. The induction of sperm-shape abnormalities in mice and humans. In: Holander A, de Series FJ, editors. Chemical mutagens: principles and methods for their detection. New York: Plenum Press, 257 – 285 p.

Zamboni L. 1992. Sperm structure and its relevance to infertility. Arch. Pathol. Lab Med., 116: 325 – 344.