EFFECT OF DIAZEPAM ON THE REPRODUCTIVE SYSTEM IN MALE RATS.

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ABSTRACT

Purpose: The purpose of this study was to determine the effects of diazepam on male fertility by assessing its effect on the weight of the reproductive organs, sperm characteristics which included the sperm count, motility, viability and morphology and its effects on serum LH, FSH and testosterone. Methods: Fifty male rats were allocated into five groups. Control (D.W.) (n=10) and test groups that received (2, 5 and 10 mg/kg/day) of diazepam by oral gavage, each group (n=10) and sulfasalazine (500mg/kg/day) for 8 weeks. Animals were kept in standard conditions. At the end of the treatment; animals were weighed before sacrificing and the testis, epididymis, seminal vesicles and prostate tissues were removed and weighed separately. While sperms were collected from cauda epididymis and the sperm count, motility, viability and morphology were determined. LH, FSH and testosterone were also measured using rat ELISA kits and the reproductive performance was also determined. Results: Animals showed an increase in weight after treatment while testis, epididymis, seminal vesicles and prostate weight were significantly reduced. Also, a significant decrease in sperm count, motility and viability with an increase in sperms abnormalities was observed. Serum LH, FSH and testosterone was also significantly decreased. Conclusion: This study observed impaired fertility in adult male rats treated with 2mg/kg/day of diazepam and higher doses.

KEYWORDS: testis, epididymis, seminal vesicles and prostate weight.

INTRODUCTION

Infertility is one of the most serious problems faced by some people around the world and the male counterpart contributes half of the infertility cases.¹ According to United States Food
and Drug Administration (FDA), infertility can be caused by androgen deficiency or low testosterone level. Testosterone deficit in men may exhibit symptoms such as decrease libido, erectile quality and low or zero sperms in semen.[2] The diagnostic testing can be done from the history of drugs taken, physical examination and of course, semen analysis.[3] According to Schulte et al. (2010) sperm characteristics assessment has been increasingly important in reproductive studies.[4]

Diazepam is a long-acting, medium-potency BZD that is used as an anticonvulsant and for anxiolysis, sedation, and myorelaxation. Diazepam is one of the most common BZDs used for anxiety.[5] The severity of diazepam induced adverse effects forces physicians to exercise caution and pay attention to side effects when prescribing this drug.[6] Recent studies found that benzodiazepines act as Ca channels antagonists as they can produce a complete inhibition of voltage-dependent Ca uptake.[7] Other suggested that the Na⁺/Ca²⁺ exchange carrier in mitochondria may be a common receptor for diazepam and calcium channel blockers.[8] The endocrine control of Leydig cell steroidogenic activity by luteinizing hormone (LH) or follicle-releasing hormone (FSH) has been exerted through their respective receptors coupled to the Ca²⁺ mediated signaling pathway.[9,10,11]

Calcium ion is implicated in diverse cellular functions in both germ cells and somatic cells in the testis, particularly, mediating the responses to endocrine hormones and local regulators in genital tracts.[12, 13] A common belief is that the Ca²⁺ influx and efflux should be tightly regulated to maintain the intracellular Ca²⁺ homeostasis, and an alteration in the Ca²⁺ transport across the cell membrane could result in a drastic impact on spermatogenesis and steroidogenesis.[14,15]

MATERIALS AND METHODS

Animals
Sprague-Dawly male rats weighing 200-250 gm. and 8 weeks old were obtained from the animal house of the College of Pharmacy- University of Baghdad. The animals were maintained on normal conditions of temperature, humidity and light/dark cycles. They were fed standard rodent pellet and they have free access to water.

Preparation of Diazepam suspension
Diazepam tablets (5mg) were powdered and dissolved in (5ml) distilled water to produce a standard solution for the preparation of different doses.
The study design
Fifty male rats were used in the present study, the study groups were divided into 5 groups:
First group (control): 10 rats were administered distilled water for 8 weeks by oral gavage.
Second group: 10 rats were used for the study of the infertility activity of diazepam in which (2mg/kg/day B.W.) of diazepam was given for 8 weeks by oral gavage.
Third group: 10 rats were used for the study of the possible infertility activity of diazepam in rat model. In this group (5mg/kg/day B.W.) dose of diazepam were used for 8 weeks by oral gavage.
Fourth group: 10 rats were used for the study of the possible infertility activity of (10mg/kg/day B.W.) diazepam was used for 8 weeks by oral gavage.
Fifth group: 10 rats were given a dose of (500mg/kg /day B.W.) of sulfasalazine for 8 weeks by oral gavage as a positive control (in this group, sulfasalazine represents standard infertility agent).

Determination of testes, epididymis, seminal vesicles and prostate weight to body weight ratio
After the end of the experiment period ; animals were weighed, anesthetized by diethyl ether, and testes, epididymis, seminal vesicles and prostate were obtained and weighed by sensitive balance after being cleaned from the accessory connective and adipose tissues and washed with normal saline.

The organ weight to body weight ratio was calculated according to the following equation: the organ weight to body weight ratio= weight of organ (gm)/ weight of animal (gm) ×100.

Epididymal tail suspension preparation
At the end of treatment; the cauda epididymis was quickly removed into a petridish that contains 10 ml of warm normal saline at 37°C and it was cut longitudinally. The sperms were released by mincing the cauda epididymis into pieces to perform the following microscopical examination on sperm characters.[16]

Determination of sperm concentration
Sperm count was determined using the haemocytometer under light microscope. A cover slip was placed on the haemocytometer before a drop of the epididymal sperm solution was
loaded under the cover slip. Sperm count was done by counting 5 RBC small squares. Sperm count was determined using the following formula:
Sperm count= total no. of sperms in 5 squares x50,000x100 (cells/ml).[17]

**Determination of sperm motility**
Sperm motility was assessed by placing a drop of the sperm suspension over a clean dry slide and covered with a cover slip and then the slide was placed under light microscope. The data were tabulated in the form of percentage using the formula:
Percentage of motile sperms =no. of motile sperms x100%/total no. of sperms (motile and immotile).[18]

**Determination of sperm viability**
In this analysis; a drop from the sperm suspension used before was mixed with one drop of eosin and then after 30 seconds, a drop of nigrosin was added and mixed. Then a smear was made. The dead sperms showed pink color of the head while the viable sperm showed colorless or whitish head based on the degree of membrane permeability, then the data were tabulated in the form of percentage using the following formula:
Percentage of viable sperms =no. of viable sperms×100%/ total no. of dead and viable sperms.[16]

**Determination of sperms abnormalities**
In this analysis; the same sperm smears made for sperm viability were observed under light microscope. The smears were examined for abnormal morphology of the head, neck and tail. Then data were tabulated using the following formula:
Percentage of abnormal sperms =no. of abnormal sperms×100%/ total no. of normal and abnormal sperms.[19]

**Effect of diazepam on the reproductive indices**
The reproductive indices were studied according to the method of Ruiz-Luna (2005) which includes fertility and pregnancy indices.[20]

At the end of the treatment; two male animals from each group were mated with four females in a ratio of (1 male: 2 females) separately in isolated cages. Before mating females were examined for the estrous cycle and mating was continuous for two week. Vaginal plug was observed which is an indication of successful mating and then females were isolated and
managed until the end of pregnancy to study the reproductive indices according to the following equations:
Fertility index= no. of pregnant animals/ no. of animals mated successfully×100.
Pregnancy index= no. of animals gives live pups/ no. of pregnant animals ×100.

Study the effects of diazepam on pituitary hormones (LH, FSH and testosterone) by using rat ELISA kits

Determination of serum LH levels
The serum levels of LH were estimated quantitatively using a readymade kit based on enzyme linked immunoassay method.[21]

Determination of rat serum FSH levels
The serum levels of FSH were estimated quantitatively using a readymade kit based on enzyme linked immunoassay technique.[22]

Determination of rat testosterone levels
The serum levels of testosterone were estimated quantitatively using a readymade kit based on enzyme linked immunoassay technique.[23]

Statistical analysis
Analysis of data was carried out using the available statistical package of SPSS-21 (Statistical Packages for Social Sciences version -21). Student t-test was used for testing the significance of difference between two groups and ANOVA between three.

The significance of difference of different percentages (qualitative data) was tested using Chi-square test.

Statistical significance was considered whenever the (p value) was equal to or less than 0.05.

RESULTS

1-Effect of diazepam on the body weight
Table (1) showed that there were no significant differences in the mean body weight at pretreatment period between T1 (2mg/kg), T2 (5mg/kg), T3 (10 mg/kg) groups and the sulfasalazine group as compared with the control group. The results were (228.5± 5.01 gm, 231± 5.67gm, 230.5±3.91gm and 230± 5.17 gm) respectively; compared to (219±5.67 gm) in the control group. While the period after treatment showed that there was a highly significant
(P<0.001) difference in the body weight between the control group and the T1 (2mg/kg), T2 (5mg/kg), T3 (10 mg/kg) and the sulfasalazine groups.

Table (1): Effect of different oral doses of diazepam suspension and sulfasalazine given to male rats in (mg/Kg) on the body weight (gm).

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>T1(2mg/kg)</th>
<th>T2(5mg/kg)</th>
<th>T3(10mg/kg)</th>
<th>Sulfasalazine (500mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment NS</td>
<td>219±5.67</td>
<td>228.5±5.01</td>
<td>231±5.67</td>
<td>230.5±3.91</td>
<td>230±5.17</td>
</tr>
<tr>
<td>After treatment</td>
<td>235±4.77</td>
<td>252.5±5.83</td>
<td>271±4.33</td>
<td>289 ±4.07</td>
<td>213±5.33</td>
</tr>
</tbody>
</table>

Data are expressed as mean (±SE); n=10 rats/group; a: t-test between control and T1, b: t-test between control and T2 , c: t-test between control and T3, d: t-test between T1 and T2, e: t-test between T1 and T3, f: t-test between T2 and T3, g: t-test between control and sulfasalazine, h: t-test between T1 and sulfasalazine, i: t-test between T2 and sulfasalazine , j:t-test between T3 and sulfasalazine, *: significant (p<0.05) difference, **: highly significant (p<0.001) difference, NS: no significant difference.

2-Effect of diazepam on the testicular, epididymis, seminal vesicles and prostate weight to body weight ratio

Table (2) showed that administration of diazepam suspension for 8 weeks caused a highly significant decrease (p<0.001) in the mean testicular, epididymis, seminal vesicles and prostate weight to body weight ratio of rats in the three treated groups T1 (2mg/kg), T2 (5mg/kg) and T3 (10 mg/kg) as well as in the sulfasalazine group compared to the control group.

Table (2) the effect of different oral doses of diazepam suspension and sulfasalazine on the testicular, epididymis, seminal vesicle and prostate gland weight to body weight ratio in male rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>T1(2mg/kg)</th>
<th>T2(5mg/kg)</th>
<th>T3(10mg/kg)</th>
<th>Sulfasalazine (500mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testes/B.W ratio</td>
<td>0.526±0.005</td>
<td>0.488±0.005</td>
<td>0.441±0.004</td>
<td>0.389±0.007</td>
<td>0.358±0.007</td>
</tr>
<tr>
<td></td>
<td><strong>a</strong></td>
<td><strong>b</strong></td>
<td><strong>c</strong></td>
<td><strong>d</strong></td>
<td><strong>e</strong></td>
</tr>
<tr>
<td>Epiddidymis/B.W ratio</td>
<td>0.202±0.004</td>
<td>0.191±0.003</td>
<td>0.161±0.002</td>
<td>0.134±0.002</td>
<td>0.119±0.002</td>
</tr>
<tr>
<td></td>
<td><em>a</em></td>
<td><strong>b</strong></td>
<td><strong>c</strong></td>
<td><strong>d</strong></td>
<td><strong>e</strong></td>
</tr>
<tr>
<td>Seminal vesicles/B. W ratio</td>
<td>0.199±0.003</td>
<td>0.185±0.004</td>
<td>0.155±0.002</td>
<td>0.143±0.002</td>
<td>0.125±0.003</td>
</tr>
<tr>
<td></td>
<td><em>a</em></td>
<td><strong>b</strong></td>
<td><strong>c</strong></td>
<td><strong>d</strong></td>
<td><strong>e</strong></td>
</tr>
<tr>
<td>Prostate gland/B.W ratio</td>
<td>0.188±0.002</td>
<td>0.170±0.001</td>
<td>0.154±0.002</td>
<td>0.134±0.003</td>
<td>0.118±0.005</td>
</tr>
<tr>
<td></td>
<td><em>a</em></td>
<td><strong>b</strong></td>
<td><strong>c</strong></td>
<td><strong>d</strong></td>
<td><strong>e</strong></td>
</tr>
</tbody>
</table>
Data are expressed as mean (±SE); n=10 rats/group; a: t-test between control and T1, b: t-test between control and T2, c: t-test between control and T3, d: t-test between T1 and T2, e: t-test between T1 and T3, f: t-test between T2 and T3, g: t-test between control and sulfasalazine, h: t-test between T1 and sulfasalazine, i: t-test between T2 and sulfasalazine, j: t-test between T3 and sulfasalazine, *: significant (p<0.05) difference, **: highly significant (p<0.001) difference, NS: no significant difference.

3-Effect of diazepam on the sperm concentration
Table (3) showed that the sperm concentration decreased highly significantly (p<0.001) in T1 (2mg/kg), T2 (5mg/kg), T3 (10mg/kg) and in the sulfasalazine group compared to the control group; the results were (529.9± 6.56 million/ ml; 400.2± 8.49 million/ ml, 292.4±9.30 million/ ml and 211±9.19 million/ml) respectively, compared with (644.5±16.29 million/ml) in the control group.

Table (3): Effect of different oral doses of diazepam suspension and sulfasalazine on the sperm characteristics of male rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Controls</th>
<th>T1(2mg/kg)</th>
<th>T2(5mg/kg)</th>
<th>T3(10mg/kg)</th>
<th>Sulfasalazine (500mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm conc. (million/ml)</td>
<td>644.5±16.29</td>
<td>529.9±6.56 <strong>a</strong></td>
<td>400.2±8.49 <strong>b</strong></td>
<td>292.4±9.30 <strong>c</strong></td>
<td>211±9.19 <strong>g****h</strong></td>
</tr>
<tr>
<td>Motility (%)</td>
<td>91.7±0.86</td>
<td>86.5±1.79 <strong>a</strong></td>
<td>77.9± 1.83 <strong>b</strong></td>
<td>64.7± 1.85 <strong>c</strong></td>
<td>58± 1.38 <strong>g****h</strong></td>
</tr>
<tr>
<td>Viability (%)</td>
<td>84.9± 1.33</td>
<td>81.1± 1.5 Ns</td>
<td>70.8± 1.65 <strong>b</strong></td>
<td>67.7± 1.01 <strong>c</strong></td>
<td>65.8± 1.16 <strong>g****h</strong></td>
</tr>
<tr>
<td>Abnormality (%)</td>
<td>5.13±0.363</td>
<td>9.88±0.436 <strong>a</strong></td>
<td>28.24±0.923 <strong>b</strong></td>
<td>30.74±1.42 <strong>c</strong></td>
<td>31.62±0.648 <strong>g****h</strong></td>
</tr>
</tbody>
</table>

Data are expressed as mean (±SE); n=10 rats/group; a: t-test between control and T1, b: t-test between control and T2, c: t-test between control and T3, d: t-test between T1 and T2, e: t-test between T1 and T3, f: t-test between T2 and T3, g: t-test between control and sulfasalazine, h: t-test between T1 and sulfasalazine, i: t-test between T2 and sulfasalazine, j: t-test between T3 and sulfasalazine, *: significant (p<0.05) difference, **: highly significant (p<0.001) difference, NS: no significant difference.

4-Effect of diazepam on the sperm motility
Table (3) showed that after 8 weeks of diazepam suspension treatment, the sperm motility in the T1 (2mg/kg), T2 (5mg/kg), T3 (10 mg/kg) and the sulfasalazine group decreased highly significantly (p< 0.001) compared to the control group. The results showed a significant...
decrease (p< 0.05) in sperm motility in the T1 (2mg/kg); (86.5±1.79%) group as compared to the control group (91.7±0.86%) while the T2 (5mg/kg), T3 (10mg/kg) and the sulfasalazine groups showed a highly significant (p< 0.001) decrease as compared to the control group. The results were (77.9± 1.83%; 64.7± 1.85% and 58± 1.38%) respectively compared to (91.7±0.86%) in the control group.

5-Effect of diazepam on the sperm viability
The effect of diazepam suspension on the percentage of dead sperms is shown in table (3). There was no significant difference in the viability percentage between the control (84.9±1.33%) group and T1 (2mg/kg); (81.1± 1.5%) group. But, there was a highly significant (p< 0.001) decrease in the T2 (5mg/kg), T3 (10 mg/kg) and the sulfasalazine groups as compared to the control group, the results were (70.8± 1.65% ; 67.7± 1.01%  and  65.8± 1.16%) as compared to (84.9± 1.33%) in the control group. Figure (1) shows sperms viability.

Figure (1) Sperm viability, (Pink head: dead sperm, colorless head: viable sperm).

6-Effect of diazepam on the sperm abnormality
The results of the abnormal sperms morphology are shown in table (3). After 8 weeks of the treatment; the percentage of the morphologically abnormal sperms increased highly significantly (p< 0.001) in the T1 (2mg/kg), T2 (5 mg/kg), T3 (10 mg/kg) and the sulfasalazine groups; the results were (9.88±0.436%, 28.24±0.923%, 30.74±1.42% and 31.62±0.648%) respectively compared to the control (5.13±0.363%) group.
2-Normal sperms

3- Tailless sperms.

4- Headless sperms.

5-Bent mid-tail sperms.

6-Abnormal long tail sperms.

7-coiled tail sperms.

Figure (2): showed normal shaped sperms and figures (3,4,5,6,&7) showed different abnormal sperms caused by diazepam treatment.

7- Effect of diazepam on serum LH concentration

Table (3) showed that serum LH levels was highly significantly (p< 0.001) reduced in the three treatment doses T1 (2mg/kg), T2 (5mg/kg), T3 (10mg/kg) and in the sulfasalazine group compared to the control group. The results were (9.59± 0.193ng/ml, 5.28±0.152 ng/ml,
2.7±0.149 and 2.24±0.85 ng/ml) respectively; compared to the control (12.02±0.235ng/ml) group.

**Table (4): Effect of different oral doses of diazepam suspension on the serum levels of hormones in male rats.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>T1(2mg/kg)</th>
<th>T2(5mg/kg)</th>
<th>T3(10mg/kg)</th>
<th>Sulfasalazine (500mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH (ng/ml)</td>
<td>12.02±0.235</td>
<td>9.59±0.193</td>
<td>5.28±0.152</td>
<td>2.7±0.149</td>
<td>2.24±0.85</td>
</tr>
<tr>
<td></td>
<td><strong>a</strong></td>
<td><strong>b</strong></td>
<td><strong>a</strong></td>
<td><strong>c</strong></td>
<td><strong>g</strong></td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
<td>20.99±0.468</td>
<td>18.34±0.282</td>
<td>16.58±0.254</td>
<td>14.27±0.324</td>
<td>12.16±0.233</td>
</tr>
<tr>
<td></td>
<td><strong>a</strong></td>
<td><strong>b</strong></td>
<td><strong>a</strong></td>
<td><strong>c</strong></td>
<td><strong>g</strong></td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>5.13±0.221</td>
<td>4.0±0.092</td>
<td>3.16±0.085</td>
<td>2.52±0.103</td>
<td>1.8±0.129</td>
</tr>
<tr>
<td></td>
<td><strong>a</strong></td>
<td><strong>b</strong></td>
<td><strong>a</strong></td>
<td><strong>c</strong></td>
<td><strong>g</strong></td>
</tr>
</tbody>
</table>

Data are expressed as mean (±SE); n=10 rats/group; a: t-test between control and T1, b: t-test between control and T2 , c: t-test between control and T3, d: t-test between T1 and T2, e: t-test between T1 and T3, f: t-test between T2 and T3, g: t-test between control and sulfasalazine, h: t-test between T1 and sulfasalazine, i: t-test between T2 and sulfasalazine, j: t-test between T3 and sulfasalazine, *: significant (p<0.05) difference, **: highly significant (p<0.001) difference, NS: no significant difference.

**8- Effect of diazepam on serum FSH concentration**

Table (3) showed that serum FSH levels was highly significantly (p<0.001) reduced in the T1 (2mg/kg), T2 (5mg/kg), T3 (10mg/kg) and the sulfasalazine groups; the results were (18.34±0.282 mIU/ml, 16.58±0.254 mIU/ml, 14.27±0.324 mIU/ml and 12.16±0.233) respectively; compared to the control (20.99±0.468 mIU/ml) group.

**9- Effect of diazepam on serum testosterone concentration**

Table (3) showed also that serum testosterone levels was highly significantly (p<0.001) reduced in the T1 (2mg/kg), T2 (5mg/kg), T3 (10mg/kg) and in the sulfasalazine group as compared to the control group; the results were (4.0±0.092ng/ml, 3.16±0.085ng/ml, 2.52±0.103ng/ml and 1.8±0.129 ng/ml) respectively as compared to the control (5.13±0.221ng/ml) group.

**10- Effect of diazepam on the fertility index**

Table (4) showed that the fertility index was significantly (p<0.05) decreased in the T1 (2mg/kg), T2 (5mg/kg), T3 (10 mg/kg) and the sulfasalazine group compared to the control
group; the results were (50%, 25%, 25% and 25 %) respectively compared to the control (100%) group.

Table (5): Effect of different oral doses of diazepam suspension on the fertility index.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>T1(2mg/kg)</th>
<th>T2(5mg/kg)</th>
<th>T3(10mg/kg)</th>
<th>Sulfasalazine (500mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of females mated successfully</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>No. of pregnant animals</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Fertility index (%)</td>
<td>100%</td>
<td>50%*a</td>
<td>25%<em>b</em>e</td>
<td>25%<em>c</em>f NS</td>
<td>25%<em>d</em>gNS</td>
</tr>
</tbody>
</table>

Data are expressed as (%), a: t-test between control and T1, b: t-test between control and T2, c: t-test between control and T3, d: t-test between control and sulfasalazine, e: t-test between T1 and T2, f: t-test between T1 and T3, g: t-test between T1 and sulfasalazine, *: significant (p<0.05) difference, NS: no significant difference.

11- Effect of diazepam on the pregnancy index

The results referring to pregnancy index are shown in table (5). The results showed that there was no significant changes among the three groups (p>0.05) as compared to the control group and the pregnancy index was 100% in all groups.

Table (6): Effect of different oral doses of diazepam suspension on the pregnancy index.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>T1(2mg/kg)</th>
<th>T2(5mg/kg)</th>
<th>T3(10mg/kg)</th>
<th>Sulfasalazine (500mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of pregnant animals</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>No. of pregnant animals gives live pups.</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pregnancy %</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

DISCUSSION

The results of the present study had shown that diazepam treatment caused an increase in body weight (table 1) this was due to the antiserotonin action of diazepam, this is inconsistent with that demonstrated by Haleem et al. (1996) who found that daily administration of
diazepam decreased serotonin level in the hippocampus and its metabolites in the hypothalamus and the level of both was decreased in the striatum and this will lead to increase in food intake and increase in body weight. The present study showed a decrease in testes, epididymis, seminal vesicles and prostate weight in treated rats with diazepam (table 2), this could be attributed to a decrease in testosterone level, where testosterone has an important role in increasing the weight of the reproductive organs including the testes, epididymis, seminal vesicles and prostate. The reduction in the weight of the reproductive organs in the treated groups may be due to the lowered availability of pituitary LH as the conversion of cholesterol to pregnenolone is dependent upon pituitary LH. Pituitary FSH has been shown to increase the testicular size. Our results showed a significant reduction in serum FSH levels in diazepam treated rats (table 4). Hence, lack of pituitary FSH can serve as a reason of decrease absolute testicular weight and epididymis weight in diazepam treated rats.

The present results were in agreement with that observed by Cook et al. (1979) who investigated the effect of diazepam and found that diazepam administration was associated with a significant reduction in both the weight of the prostate of treated rats and the serum testosterone levels. It was suggested that the above observations were induced via direct suppression of the interstitial cells of the testes. On the other hand, one of the possible causes of a drop in the weight of the testes and epididymis by the effect of diazepam is by suppressed spermatogenesis; in the absence of any known pathology, testes weight is highly related to daily sperm production.

The present study showed a significant decrease in the sperm concentration (table 3), this is may be due to either a decrease in testicular weight or inhibiting the spermatogenesis process. Spermatogenesis is influenced by the hypothalamic- pituitary- testicular axis relating gonadotropin releasing hormone, LH, FSH and androgens. Thus, the effects evoked by diazepam on sperm concentration might be strongly linked with status of LH and FSH hormones which are also reduced and greatly affect sertoli cells functions in the testes specially sperm production.

This implies that the decrease in sperm count caused by the drug in the treated rats was as a result of a decrease in plasma level of testosterone, because this hormone has been reported to be important in initiation and maintenance of spermatogenesis. Table (3) also showed a significant decrease in sperm motility. It is known that the structure and function of the
epididymis are dependent on androgens.\textsuperscript{[35]} In this study, a dose related suppression of the epididymis sperm motility in treated rats suggests an under supply of testosterone to epididymis and therefore an impaired epididymal function. The impaired epididymal function may also be due to reduced activity of the testes which affects the normal passage of the testicular fluid into the epididymis.\textsuperscript{[36, 37, 38]} This is also confirmed by the reduced epididymal weight.

This study referred to highly significant decrease in sperm viability with increasing the dose of diazepam (table 3 and figure 1), this is in consistent with that indicated by Mohana et al. (2013) who found that diazepam decreased significantly the motility and viability of goat epididymal sperms.\textsuperscript{[39]} They suggested that diazepam induced oxidative stress in goat epididymal sperms and that it had a significant role in disturbing the balance between oxidative stress and antioxidant system. They concluded that the use of diazepam could be a considerable factor in causing infertility in human males.

A significant increase in the percentage of morphologically abnormal sperms showed in table (3) and figures (3-7) may be due to the drug interference with the spermatogenic processes in the seminiferous tubules, epididymal functions and testosterone activity on the hypothalamic release factor and anterior pituitary secretion of gonadotropins which may result in alteration of spermatogenesis.\textsuperscript{[40,41]}

Kar and Das (1983) demonstrated a significant incidence of abnormalities induced by diazepam on sperms involving both shape and size of the sperm head and tail.\textsuperscript{[42]} This is in consistent with this study which had shown a significant increase in sperm abnormalities. Testosterone hormone is necessary for development and divisions of spermatogonia.\textsuperscript{[43]} The sperm morphology is also an important characteristic of sperm for evaluating male fertility.\textsuperscript{[44]} The sperm motility is the most important parameter in determining fertilization rate.\textsuperscript{[45]} Therefore, all of these parameters leading to decrease fertility capacity in treated rats. (Tables 5 and 6).

The development and growth of male reproductive ducts and seminiferous tubules is dependent on the increase of testosterone concentration.\textsuperscript{[46]} Testosterone is essential for the survival of the spermatogenic endothelium and the significant decrease in testosterone hormone causes an increase in sperms abnormal morphology and decrease in sperm viability.\textsuperscript{[47]}
The results of the present study had shown that treatment with diazepam suspension led to significant decrease in testosterone level in serum of treated animals (Table 4). Testosterone hormone is the principle male hormone; it is synthesized by Leydig cells from cholesterol. This decrement of testosterone level may be due to the effect of diazepam on serum cholesterol which is a precursor of testosterone synthesis by its action on the Leydig cells.

Therefore, the decreased testosterone concentration produced by the drug could be explained either by direct effect at Leydig cell level or an indirect effect by disturbing the hormonal milieu at hypothalamic-pituitary axis.

Bourguignon et al (1987) showed that in the presence of calcium channel blockers, the release of GnRH from hypothalamic neurons was markedly and reversibly reduced. GnRH induced LH secretion from pituitary gonadotrophs is also calcium dependent because the induction-repression of LHβ gene is dependent on calcium influx.

On the other hand FSH and LH exert their action on steroidogenesis through voltage dependent calcium channels (VDCCs) in sertoli cells and Leydig cells; thus, it is possible that any malfunction of Ca channels in somatic cells may result in drastic attenuation of testosterone level. Collectively, the decrease in serum concentration of LH might result in corresponding decrease in testosterone production by Leydig cells. This is because Ca ions are implicated in the process of LH secretion.

Testosterone is synthesized in Leydig cells. One of the genes directly responsible for cholesterol transport and testosterone synthesis in rat testes is the steroidogenic acute regulatory (StAR) gene.

StAR is necessary for the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane in Leydig cells, and this step is the rate-limiting regulated step in steroidogenesis. The inhibition of StAR expression results in a dramatic decrease in steroid biosynthesis. Several mechanisms underlying the reduction in mRNA expression of StAR gene caused by the drug closely correlated with the fact that Ca affects the transfer of cholesterol to the inner mitochondrial membrane, the rate-limiting step in steroidogenesis.
As Ca ions are required for several steps of steroidogenesis, diazepam as Ca channel antagonist would be expected to have an effect on StAR protein expression.

CONCLUSION
Diazepam had antifertility effects on male rats through attenuating steroidogenesis and testosterone production by inhibiting the pituitary gonadal axis hormones and the StAR gene expression through its effects on calcium ions.

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