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The Impact of Gemcitabine-induced Reproductive Toxicity on The Sperm count and Morphology of Albino Rats

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Abstract

Cancer treatments can affect sperm production and a significant percentage of cancer patients may develop permanent azoospermia or severe oligozoospermia after chemotherapy. To investigate the influence of Gemcitabine toxicity on the reproductive system of albino male rats (sperm count and morphology). An experimental animal study conducted in the zoology department, College of Science, King Saud University during the period from June to October 2014 using albino rats (Rattusnorvegicus) (Wistar strain). Males were divided into four different groups (control" 0 mg/kg",7 mg/kg,14 mg/kg, and 21 mg/kg). The reproductive organs, testicles and epididymis decreased in weight and atrophied in most of the animals treated with the drug in various doses. The mean absolute and relative epididymal weights were also significantly decreased. In the drug-effects recovery group, neither the testicles nor the epididymis in the animals treated with the three doses recovered fully normal weight. The testis's efficiency in producing sperm was significantly decreased at all doses. In the recovery group, the testis regained its efficiency, as no significant difference was recorded between the drug-treated groups and the control group. The drug caused complete loss of sperm, in a rat treated with the big dose. Gemcitabine caused a significant increase in the percentage of deformed sperms in all treated animals. Gemcitabine drug has high toxicity on the reproductive system of rats with a dose tenth of human dose, with a massive decrease in the count and quantity of sperm, which means that this drug can have more toxicity effects on human.

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INTRODUCTION

Cancer treatment focuses on curing the disease itself, whereas people insist on treatment safety and efficiency. The American Cancer Society estimated that 10,380 new cancer cases and 1,250 deaths from cancer occurred in 2016 among males and females aged 0 to 14 years (Okada and Fujisawa, 2019). Infertility is a critical long-term adverse effect in males even with latest progresses in treatments for malignancies that may cure young cancer patients (Chan, 2013; Siegel *et al.*, 2016). Progressively many young adults are long-term survivors of cancer. Patients younger than 15years of age undergoing cancer treatment have a 5-year cancer survival rate of 75% (Trost and Brannigan, 2012). More than 50% of these young male survivors will desire fatherhood after treatment (Hryciuk *et al.*, 2017).

Unfortunately, the toxic effect of cancer treatments can damagingly affect sperm production and a significant percentage of cancer patients up to 24%

may develop permanent azoospermia or severe oligozoospermia. after chemotherapy or radiotherapy. However, due to the fact that many post-treatment cancer patients have a complete return of sperm production, an important question was presented whether post-therapy spermatozoa are a proper choice for conception, either naturally or through assisted reproductive technologies.

Gemcitabine (GCB) is an anticancer agent used exceedingly alone or in combination with other anticancer agents in the treatment of various malignancies (Okada and Fujisawa, 2019). Usually, GCB toxicity is mild, transitory, and infrequently dose-limiting. The common side effects were laboratory changes (myelosuppression, mild proteinuria trans¬aminase elevation, and hematuria) where symptomatic toxicities are generally well-controlled and not life-threatening (Chan, 2013; Siegel *et al.*, 2016; Pectasides *et al.*, 2009). The main factors raising GCB toxicity were; liver and kidney diseases, combination with platinum derivatives or taxanes, and alcohol abuse (Trost and Brannigan, 2012; Hryciuk *et al.*, 2017). There is no clear evidence about dose adjustment of GCB, and clinical decisions are mainly made based on experimental bases. The aim of the present study was to investigate the influence of Gemcitabine toxicity on the reproductive system of albino male rats (sperm count and morphology).

MATERIALS AND METHODS

This experimental animal study was conducted at the Zoology Department, College of Science, King Saud University.

Animals

Sexually mature male and female albino rats (Rattusnorvegicus) of the Wistar strain, with age, ranged between 8-10 weeks and weights of 220-250 gm, were obtained from Animal House, College of Pharmacy, King Saud University which fulfilled the following criteria:

Drugs

Gemcitabine is available from the manufacturer in packages containing either 200 mg or 1 gram of salicetabine hydrochloride prepared with mannitol (200 mg and 1 g respectively) and sodium acetate (12.5 mg and 62.5 mg respectively (Casciato, 2004; AdisInsight, 2006).

It was dissolved in a 0.9% solution of sodium chloride and remained stable for 24 hours at room temperature. The drug should not be cooled in the refrigerator after it has been thawed, as this may lead to crystallization (AdisInsight, 2006; Mini *et al.*, 2006).

Experimental Design

Animals were dealt with and the various experiments and tests were designed in general according to the guidelines and standards used in estimating the toxicity of chemical compounds on the reproductive system (Clegg *et al.*, 2008). Males were divided into four different groups (control" 0 mg/kg",7 mg/kg,14 mg/kg, and 21 mg/kg). Each group of the four groups included 20 rats, which were divided into two subgroups, each of which included ten rats so that the different tests were applied to the first subgroup immediately after the end of the treatment period (9 weeks) in order to find out the harm caused by the drug, while different tests were applied to the group. The second subset after one of the spermatogenesis cycles has passed, with the aim of knowing whether or not the reproductive system functions recover and return to a normal state.

The animals injected each group into the peritoneal cavity "IP" once a week for a period of 9 weeks. The control group was injected with a physiological solution, while the three treated groups were injected with a physiological solution in which the drug was dissolved according to the specified dose.

During the injection period, the animals were monitored daily in order to follow up the appearance of disease signs resulting from drug toxicity or mortality, and their weights were recorded at the beginning of the experiment and then weekly. Immediately after the end of the injection period (10 males from each group) or after a complete sperm cycle equivalent to nine weeks (10 males - recovery group), then the males were mated with healthy females at a rate of (1: 2).

The males were sedated after the end of the treatment period and mating with ether, then killed, and blood was collected from the arterial stem in the neck and left to coagulate at room temperature for an hour, then the serum was collected after centrifugation at 3000 rpm for 20 minutes and kept at -80 ° C. To measure testosterone concentration at a later date (Foster and Harris, 2005). After killing males and collecting blood, the abdomen was incised and the internal organs were fully exposed and examined, and any anatomical or pathological changes were recorded. The organs of the reproductive system consisting of the testes, epididymis, prostate gland, seminal vesicles, and associated clotting glands were removed, and the adipose tissue attached to them was trimmed and weighed all. The weight of the different organs was expressed as absolute weight and as relative weight to body

weight, which was calculated according to the formula (member weight/bodyweight x 100) (Andrade *et al.*, 2002; Yu *et al.*, 2009). The right testicle and right epididymis were frozen at -80 ° C in order to estimate the daily production of sperm and the epididymal stock of sperm at a later time. The left testis and left epididymis were used to assess the toxic effect of the drug on histopathology.

The percentage of sperm abnormalities was estimated by examining 200 sperms for each experimental animal by light microscopy at a magnification of 400 ×. Sperm was classified into healthy or malformed, and head deformities included: straight head (hookless), banana-like head, amorphous head, pinhead, detached head, bent neck, double head, and head Macrocephaly Big, Microcephaly Small Head. Tail abnormalities included: Bent tail, Coiled tail, and double tail (Chandra *et al.*, 2007).

Daily sperm production (DSP) is the number of sperm produced by the testicle or testicles per day in an organism, and the efficiency of sperm production is expressed as the number of sperm produced by one gram (or a certain unit of volume) of Testicular parenchyma (Amann, 1981). During later stages of spermatogenesis, nucleoproteins in spermatocytes become highly condensed and highly resistant to chemical or mechanical cracking, so tissue milling destroys all cells and nuclei in the testicle except for the nuclei of the spermatids that are resistant to grinding or sperm when epididymal tissue is ground (Amann, 1986).

Elongated spermatids and grinding-resistant sperms were counted using a Hemocytometer. The count was done twice and on average. The daily production of DSP in the whole testis was calculated by dividing the total number of sperm cells resistant to grinding in the testis by 6.1, which is the number of days it takes for spermatids resistant to grinding during the process of spermatogenesis. The efficacy of DSP sperm production was estimated by dividing the entire daily testicular sperm production by the weight of tissue remaining after removal of the capsule in grams. Estimate the number of sperms in the epididymis in a manner similar to that used to estimate daily production.

Determine the testosterone hormone in the serum using the Enzyme Immunoassay (Pro-View Testosterone ELISA) kits from Alfa Scientific Designs In. Poway CA 926064, USA, which depends on the principle of competitive association between the testosterone hormone present in the sample and the compound Testosterone-HRP associated with a specific amount of rabbit-derived testosterone antitestosterone. The method was followed according to the manufacturer's instructions that came with the kit, and the Anthos 2020 colour absorption reader from (Anthos Labtec Instruments, Austria) was used to read the samples and estimate the hormone concentration in 8 individuals from each group.

Statistical Analysis

The obtained data were represented as mean ± standard error of mean ± SE and a significant level of P 0.05 and P 0.01 was adopted. For the statistical analysis of the data, both SPSS version 16 and SigmaStat version 3.5 were used. The data were analyzed using One Way ANOVA with the aim of determining the existence of differences between groups, and in case of differences between groups, they were dimensionally compared using Dunnett's test for multiple comparisons or Fisher LSD test in the case of data homogeneity. In the case of inconsistency in the data, the Kruskal-Wallis One Way ANOVA test was used to analyze the differences between groups, and then Dunnett's test was used for the dimensional comparisons (Chen, 2006).

RESULTS

Observations and Mortality

There was no death in the control group animals or the treatment with a dose of 7 mg/kg, while one of the subjects of the larger dose (21 mg/kg) was found dead after the fifth dose, and after the sixth dose, one of the subjects of the average dose (14 mg/kg) died. Immediately after the end of the treatment period (after the tenth dose), one of the members of the average dose was found in a dying state and therefore he was directly autopsy without undergoing a mating test and was examined internally and the presence of ulcers was observed A pale white spread in the lungs, and his testicles were dark in colour, indicating that there was bleeding inside them. A few other animals with the medium and high doses also suffered from general wasting, weight loss, and hair erection and thinning, especially in the facial area.

During the autopsy, no signs of disease were observed on the internal organs, except for the presence of some light bleeding in the lungs in a number of animals in the treated and control groups alike. As for the reproductive organs, testicles and epididymis decreased in weight and atrophied in most of the animals treated with the drug in various doses.

The effect on body weights

Final body weight decreased in the animals treated with Gemcitabine at different doses used. Also, slow

Table 1: Effect on the mean final of gemcitabine and gained body weights, and averages of relative and absolute male reproductive system weights (Mean ± SE)

Parameters	Dose			
	Control	7 mg	14 mg	21 mg
Starting body weights	264.00 ± 3.39	256.50 ± 6.33	254.09 ± 5.79	253.11 ± 5.19
Final body weights	413.60 ± 8.70	395.90 ± 14.25	376.45 ± 14.2*	383.00 ± 13.29
Weight gain	149.60 ± 8.69	139.40 ± 12.66	122.36 ± 12.10	129.89 ± 12.79
Absolute organ weights				
Testes	3.50 ± 0.08	2.01 ± 0.16**	1.82 ± 0.09**	1.60 ± 0.09**
Epididymides	1.14 ± 0.03	0.82 ± 0.03**	0.77 ± 0.02**	0.73 ± 0.08**
Seminal vesicles	1.77 ± 0.13	1.93 ± 0.16	1.63 ± 0.14	1.74 ± 0.13
Prostate	0.64 ± 0.02	0.61 ± 0.04	0.60 ± 0.04	0.52 ± 0.05
Relative organ weights				
Testes	0.85 ± 0.02	0.51 ± 0.04**	0.50 ± 0.04**	0.42 ± 0.03**
Epididymides	0.28 ± 0.01	0.21 ± 0.01**	0.21 ± 0.01**	0.19 ± 0.01**
Seminal vesicles	0.43 ± 0.03	0.49 ± 0.03	0.43 ± 0.03	0.46 ± 0.04
Prostate	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.14 ± 0.1

*Significantly different from control($p \leq 0.05$); ** Significantly different from control($p \leq 0.01$).

Table 2: Daily Sperm Production, Sperm Count, Sperm Motility, and sperm abnormality

Parameters	Dose			
	Control	7 mg	14 mg	21 mg
Daily sperm production (10^6)				
Per Testis	36.5±1.6	9.6±3.7**	5.7±1.8**	4.6±1.5**
Per gram testis	24.2±0.91	10.5±2.9**	8.4±2.4**	8.4±2.7**
Sperm count(10^6)				
Per cauda	162.3±23.4	25.5±9.6**	16.5±6.9**	10.4±4.0**
Per gram cauda	719.2±80.5	147.6±54.9**	99.8±36.0**	68.2±24.7**
Sperm abnormalities				
% abnormal sperms	5.5±0.71	41.47±7.4**	53.77±6.08**	58.21±9.77**
Sperm motility				
% motile sperms	61.21±3.74	16.27±4.73**	10.25±3.48**	7.41±2.5**

Significantly different from control ($p \leq 0.01$).

Table 3: Daily Sperm Production, Sperm Count, Sperm Motility, and sperm abnormality among recovery group after stopping the drug for a complete cycle(9 weeks)

Parameters	Dose			
	Control	7 mg	14 mg	21 mg
Daily sperm production(10^6)				
Per Testis	37.81±1.12	31.52±5.21	27.07±4.09*	29.23 ±2.17
Per gram testis	23.29±0.79	21.95±2.7	23±3.24	24.01±1.09
Sperm count (10^6)				
Per cauda	146.6± 14.4	85.2±19.9*	62.9±16.7**	77.1±11.3**
Per gram cauda	664.1±51.2	424.7 ± 84.5*	374.7 ± 96.4*	471.4 ± 65.3
Sperm abnormalities				
% abnormal sperms	7.95±1.79	19.02±3.86	23.24± 11.01	22.1±5.3
Sperm motility				
% motile sperms	53.8±2.54	31.86±4.69**	36.43±7.48*	31.96 ±2.76**

* Significantly different from control ($p \leq 0.05$); ** Significantly different from control ($p \leq 0.01$).

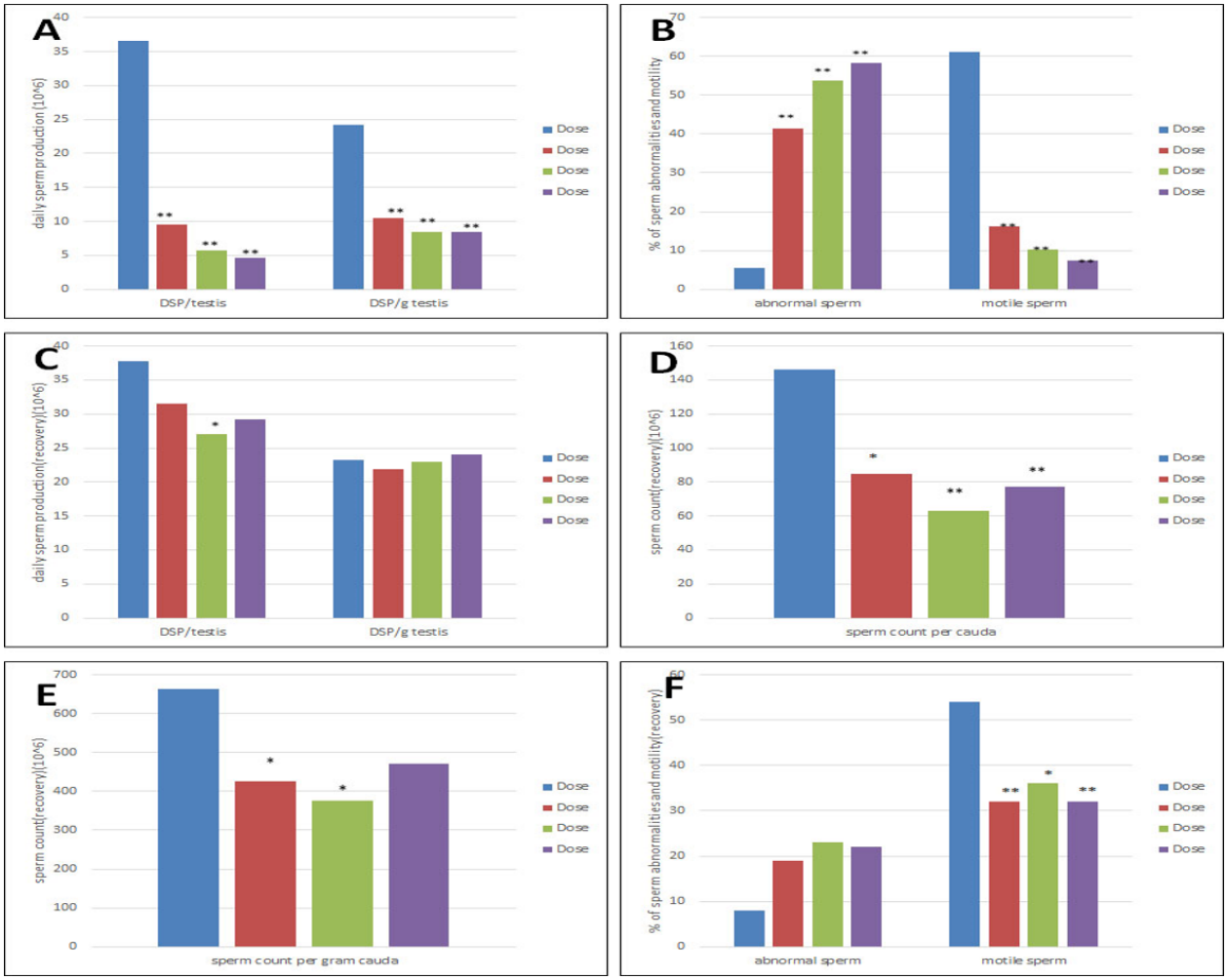

Figure 1: The effect of gemcitabine on sperm

Table 4: Testosterone levels

Dose			
Control	7 mg	14 mg	21 mg
After treatment termination			
2.43±0.38	1.97±0.29	2.38±0.55	2.43±0.44
Sperm count(10^6)			
2.75±0.51	3.07±0.46	2.53±0.55	2.26±0.53

weight gain was observed in the treated groups compared to the control group, but the reduction in final weight or weight gain was not related to the dose administered. At the end of the experiment, the mean weights of the 7, 14 and 21 mg/kg dosed group members, relative to that of the control group, were about 96%, 91% and 93%, respectively. The mean weights gained relative to that of the control group were about 93%, 82% and 87%, respectively. The statistical analysis did not show that this decrease in final or gained weight was significant except for the final weight at the dose of 14 mg/kg ($p \leq 0.05$) (Table 1).

The effect on organ weight

The mean absolute and relative epididymal weights were also significantly decreased ($p \leq 0.01$). The mean absolute weights at the 7, 14 and 21 mg/kg doses, relative to that of the control group, were about 72%, 68% and 64%, respectively. While the mean relative weights, relative to that of the control group, were about 75%, 74% and 69%, respectively. As for the drug effect recovery group, the mean final and gained weights for the members of the lower and intermediate doses were equal to that of the control group, while the mean final and gained weight in the group treated with the maximum dose

Figure 2: Normal sperms of the control group

was less than their two counterparts in the control group. Also, in the drug-effects recovery group, neither the testicles nor the epididymis in the animals treated with the three doses recovered fully normal weight.

The effect on daily sperm production

Table 2 and Figure 1 show a significant decrease in daily sperm production in rats treated with Gemcitabine. It is evident from the table data that this decrease was significant ($p \leq 0.01$) at all three doses of the drug. The testis's efficiency in producing sperm was significantly decreased (p 0.01) at all doses. Average daily sperm production in rats treated at doses of 7, 14 and 21 mg/kg, relative to that of the control group, was about 26%, 16% and 13%, respectively, while the average testicular efficiency in producing sperms was about their counterparts in the control group, approximately 43%, 34% and 34%, respectively. In the recovery group, testes in the drug-treated rats regained their ability to produce sperm at rather high rates, although not in the control group, and production levels remained significantly low (p 0.05) in the 14 mg/kg dose-treated group. The average testicular production in the three-dose drug-treated rats, relative to that of the control group, was about 83%, 72% and 77%, respectively. As for the efficiency of sperm production, the testis regained its efficiency, as no significant difference was recorded between the drug-treated groups and the control group (Table 3).

The effect on sperm count

There is a significant (p=0.01) reduction in the number of sperms in the tail of the epididymis of rats treated with different doses of Gymsitabine even after it was expressed in relation to the tail weight of the epididymis. The averages of this number in the three doses, relative to that of the control group, were about 16%, 10% and 6%, respectively, while the mean number of sperms per gram of epididymal tail in the three doses, relative to that of the control group, was about 20 %, 13% and 9%, respectively. The drug also caused complete loss of sperm, Azoospermia, in one of the group members treated with the big dose (Table 2). In the drug-effect recovery group, the decrease in the number of spermatozoa in the tail of the epididymis continued, which was significant at (p 0.05) with respect to the dose of 7 mg/kg and at (p 14 0.01) for the 14 and 21 mg/kg doses, although this was not The decrease sharply is that which immediately followed the completion of the transaction. The average number of sperms in the tail of the epididymis in the three doses, relative to that in the control group, was about 58%, 43% and 53%, respectively, while the average number of sperms per gram of epididymis tail, relative to that of the control group, was about 64 %, 56% and 71%, respectively.

The effect on sperm motility and abnormalities

The effect of treatment with the drug gemcitabine on sperm motility in rats was studied. This table shows the significant and significant decrease (p) 0.01) in the percentage of motile sperms at all doses used. Sperm motility averages at doses of 7, 14 and 21 mg/kg, relative to that of the control group, were approximately 26%, 18% and 12%, respectively (Table 2).

In the recovery group, sperm motility improved significantly, although it remained low than the control group, with a significant decrease at (p 0.01) for doses 7 and 21 mg/kg and at (p 0.05) for the dose of 14 mg/kg. Their counterparts are in the control group, approximately 59%, 68% and 59%, respectively.

The treatment with the drug Gemcitabine caused a significant increase in the percentage of deformed sperms in all the treated animals ($p \leq 0.01$). The mean percentages of sperm abnormalities in the control group were 5.5%, while at doses 7, 14 and 21 mg/kg it reached 41.47%, 53.77% and 58.21%, respectively (Figures 2 and 3).

In the recovery group, the sperms recovered relatively, as they recorded fewer abnormalities than before, although they remained higher than their counterparts in the control group, even if they were not significant. The mean percentages of sperm abnormalities in the control group were 7.95%, while at doses 7, 14 and 21 mg/kg it was 19.02%, 23.24% and 22.1%, respectively.

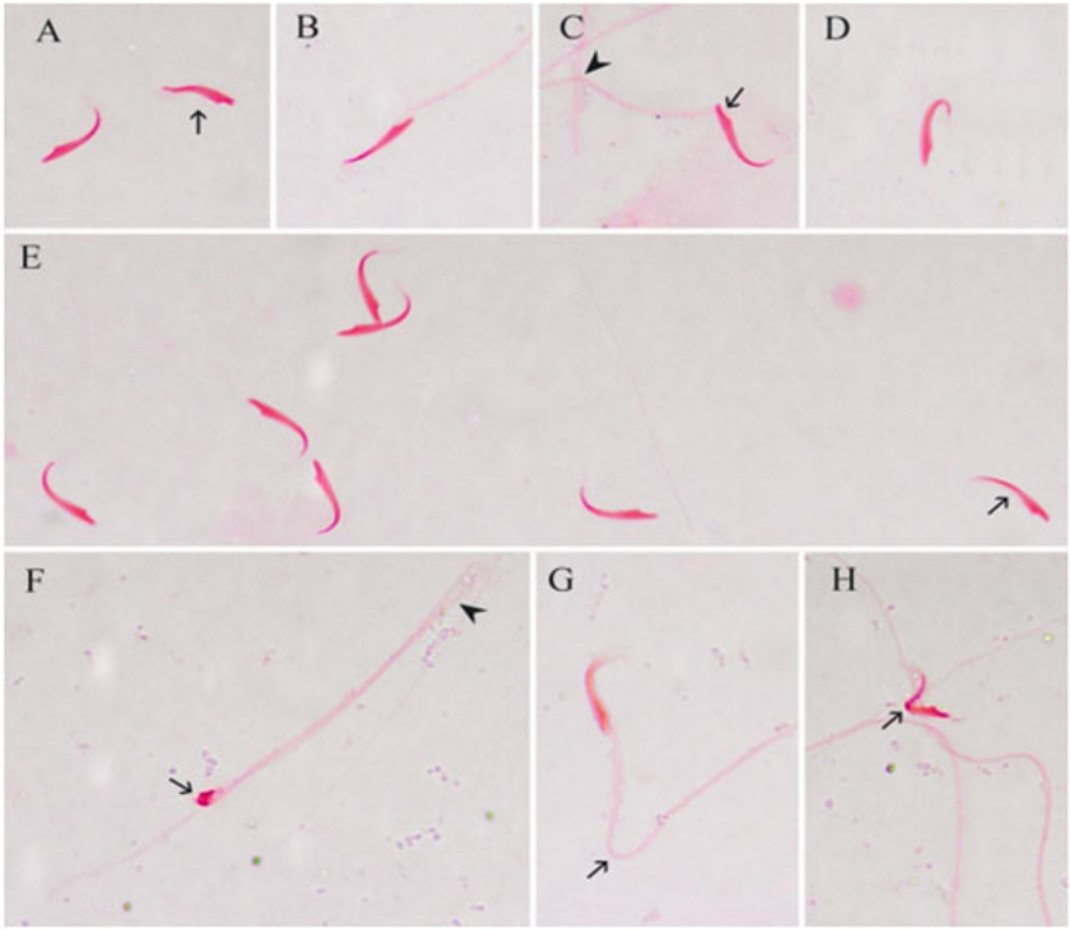

Figure 3: Abnormal sperms of the group treated with gemcitabine

Effect of treatment with gemcitabine on testosterone level

The drug administration did not lead to any significant differences in testosterone concentration levels in rat blood serum. The different concentrations of this hormone in the treatment groups immediately after the end of the injection period and the groups recovering from the effects of the drug (Table 4).

Figure 1 shows the effect of gemcitabine on sperm production (A), motility and abnormalities (B), sperm production (C), tail epididymis sperm count (D), sperm count in relation to epididymal weight (E), sperm abnormalities (F) during the recovery.

Figure 2 shows a group of normal sperms of the control group (x1000).

Figure 3 shows separated sperm heads (A), the straight head is attached to the tail (B), folded neck (arrow) (C), separate head with an unnatural hook (D), many separate heads (E), pinhead (arrow), twisted tail (arrowhead) (F), bent tail (G), deformed head (H) (x1000).

DISCUSSION

Previously, cancer patients and their physicians were most concerned about cure rates and relapse rates after a reduction in disease severity. Today, with the development of effective treatments against many types of cancer, survival rates have risen and improved steadily and steadily, shifting attention from cancer treatment itself to quality of life after recovery. Perhaps one of the most prominent things that may make a person's life disturbed is his loss of fertility as a result of the toxic effects of cancer treatment (Sabanegh and Ragheb, 2009).

The current study aimed to investigate the toxic side effects of one of the most prominent relatively new anti-cancer drugs on the reproductive system of male rats, as an animal model through which similar effects can be predicted on humans in the absence of any information or studies dealing with that. It is known that the process of spermatogenesis is similar to a large extent in many of its characteristics between humans and experimental animals, especially mice and rats (Meistrich, 2013).

The results of the present study showed that the

drug gemcitabine caused a significant and severe decrease (p 0.01) in the daily production of sperms in the testes of rats treated with different doses of it. This indicates its high toxicity towards germ cells in the testis.

In the current study, the histological examination of the testicular segments did not show any evidence of obstruction in the outgoing ducts or the occurrence of cell ingestion of sperm in them, while the occurrence of retention of spermatids in the lower dose animals, which is believed to have partially contributed to a decrease in the number of sperms in the epididymis. The major contribution to this decline came from the decrease in the production of sperm, as indicated by the estimate of the daily production of sperm, which decreased to very low levels at all doses used.

In the current study, a significant decrease in the ratios of motile sperms was observed in the groups treated with gemcitabine when compared to the control group. These percentages were 16.27% in the low dose, 10.25% in the average dose and 7.35% in the larger dose compared to 61%. , 21% in the control group.

The results of the present study showed a significant increase in the rates of sperm abnormalities as a result of the treatment with the drug Gemcitabine, with an average of 41.47%, 52% and 45.82% at doses 7, 14 and 21 mg/kg, respectively, compared to 5.5% in the control group. This is consistent with the toxic effects recorded for other types of anti-cancer drugs, as in addition to reducing the number of sperms, these drugs can cause permanent changes in the quality of the sperm produced. Abnormalities in the sperm heads and separation of the head from the tail have been recorded in the sperm, taken from the epididymis in mice after a long time treatment with adriamycin (Meistrich, 1986).

It was observed in this study that the number of head-separated sperms increased dramatically, as this type constituted the largest proportion of the abnormalities that were recorded in animals treated with gemcitabine. This type of deformation has been associated with several chemicals that cause sperm abnormalities, and it is believed that it occurs due to damage caused by these substances to the medium piece (Takahashi *et al.*, 2006). Some researchers also believe that it is caused by the degeneration of sperm stored in the epididymis as a result of being deprived of testosterone (Ramesh *et al.*, 2008). As for the current study, there was no change in the level of testosterone in the blood, but the histological composition of the epididymis was significantly affected, which may be the reason for the degradation of the sperm stored in it in a manner similar to that caused by the low level of testosterone in the blood.

The results of the current study did not demonstrate any effect on blood serum testosterone levels. This result is consistent with no decrease in the average auxiliary gonadal weights, as their secretory functions are mainly dependent on androgens and are very sensitive to the concentration of testosterone in the blood (Creasy, 2001).

This is in agreement with what is known about chemotherapy, which usually causes a decrease in the number of sperms or sometimes even loss without affecting the production of testosterone by the Ledge cells in most cases (Brougham *et al.*, 2003).

CONCLUSION

The results of this study showed that Gemcitabine drug has high toxicity on the reproductive system of rats with a dose represent a tenth of human dose, where huge damage happened in the structure of testicle with a massive decrease in the count and quantity of sperm this means that this drug can have more toxicity effects on the reproductive system of human.

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Conflict of Interest

The author declares that he has no conflict of interest for this study.

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