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Effect of Cisplatin drug on sperm characterizes, spermatogenesis and sex hormones levels of male mice *Mus musculus* L.

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Abstract

The present study has aimed to investigate the effect of the anticancer drug cisplatin in sperm count, sperm abnormalities, spermatogenesis and sex hormones in male mice were divided into three groups consisting eight animals in each group. The first group was served as control and received 0.9% of normal saline. The second group was received cisplatin (1 mg/kg). The third group was received cisplatin (2 mg/kg). The mice were treated 8 times of cisplatin (i.p.) for 16 days at intervals of 48 hr between treatments. The animals were sacrificed after 16 days of last injection .The sperm count significantly decreased in the treated with two doses (1and 2mg/kg) at ($p<0.01$) .Cisplatin induced tail abnormalities, but a significant increase was seen in the high dose only. The spermatogonia, primary spermatocyte, germinal epithelial thickness and seminiferous tubular diameter decreased significantly in the both doses, also elongated spermatids decreased significantly in the treated mice with high dose only. Drug-induced germinal epithelial sloughing and vacuoles in the seminiferous tubules for testis . Luteinizing hormone (LH) and testosterone levels decreased significantly in male mice with (1 and 2 mg/kg) doses of cisplatin compared with control group, whereas, there was no significant difference in the serum follicle-stimulating hormone (FSH) of male mice treated with two doses of cisplatin.

Keywords Cisplatin ; sperm count ; ; Sperm abnormalities ; Spermatogenesis ; Testosterone ; FSH ; LH.

1. Introduction

Chemotherapy involves the use of chemical agents to stop the growth and eliminate cancer cells even at distant sites from the origin of primary tumor. However, it does not distinguish between a cancer and normal cells, and eliminates not only the fast-growing cancer cells but also other fast-growing cells in the body [1]. The recent studies on secular trends in male reproductive health have provided conflicting evidence with some investigations suggesting that sperm counts have declined significantly during the past 50 years. A number of possible causes including exposure to chemotherapeutic drugs have been suggested by some of these investigations. Moreover many chemotherapeutic drugs often can cause severe alterations in spermatogenesis [2].

The testis is a known target organ for injury resulting from exposure to both chemotherapeutic and toxic environmental agents. Chemotherapy induced physiological damage to male germ cells in the testis has been associated with fertility, which is monitored by parameters of semen quality. Exposure to more than 100 chemicals individually or as mixtures including chemotherapeutic are known to induce detrimental effects on semen quality [3] . Platinum-containing drugs, including cisplatin, are widely used in the treatment of solid tumors such as ovarian, testicular, bladder, and lung cancers. In particular, cisplatin has had some of its greatest therapeutic successes in the management of nonseminomatous germ cell tumors of the testis [4].Cisplatin dose has negative effects on the normal function of the testis . Sterility is one of the long-term adverse effects of cisplatin [5] and extension of survival is accompanied by problems of fertility [6] .Additional studies should be conducted to find ways to prevent the loss of fertility in patients receiving chemotherapy .To meet this goal, mechanisms of cytotoxicity of cisplatin in the testis must be examined in detail.

Spermatogenesis is often impaired in testicular cancer patients prior to chemotherapy as a result of the cancer itself [7] . In addition, Bleomycin, Etoposide and Cisplatin BEP drugs have substantial detrimental effects on spermatogenic function; most patients are rendered temporarily azoospermic or oligozoospermic, depending on the dose and duration of treatment [8] . Normal spermatogenesis recovers in about 50% of the patients after 2 years and in the large majority (80%) 5 years after the completion of chemotherapy [9, 10, 11]. However, in some patients, sperm production does not reinitiate and the permanent infertility ensues. Thus, fertility is a major concern for testicular cancer patients undergoing BEP chemotherapy.

In animal models, previous studies have shown that either acute or chronic administration of the chemotherapeutic drugs cisplatin or etoposide induces adverse effects on various male reproductive parameters shortly after exposure. For instance, sub chronic administration of cisplatin over a 9-week period resulted in a decrease in reproductive organ weights, including testes and epididymides, decreased sperm motility, and increased preimplantation and post implantation loss; malformed and growth-retarded fetuses were observed among the progeny sired by cisplatin-treated males [12]. However many reports available on cisplatin caused toxicity in various tissues, but very limited studies demonstrated the antifertility effects caused by cisplatin anti-cancerous drugs.

Androgens play a vital role in initiating and maintenance of male reproductive function or testicular function which includes spermatozoa production. The main testicular androgen i.e. testosterone is produced by leydig cells under the stimulation of pituitary LH, which is essential for spermatogenesis, fertility and maintenance of the male phenotype. Spermatogenesis depends on the action of testosterone which is produced by leydig cells in the testis [13]. Spermatogenic failure has been a recognized consequence of treatment with chemotherapeutic agents [14]. Hence, an attempt has been made in the present study to investigate the effect of Cisplatin anticancer drugs on epididymal sperm count , spermatogenesis and sex hormones in male mice.

2. Materials and Methods

2.1. Drug

Cisplatin drug was purchased in vial contain 50 mg powder dissolved in 50 ml solution produced by Merck company / France, manufacture number : 3096.

2.2. animals

Male mice *Mus musculus* L. strain Balb/C were used for the present study. These mice bred and housed in the animal house of the Biology Department / College of Education/ Basrah University. Animals were maintained in light- controlled room and at a temperature of (22±3c°) through out the experiment . The food was prepared in the laboratory by mixing crude protein, ground Soya bean, wheat flour, wheat bran, milk powder, mineral and vitamins.[15]

2.3. Animal experiment – dosing design

Twenty four intact male mice (7-8) weeks of age and (22-25)g body weight were used in the present study. The male mice divided into 3 different treatment groups (n =8): a control group (0.1 ml of normal saline), second group treated (I.P.) with low dose 0.1 ml of Cisplatin solution (1 mg/ kg) and third group treated (I.P.) with high dose 0.l ml of Cisplatin solution (2 mg/ kg). The mice treated with cisplatin drug of 8 doses , one dose of each 48 hr.

2.4. sperms count

Male mice were sacrificed by using chloroform on the 16^{th} day after the last injection, and the epididymis were collected and minced in 1ml phosphate buffered saline (PBS, pH 7.2). The suspension was mixed with 1% aqueous eosin Y (10:1) and kept for 30 min for the staining of sperms. Then an aliquot of stained the filtrate was taken in white blood cell pipette up to the 0.5 mark and diluted further up to mark 11 with PBS. The mixture was shaken and charged into Neubauer`s chamber and sperm count was performed as per standard procedure. The sperm count in 8 squares of $1mm^2$ each area except the central erythrocyte counting area of Neubauer`s chamber was performed and multiplied by 5 X 10^4 factor to calculate the total number of sperms [16].

2.5. Percentage of normal and abnormal sperms

The Method of [17] was used in this study. The epididymis was put in a Petri dish containing 5 ml (0.9%) physiological saline then it was cut into six parts or more by using sharp razor and appointed tong . After that, drops of the final produced solution were spreading on the slide and dried. The slides were stained by 1% eosin for 5-10 minutes and then left to be dried. For each sample,100 sperms were counted on each slide (five slides) and then the normal and abnormal percentage of sperm was determined.

2.6. Histological examination

For histological studies, the testis was fixed overnight in Bouin's fluid, dehydrated in ethanol, and embedded in paraffin. Tissue sections (6 µm-thick) were cut on a microtome , mounted on glass slide. Staining of section with hematoxylin and eosin (HE) . The Seminiferous tubular diameter and germinal mass thickness were measured and the number of spermatogonia, spermatocytes, and spermatids were counted in each group and examined under a light microscope [18].

2.7. Hormone assays

Serum concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone were measured by enzyme-linked immune sorbent assay (ELISA) as described in the instructions provided by manufacturer's kits (Human Co, Germany).

2.8. Statistical analysis

All statistical analysis was conducted using SPSS program version 11. One-way ANOVA was applied and $p<0.01$ was considered statistically significant.

3. Results

3.1. Effect of Cisplatin on sperm count and sperm abnormalities .

The effect of cisplatin drug on sperm count and sperm abnormalities of the male mice are illustrated in table 1. The results showed a significant decrease ($p<0.01$) in sperm count of male mice treated with two doses 1 and 2 mg/kg of cisplatin compared with the control group. Also, there was a significant increase ($p<0.01$) in abnormal sperms of the male mice treated with high dose only 2 mg/kg compared with the control group. The majority of abnormalities included the changes of tail shape as shown in figure 1.

Table 1. Effect of Cisplatin on the abnormalities and count of the mice sperms
(N=8) (Mean ± standard error)

Treatments	Sperm count ($mm^3 \times 10^6$)	Normal sperm (%)	Abnormal sperms	
			Abnormal head (%)	Abnormal tail (%)
Control group normal saline	8.34 ± 0.36	85.37 ± 2.31	6.36 ± 0.31	8.22 ±0.50
Cisplatin 1 mg/kg	*****4.11 ± 0.57	82.57 ± 1.50	6.95 ± 0.25	10.48 ± 0.46
Cisplatin 2 mg/kg	*****3.44 ± 0.59	*71.70 ± 2.94	8.08 ± 0.80	*20.62 ± 1.13

* There is a significant difference($p<0.01$) compared with the control group .

3.2. Effect of Cisplatin on spermatogenesis.

Table 2 represented the results of Cisplatin effects on spermatogenesis as shown in figure 2 . The results showed a significant decrease ($p<0.01$) in the spermatogonia and primary spermatocyte in the mice treated with 1 and 2 mg/kg of cisplatin, while there is a significant decrease in spermatide in the mice treated with 2 mg/kg only, as well as, there is significant reduction in the Seminiferous tubular diameter and germinal mass thickness in the males treated with both doses compared with the control group . Another effect of the drug was the distortion of seminiferous epithelium in terms of sloughing.

Table 2. Effect of Cisplatin on spermatogenesis of male mice

(N=8) (Mean ± stander error)

Treatments	Seminiferous Tubular diameter (µm)	germinal mass thickness	Spermatogonia count	Primary spermatocyte count	Elongated Spermatide count
Control group normal saline	159.38 ± 3.55	60.31 ± 2.46	29.38 ± 1.20	31.81 ± 1.34	53.74 ± 2.52
Cisplatin 1 mg/kg	*142.44 ±3.55	*44.40 ± 2.33	*11.22 ± 1.04	*10.17 ± 1.08	43.32 ± 3.25
Cisplatin 2 mg/kg	*129.13 ± 3.61	*42.26 ± 3.02	*8.38 ± 0.59	*7.78 ± 0.90	*38.63 ± 3.18

* There is a significant difference($p<0.01$) compared with control group.

3.3. Effect of Cisplatin on sex hormones.

The treated of male mice with 1 and 2 mg/kg of cisplatin caused a significant decrease in testosterone and Luteinizing hormone LH levels compared with control group , while follicle stimulating hormone FSH level in male mice was not affected by the treatment with two doses compared with the control group as shown in table 3.

Table 3. Effect of Cisplatin on sex hormones of male mice (N=8) (Mean ± stander error)

Treatments	Luteinizing hormone LH lU/l	Follicle simulating hormone FSH lU/l	Testosterone ng/ml
Control group normal saline	6.77 ± 0.42	3.39 ± 0.22	4.41 ± 0.45
Cisplatin 1 mg/kg	*2.14 ±0.27	3.32 ± 0.34	*1.88 ± 0.37
Cisplatin 2 mg/kg	*1.71 ± 0.17	2.68 ± 0.35	*0.94 ± 0.12

* There is a significant difference($p<0.01$) compared with the control group .

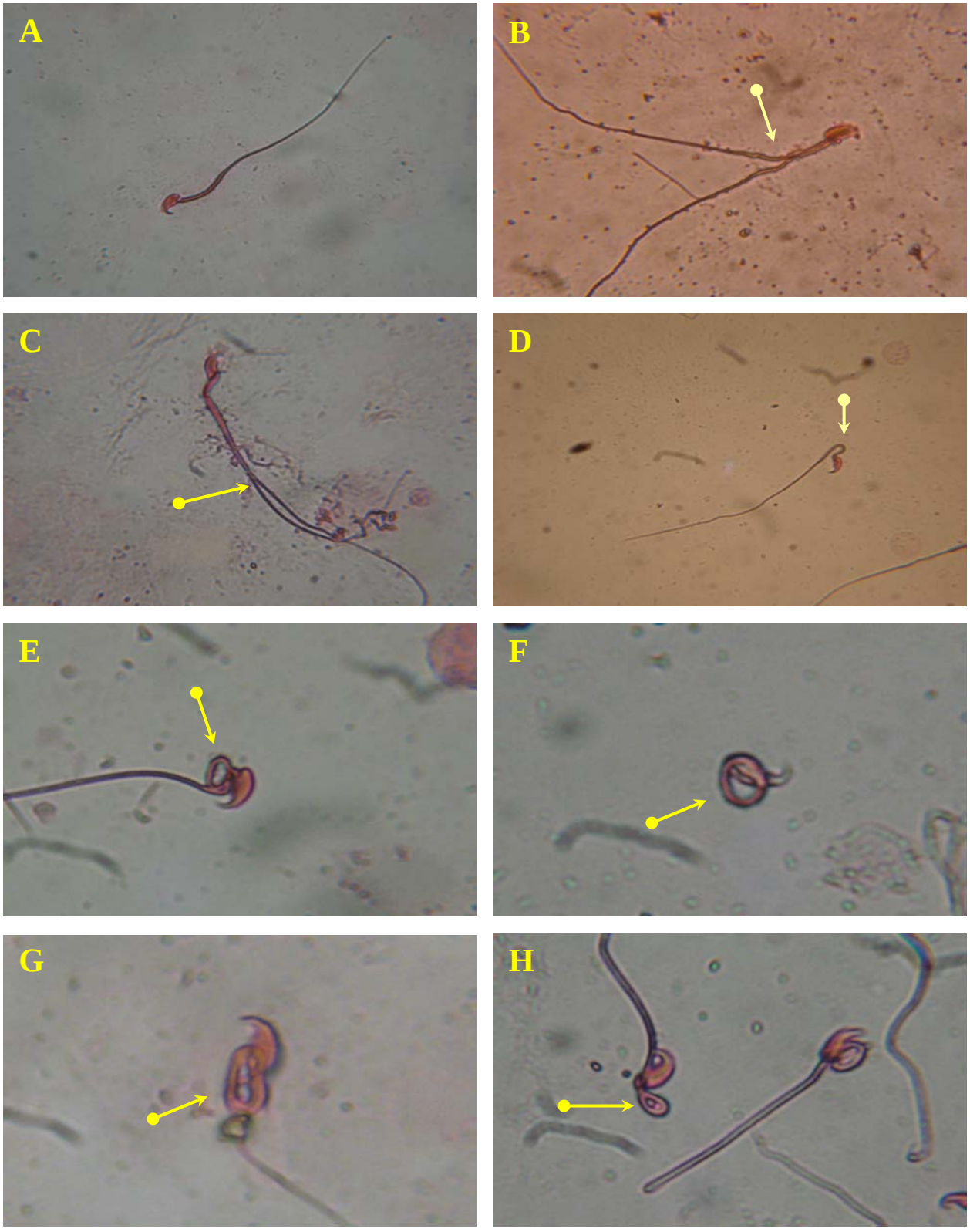

Fig. 1. Cisplatin induced sperm shape abnormalities in mice. (A) normal sperm with characteristic hook, (B) two-tailed sperms,(C) a schizoid tail sperm, (D) folded-tailed sperm, (E) & (H) highly coiled- tailed sperm, (F) a ring-shaped sperm, & (G) infinity -shaped sperm . 1% eosin Y; 400×

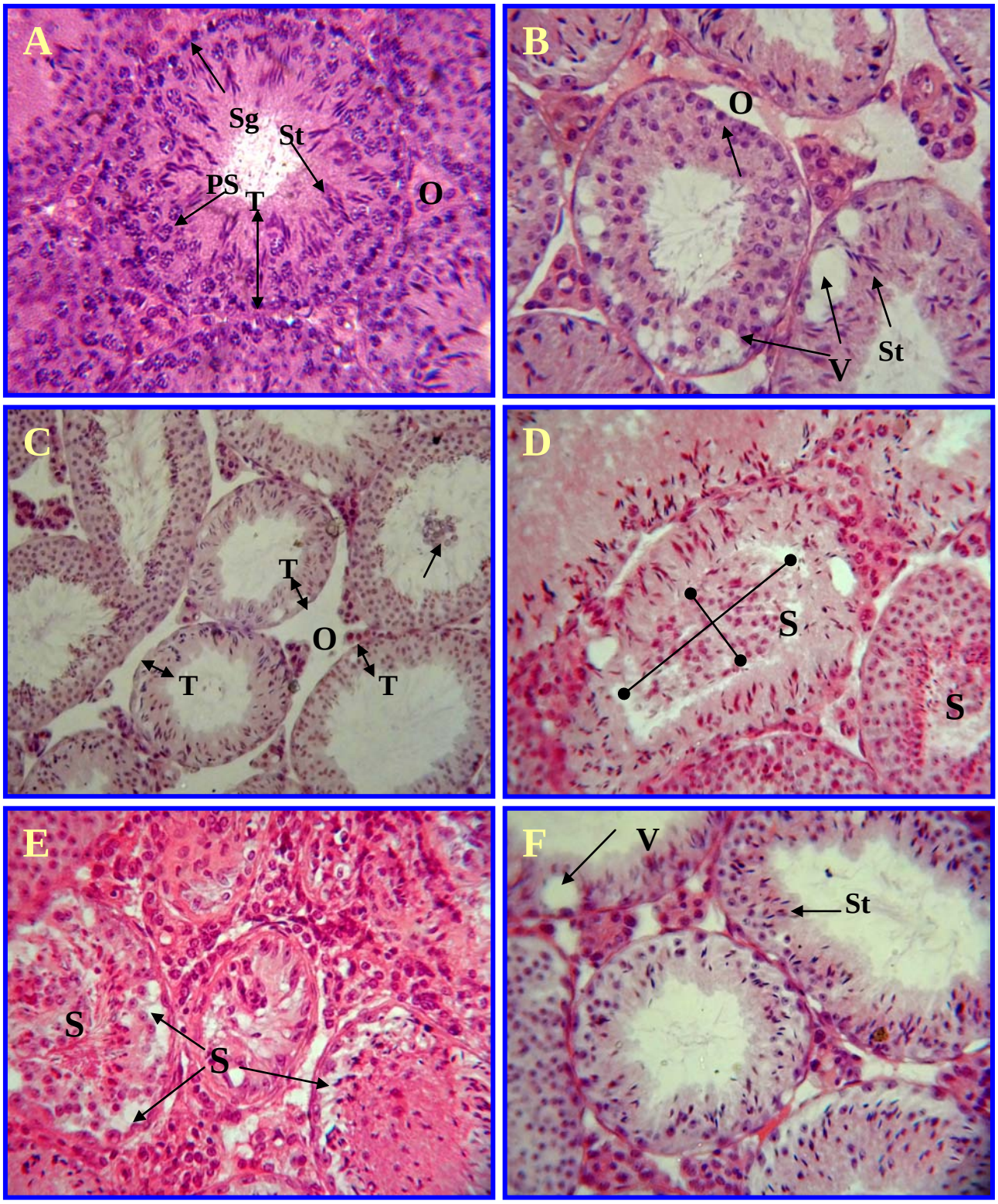

Fig. 2. Testicular structure in control and Cisplatin treated mice. A) A photomicrograph of testicular section from a control mouse showing normal germinal mass thickness (T), and interstitial tissue (O), Spermatogonia (Sg), Primary spermatocyte (Ps), Elongated spermatide(St), original magnification, B) a photomicrograph of testicular section from a Cisplatin treated mouse(2mg/kg on day 16) showing decrease in number of Spermatogonia , Primary spermatocyte and Elongated spermatide , and vacuolization (arrowhead V) with destruction in the interstitial tissue (O) were observed. (C) Photomicrograph of a testicular section showing sloughed germ cells aggregated in the lumen of a tubule (S) and measurements of seminiferous tubule diameters and germinal mass thickness (T) is reduced . D) A photomicrograph of a testicular section from a Cisplatin treated (2mg/kg) mouse showing epithelial sloughing (S), original magnification, and E) a photomicrograph of testicular section from a Cisplatin (2mg/kg) treated mouse showing epithelial sloughing (S) and distorted seminiferous epithelium, and F) a photomicrograph of testicular section from a Cisplatin (1mg/kg on day 16) treated mouse showing normal of number elongated spermatids. 200×, H&E.

4. Discussion

Mouse model provides an attractive alternate for humans to carry out the physiological study of cancer drugs involved in male mice reproductive functions. The decrease in sperm count is an important indicator of male infertility [19]. In the present study a reduction of sperm count spermatogenesis were observed in two doses at day 16th indicating the cytotoxicity of cisplatin on all types of germ cells spermatogenesis. It is well known that, cytotoxic drugs depress spermatogenesis in mammals [20], by causing the death of the developing germ cells in the seminiferous tubules. This results in the elimination of active cells of spermatogenesis and thereby results in the reduction in daily sperm production [21].

Spermatogenesis is a highly regulated differentiating system, both temporally and spatially. Germ cells, in particular, differentiating spermatozoa are extremely susceptible to cytotoxic agents because of their rapid proliferation. The non-proliferate leydig cells and sertoli cells survive most cytotoxic therapies but could suffer functional damages [22] . The decreasing in testosterone levels found in present study might be responsible for the reduction in decreasing sperm count and spermatogenesis in mice injected with cisplatin drug to decrease the sperm count. The reduction in testosterone level depletes the germ cells at specific stages of the seminiferous epithelial cycle – in particular – pachytene spermatocytes and spermatids in stages VII and VIII tubules, and the conversion of spermatids from step 7 to 8 is considered to be intimately related to testosterone concentration [23].

Within the testis, the main target cells for toxicants that disrupt spermatogenesis are the somatic cells, (leydig and sertoli cells) and the germ cells. In animal models, each of these cell types can be selectively targeted by specific toxicants, resulting in apoptosis. Detailed investigations in animal models on the testis indicate that platinum compounds have broad activity, targeting leydig cells, sertoli cells and germ cells [3]. Germinal epithelial damage leading to azoospermia has long been recognized as a consequence of treatment with chemotherapeutic agents, and there is also an evidence of leydig cell impairment following treatment. Chemotherapy may have a direct toxic effect on the leydig cells. Histological examinations in testes further indicate significant damage to sertoli cells, leydig cells and germ cell populations induced by cisplatin [24]. Cisplatin based chemotherapy (Cisplatin, vinblastine, and bleomycin) led to persistent impairment of fertility and leydig cell function in the majority of patients with testicular cancer accompanied with significant reduction in sperm production [25]. Cai *et al.* [26] demonstrated an involvement of Cisplatin, that induced germ cell apoptosis, in the occurrence of azoospermia . Several studies also showed that antitumor drugs such as cisplatin, etoposide, and Cyclophosphamide induce apoptosis in male germ calls [27, 28, 26]. Cisplatin treatment induced a significant increase of germ cell in apoptosis in mouse testes [29], which might contribute to the decrease in sperm count, spermatogonia, primary spermatocyte and spermatide.

Cisplatin induced the histopathological changes in the testis by inducing the vacuoles in the seminiferous epithelium. The presence of vacuoles and sloughing of the seminiferous epithelium is an indicator of Sertoli cell damage owing to microtubule disruption [30, 31]. The reduction in germinal epithelium thickness and seminiferous tubular diameter might be due to the inhibition of spermatogenesis, which must also be a contributor to decrease the sperm count.

In the present study, it was observed that the exposure to cisplatin caused a significant increase in sperm abnormalities at high dose only. Anticancer drugs induced the formation of abnormal sperms indicating its genotoxicity to germ cells [14 , 20 , 32]. Mainly, the sperm abnormalities

were seen on day 16th indicating that cisplatin affected the morphogenesis of spermatids and spermatocytes, respectively during treatment [20]. During meiotic phase of spermatogenesis, the spermatocytes are in the G2 phase of the cell cycle [33], and cisplatin is known to arrest the cell cycle at G_2 and M_2 phases [34] , therefore this mechanism may be involved in cisplatin induced abnormal sperm morphology. It is known that a variety of chemicals induces sperm abnormalities [35, 20], most likely due to point mutations that occurs in the germ cells [35, 20 , 32]. The increase in sperm abnormalities in this case indicates that cisplatin induced the DNA damage in germ cells leading to altered sperm morphology [36] .

Our results showed that FSH was not significantly altered in cisplatin treated mice in both doses, whereas, a significant decrease in serum testosterone and LH were observed in the two doses. Alternation in steroid metabolism can be attributed in part to the suppression of the serum testosterone levels that are associated with the administration of cisplatin [37]. It has been suggested cisplatin – mediated reduction in serum testosterone levels might be caused by reducing responsiveness to gonadotropin of the testicular leydig cells and accompanying depression of steroid side-chain cleavage activity [38] . In addition, the results indicate that hormonal perturbation caused by cisplatin are mediated by its effects on the hypothalamic-pituitary-gonadal axis [39] . In another study, it was shown that the low level of testosterone associated with treatment cisplatin is dependent upon depression of numbers of LH receptors and level cytochrome P-450 in rat testicles [38]. Aydiner *et al.* [37] reported a similar decline in microsomal concentration of P-450 in rat testicles. With this decline , plasma testosterone levels were also found to be low.

The utrastructural changes in leydig cells , such as increasing number of lipid inclusion in the leydig cells, disorganizing mitochondrial cristae and losing of the matrix, suggest that the hormonal changes might be partly dependent on toxicity of cisplatin to these cells [37]. The changes induced by cisplatin reflect the decline in the rates of biosynthesis and the secretion of testosterone. Moreover, Malarvizhi and Mathur [40] reported that the activity of 3β-hydroxy steroid dehydrogenase and 17β-hydroxy steroid dehydrogenase were decreased significantly in the leydig cells cultured with different concentrations of cisplatin in mice . These two enzymes have very important role in the synthesis of testosterone [41].

In conclusion, it was indicated that male reproductive toxicity induced by cisplatin would be augmented by decreased serum LH and testosterone levels as well as an inducing of the damage in sertoli cells, in addition to the direct Cytotoxic effect on germ cells. It is suggested that these endocrinological changes related to male reproductive toxicities can be detected in the 16th -day-treated study.

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تأثير دواء Cisplatin في خصائص النطف وعملية نشأة النطف ومستوى الهرمونات الجنسية في ذكور الفئران *Mus musculus* L.

فارس شاكر كاطع

قسم علوم الحياة/ كلية التربية للعلوم الصرفة/ جامعة البصرة / العراق

الملخص:

تهدف الدراسة الحالية للبحث في تأثير دواء الـ cisplatin المضاد للسرطان في أعداد النطف السوية وغير السوية وعملية نشأة النطف وقياس مستوى الهرمونات الجنسية. قسمت الفئران المختبرية المستخدمة في هذه الدراسة الى ثلاث مجاميع تتألف كل مجموعة من ثماني فئران , حقنت المجموعة الأولى (مجموعة السيطرة) بمحلول الفسيولوجي 0.9% و المجموعة الثانية حقنت بـ 1 ملغم /كغم من دواء cisplatin , أما المجموعة الثالثة فحقنت بـ 2 ملغم/كغم من دواء cisplatin. حقنت الفئران المختبرية في هذه الدراسة بثماني حقن من الدواء في منطقة الخلب I.P ولفترة 16 يوماً وبواقع حقنة واحدة كل 48 ساعة ثم شرحت الفئران المختبرية بعد اليوم 16 . أظهرت الدراسة الحالية وجود أنخفاض معنوي في أعداد النطف وبالجرعتين1 و2 ملغم/كغم بمستوى احتمالية ($p<0.01$) , كما سبب الدواء زيادة معنوية في أعداد النطف مشوه الذيل وبالجرعة العالية فقط 2 ملغم/كغم. أما بالنسبة الى عملية نشأة النطف فقد سبب الدواء cisplatin انخفاضاً معنوياً في أعداد خلايا سليفات النطف والخلايا النطفية الأولية مع اختزال في سمك الطلائية الجرثومية وقطر النبيبات المنوية وبكلى الجرعتين , فضلاً عن حدوث أنخفاض معنوي في أعداد خلايا طلائع النطف المتطاولة وبالجرعة العالية فقط. وسبب الدواء أيضاً حدوث انسلاخ في الطلائية الجرثومية وظهور التجاويف في النبيبات المنوية للخصى المعاملة بالدواء. وأظهرت نتائج حقن دواء cisplatin في الفئران المختبرية وجود انخفاض معنوي في مستوى هرمون اللوتيني LH وهرمون التستوستيرون وبالجرعتين , بينما لم تظهر النتائج وجود اختلاف معنوي في هرمون المحفز للجريبات FSH في مصل الفئران المعاملة بالدواء وبالجرعتين.

الكلمات المفتاحية. Cisplatin , أعداد النطف , النطف غير السوية , عملية نشأة النطف , FSH , LH , التستوستيرون .