RESEARCH ARTICLE

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HISTOLOGICAL AND ULTRASTRUCTURAL STUDIES OF THE EFFECT OF ALPRAZOLAM-INDUCED TESTICULAR DAMAGE IN MALE MICE

ABSTRACT:
Alprazolam 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), through 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 three experimental mice groups were received daily oral doses of ALP 0.5, 1.5 and 4.5 mg/kg BW, respectively 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. Experimental groups exhibited significant increase in sperm anomalies. The prominent sperm deformities were detached head, amorphous head and 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 seen. A marked increase of inactive ST with degenerated spermatids, spermatooza and Leydig cells was also noticed. 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. Accommodation 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 including 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.

KEY WORDS:
Alprazolam, epididymis, Leydig cells, spermatids, testosterone

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 showed that the epileptic discharges are 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). Montours 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.

MATERIAL AND METHODS:
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).

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 the experiment.

Experimental design:
Sixty male mice were randomly assigned into four groups (15 mice; each) according to their approximately equal mean body weight, one control (saline–treated mice) and three experimental groups. Experimental mice groups were received daily therapeutic doses of 0.5, 1.5 and 4.5 mg/kg BW, for three months respectively. The applied dose was dissolved in 0.5 ml saline solution.

Monitoring of 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.

Body weights and reproductive organs weight:
Body weights of all experimental mice were recorded weekly during the periods 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 dissected. The testes and epididymes were excised out quickly, weighed and the absolute and the relative weights were calculated according to Matousek (1969):
\[ \text{I.W.} = \frac{\text{organ weight (g)}}{100} \times \text{body weight (g)} \]

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 stored at a refrigerator at −20°C until used. Testosterone levels were assayed by an enzymimmunoassay (EIA) method, using commercial Kit, Calbiotech, Spring Velley, CA, USA (Chen et al., 1991).

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 modified Tyrode’s medium (MT6) (125 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl2–6H2O, 0.36 mM NaH2PO4–2H2O, 5.56 mM glucose, 25 mM NaHCO4, 1.80 mM CaCl2, 100 units penicillin and 4 mg/ml BSA) and left for 30 min at 37 °C for sperm release (Oliveira et al., 2009). Aliquots of spermatozoa in MT6 medium were smeared on clean, grease-free microscope slides, air-dried, and 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). Two hundred 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). All sperm abnormalities were recorded as the percentages of the total number of counted spermatozoa.

Histopathological studies:
Testis of both control and alprazolam-treated mice of the different experimental groups 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).

Transmission electron microscopy:
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. The specimens were then 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.
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 treated 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).

RESULTS:

Table 1 shows an increase in the mortality rate of the treated mice, depending on the dose level of treatment, compared with the control.

Body and the reproductive organs weight:

The applied doses (0.5, 1.5 & 4.5 mg/kg BW) of the drug treatment cause a body weight loss, and testes and epididymal weight in comparison with the control. Significant decrease is remarked at higher dose-treatment (Table 1).

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

<table>
<thead>
<tr>
<th>Doses of alprazolam (mg/kg BW)</th>
<th>Control</th>
<th>0.5</th>
<th>1.5</th>
<th>4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of male mice</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Number of dead mice</td>
<td>-</td>
<td>1</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Rate of mortality (%)</td>
<td>0</td>
<td>6.6</td>
<td>26.3</td>
<td>46.6</td>
</tr>
<tr>
<td>Mean initial body weight</td>
<td>30.9</td>
<td>33.8</td>
<td>33.5</td>
<td>32.2</td>
</tr>
<tr>
<td>± SD</td>
<td>± 4.01</td>
<td>± 3.25</td>
<td>± 2.45</td>
<td>± 2.96</td>
</tr>
<tr>
<td>Mean final body weight</td>
<td>34.3</td>
<td>32.1</td>
<td>28.1</td>
<td>17.3</td>
</tr>
<tr>
<td>± SD</td>
<td>± 2.98</td>
<td>± 3.01</td>
<td>± 2.41</td>
<td>± 1.98</td>
</tr>
<tr>
<td>Weight gain</td>
<td>11.0</td>
<td>-5.03</td>
<td>-16.2</td>
<td>-46.3</td>
</tr>
<tr>
<td>± SD</td>
<td>± 0.15</td>
<td>± 0.42</td>
<td>± 0.03</td>
<td>± 0.22</td>
</tr>
<tr>
<td>Absolute testes weight (g)</td>
<td>0.25</td>
<td>0.25</td>
<td>0.22</td>
<td>0.12</td>
</tr>
<tr>
<td>± SD</td>
<td>± 0.031</td>
<td>± 0.031</td>
<td>± 0.016</td>
<td>± 0.010</td>
</tr>
<tr>
<td>Absolute epididymis weight (g)</td>
<td>0.07</td>
<td>0.07</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>± SD</td>
<td>± 0.003</td>
<td>± 0.013</td>
<td>± 0.018</td>
<td>± 0.074</td>
</tr>
<tr>
<td>Relative testes weight (g/100 gm fresh tissue)</td>
<td>0.55</td>
<td>0.45</td>
<td>0.49</td>
<td>0.33</td>
</tr>
<tr>
<td>± SD</td>
<td>± 0.036</td>
<td>± 0.033</td>
<td>± 0.025</td>
<td>± 0.013</td>
</tr>
<tr>
<td>Relative epididymis weight (g/100 gm fresh tissue)</td>
<td>0.07</td>
<td>0.07</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>± SD</td>
<td>± 0.003</td>
<td>± 0.013</td>
<td>± 0.018</td>
<td>± 0.074</td>
</tr>
</tbody>
</table>

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 ≤ 0.05.

Testosterone level:

Figure 1 illustrates gradual decreases in serum testosterone level of mice administered with 0.5, 1.5 and 4.5 mg/kg BW ALP, comparing to the control.

Sperm morphology:

As shown in table 2, it is evident that mice administered 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 (Table 2; 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 (Table 2). These abnormalities included: amorphous head, hookless head, calyculated head, doublet heads, compact head tail with a cytoplasmic droplet, irregular tail and coiled tail (Fig. 2e-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 highly folded tail.

Table 2. Sperm shape abnormalities (%) in the epididymis of control alprazolam-treated mice, for three months

<table>
<thead>
<tr>
<th>Sperm morphology</th>
<th>Control</th>
<th>Doses of alprazolam (mg/kg BW)</th>
<th>0.5</th>
<th>1.5</th>
<th>4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head abnormalities</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Big</td>
<td>0.5</td>
<td>0.5</td>
<td>6.5</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>Amorphous head</td>
<td>1.87</td>
<td>2</td>
<td>16.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hookless head</td>
<td>0</td>
<td>0.9</td>
<td>0.4</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Banana-like</td>
<td>0.5</td>
<td>0.6</td>
<td>0.5</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>Tailless</td>
<td>4.8</td>
<td>4.9</td>
<td>13.3</td>
<td>16.9</td>
<td></td>
</tr>
<tr>
<td>Tail abnormalities %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abaxial</td>
<td>1.94</td>
<td>2</td>
<td>2.6</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>Simply bent</td>
<td>4.6</td>
<td>4.7</td>
<td>5.8</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>Strongly Folded</td>
<td>4.8</td>
<td>4.9</td>
<td>5.3</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>Coiled</td>
<td>0</td>
<td>0.3</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic droplet</td>
<td>0</td>
<td>0.2</td>
<td>2.9</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>Headless</td>
<td>1.2</td>
<td>1.3</td>
<td>2.4</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Total abnormal sperm</td>
<td></td>
<td></td>
<td>19.71</td>
<td>21.9</td>
<td>41.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>67.54</td>
<td>67.54</td>
<td></td>
</tr>
</tbody>
</table>

Ten animals per group (representing a total of 2000 sperms)
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 ≤ 0.05
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 by 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).

In ALP-treated mice, many histopathological changes were observed, depending on the dose of treatment. At low doses, most seminiferous tubules are 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).
Histological and Ultrastructural Studies of the Effect of Alprazolam-Induced Testicular Damage in Male Mice

Figs 6 – 8. Light micrographs of testis of mice treated with 0.5 mg/kg BW alprazolam, showing: (6): well organized ST with an orderly arranged spermatogenic stages; Leydig cells (L), H& E, X 200; (7): well organized ST with an orderly arranged spermatogenic stages; Leydig cells (L), H& E, X 400; (8): intratubular degeneration in spermatogenic epithelium (arrow), 400.

These changes are severe and more pronounced in testes of mice administered 1.5 and 4.5 mg/kg BW ALP. Most seminiferous tubules are separated from each other; leaving wide intratubular spaces, have irregular outlines and greatly depleted germ cells (Figs 9-10). Furthermore, detachment and sloughing of spermatogenic epithelium are apparently seen in many of these ST (Fig. 11). In other tubules, there are loss of elongated spermatids and spermatozoa. In addition, severe degeneration in Leydig cells is observed in the loosely packed interstitial tissues among ST (Figs 10-12).

Figs 9 – 12. Light micrographs of testes of mice treated with 1.5 mg/kg BW alprazolam, showing: (9): wide intertubular spaces (arrows) of ST, H& E, X 200; (10): loss of spermatogenic stages, loss of elongated spermatids, degenerated Leydig cells (L) H& E, X 400; (11): loss of spermatogenic stages, loss of elongated spermatids, detachment and sloughing epithelium (arrows), degenerated Leydig cells (L), H& E, X 400; (12): vacuolation in the intratubular ST (arrows), X 630.

Sections of testes of mice treated with 4.5 mg/kg BW ALP display more advanced stages of injury and marked degree of vacuolar degenerative changes in the spermatogenic epithelium, most ST are separated from each other, leaving wide intertubular spaces. In some ST, most spermatogenic cells are are pyknotic (Fig. 13). The spermatogenic epithelium has a slightly loosened organization represented by intratubular vacuolation (Fig. 14). Many of these tubules show low height of germinal epithelium, and induce incomplete spermatogenesis. The lumens of these tubules are severely devoid of spermatozoa (Figs 14 & 15), and there is a disruption in the continuous sheath of myoid cells. The tunica albuginea is much thicker and contains vacuolized areas. Severe degeneration of Leydig cells is observed in the interstitial tissues among ST (Fig. 16).
Figs 13-16. Light micrographs of testis of mice treated with 4.5 mg/kg BW alprazolam, showing: (13): Seminiferous tubules (ST) are separated by wide intertubular spaces (arrows), pyknotic spermatogenic cells (arrowheads), H& E, X 200; (14): intratubular vacuolation (●) of ST; devoid of spermatozoa, X 400; (15): degenerated spermatogenic epithelium with incomplete spermatogenesis, devoid of spermatozoa H& E, X 400; (16): thick tunica albuginea (TA) containing vacuolated area (●), severe degeneration of Leydig cells (arrows), X 630.

The electron microscopic results:

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

The secondary spermatocytes are rarely observed among the germinal epithelium. They are spherical in shape and their nuclei are almost ovoid 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 ones are round 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 are 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).
Figs 17-21. Electron micrographs of testes of control mice 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; (21): slightly irregular nucleus (N) of Leydig cell (L), the cytoplasm contains many ovoid-shaped mitochondria (m), small vacuoles, smooth ER (sER), X 5000.

Few cytoplasmic alterations are detected in the primary and secondary spermatocytes. Some primary spermatocytes possess clumping of chromatin with signs of pyknosis (Fig. 22). The secondary spermatocytes are almost spherical in shape and covered by the acrosomal vesicle (Fig. 23). Many of the spermatids become smaller in size and show vacuolated cytoplasm. Spermatozoa are irregularly arranged in haphazard fashion in relation to the surface of the supporting Sertoli cells (Fig. 24).

The interstitial Leydig cells are large in size, polyhedral in shape and containing irregularly-shaped nuclei with peripherally-placed nucleoli. The cytoplasm contains many mitochondria with conspicuous cristae. Also, few small dense lysosomal particles and short abundant smooth endoplasmic reticulum are observed (Fig. 25).
Figs 22-25. Electron micrographs of testes of mice treated with 0.5 mg/kg BW 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 body; (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.

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

Different stages of spermatozoa formation with abnormal formation of flagellum and wide cytoplasmic regions are seen. Among the commonest head anomalies are the deformed head shape, and the abnormal chromatin condensation. The heads of most late spermatids are broken and detached from the middle piece (Figs 27 & 28). They are 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 are apparently pyknotic (Fig. 29).
One of the most important ultrastructural alterations observed in sections of testes of mice administered 4.5 mg/kg BW ALP is that the intercellular spaces of spermatogenic stages became abnormally wide. The primary spermatocytes show marked vacuolation in their cytoplasm (Fig. 30), and the secondary spermatocytes are the most affected stages where the mitochondria in these cells are numerous; contain wide intercristae spaces and they form dense masses of intermitochondrial cement. Accumulation of myelin-like inclusion in cytoplasm of these cells is observed (Fig. 31). Spermatids have a tendency to show more severe ultrastructural changes, where they have deformed nuclei, and irregularly-shaped acrosomes (Fig. 32). In other spermatids, the acrosomes are stretched out unusually, and different stages of spermatozoa formation with abnormal formation of flagellum and wide cytoplasmic region are detected (Figs 33 & 34).

The Sertoli cells maintained little morphological characteristics as an indented nucleus and evident nucleolar associated chromatin. Also, they have marked decrease in cytoplasmic area and very thin cytoplasmic extensions. The cytoplasm shows a marked loss of cytoplasmic organelles and large lysosomes-like vacuoles are observed (Fig. 31).

Concerning the interstitial Leydig cells, their nuclei are apparently pyknotic, having prominent nuclear irregularity and contain small patches of chromatin in the electron lucent nucleoplasm. The cytoplasm contains few mitochondria, and other organelles are not clearly observed (Fig. 35).
10

Figs 30 – 35. Electron micrographs of testes of mice treated with 4.5 mg/kg BW 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 (arrow); (32): deformation in the nuclei of most spermatids (SP) and the acrosomes are stretched out unusually; (33): abnormal head formation of spermatozoa (arrows); (34): abnormal formation of spermatozoa (arrows); (35): pyknotic nucleus (N) of the interstitial Leydig cell (L), the cytoplasm contains few mitochondria (m), X 5000.

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, the present study suggests 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 confirm 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 (McLachlan 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 a 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 myoid 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) reported that the main morphologic responses of Sertoli cells are 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) 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 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) reported that defects in the sperm head morphology could reflect abnormalities in spermatogenesis. Also, Lister and McLean (1997) 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 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; de Souza Predes et al., 2011).

Isojärvi et al. (1993) suggested that the partial epilepsy might affect sperm concentration, morphology and motility; and that the 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 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) 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 was dispersed, and their nucleoli disappeared suggesting their atrophy (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 possesses 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:


دراسات نسيجية وتكيبية دقيقة عن تأثير عقار الأليبرازولام المحت للضرر في حضية ذكور النثر

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عقار الأليبرازولام واحد من مجموعة النتروباريين، يستخدم في علاج الكثير من أنواع الاضطرابات، بما في ذلك نوبات الهلع والقلق العام والأكتاني. وقد أجريت العديد من الدراسات على هذا العقار لدراسة التأثير السريري له على القشرة الإقراضية عند الذكور، وقد أدت بعض الدراسات إلى تأثير عقاري على التركيب النسيجي والمنطق لنسخة الخصية عند ذكور الفئران بعد تعرض لثلاث جرعات مختلفة لمدة ثلاثة أشهر متتالية. كذلك تم تعيين مرضى هرمون التستوستيرون في سيرم هذه الذكور كدراسة على تقييم الخصوبة المجمعة، كما تم دراسة مظهر الجينات النموية من حيث اختلاف نحوهما. تم تقسيم ذكور الفئران إلى أربع مجموعات (15 فارا لكل مجموعة). المجموعة الأولى وهي المجموعة التي أعطيت 0.5 مل من المحلول الملحي. أعطيت مجموعات من الفئران 0.5 مل بجرعات 0.5، 1.5، و 4.5 مل/كجم/يوم لمدة ثلاثة أشهر. أظهرت نتائج هذه الفئارات تأثيرات كبيرة في التسرع في الخصوبة عند الذكور، لذلك نصح بعدم استخدام هذا العقار إلا تحت إشراف الطبيب المعالج.

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