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Effect of Cyclophosphamide Treatment During the Embryonic Period on Fertility of Adult Male Mice

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Abstract

The cyclophosphamide is commonly used for the treatment of cancer and immunosuppressive diseases in young and old age and can induce oxidative stress in reproductive organs, therefore has adverse effects on sperm quality and quantity. In this study, the effects of a single dose of cyclophosphamide on sperm parameters in adult male mice that treated with 10 mg/kg of this drug on the 11th embryonic day were investigated. Adult female pregnant NMRI mice were divided into 2 groups; the control group received saline and the cyclophosphamide group received cyclophosphamide at a dose of 10 mg / kg on day 11th of gestation (i.p). 60 days after the birth of the infants, male mice were sacrificed and the sperm collected from cauda of epididymis for sperm parameters study. Cyclophosphamide administration resulted in a decrease in body and testicular weight, viability, motility, and count of sperm ($p \leq 0.05$), and an increase in sperm head, neck and tail abnormality ($p \leq 0.05$). It was concluded that the cyclophosphamide, has long-term destructive effects on sperm parameters and these effects can be continuing after puberty.

Keywords: Mouse, cyclophosphamide, sperm parameters.

تأثير عقار السايكلوفوسفاميد خلال المراحل الجنينية على خصوبة ذكور الفئران البيض البالغين

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الخلاصة

يستخدم عقار السايكلوفوسفاميد لعلاج السرطان والأمراض المثبطة للمناعة ، ويمكن أن يحفز الإجهاد التأكسدي في الأعضاء التناسلية ، وبالتالي لديه آثار ضارة على نوعية وعدد الحيوانات المنوية. في هذه الدراسة ، تم دراسة آثار جرعة واحدة من السايكلوفوسفاميد على الحيوانات المنوية في ذكور الفئران البالغين المعاملة ب 10 ملغم / كغم من هذا الدواء في اليوم الحادي عشر الجنيني. تم تقسيم الفئران الإناث الحوامل NMRI إلى مجموعتين. جرعت مجموعة السيطرة بالملح الفسيولوجي وجرعت مجموعة السايكلوفوسفاميد بجرعة 10 مغ / كغ من السايكلوفوسفاميد تحت الصفاق في اليوم الحادي عشر من الحمل ، بعد 60 يومًا من ولادة الرضع ، شرحت ذكور الفئران وتم جمع الحيوانات المنوية من البربخ لدراسة تشكل واعداد الحيوانات المنوية. تسبب السايكلوفوسفاميد بانخفاض في وزن الجسم والخصى ، حيوية ، حركة ، وعدد الحيوانات المنوية ($p \leq 0.05$)، وزيادة في تشوها رأس,عنق وذيل الحيوانات المنوية ($p \leq 0.05$). يمكن الاستنتاج بأن

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السايكلوفوسفاميد ، له آثار مدمرة على المدى الطويل على معايير الحيوانات المنوية وهذه الآثار يمكن أن تستمر بعد سن البلوغ وتسبب العقم.

Introduction

Cyclophosphamide (CP) is an oxazaphosphorine agent recommended by the Food and Drug Administration (FDA) for treating a number of rheumatologic conditions, benign and malignant diseases [1]. As, cyclophosphamide, considered as an antimitotic agent, is known to affect the cells proliferation, also it may interfere with the migration as well as a proliferation of primordial germ cells (PGCs) from the yolk sac to the genital ridges [2]. Cyclophosphamide is metabolically activated in hepatic cells by cytochrome P450 enzymes to the reactive intermediates, phosphoramide mustard and acrolein [3, 4]. The active metabolites of this drug are alkylating agents which cross-link DNA, thus interfering with both DNA synthesis and RNA transcription [2-5]. The cytotoxic effect of cyclophosphamide targets rapidly dividing cells. The spermatogenic lineage cells are particularly susceptible to cyclophosphamide damaging effects due to its constantly turnover from the germline cell pool and the impairment of new Leydig cells maturation [6, 7]. Studies on human displayed a long-term male gonadal damage after chemotherapy by cyclophosphamide, including a reduction in production of hormone and infertility due to depletion of spermatogonia [8, 9]. Toxic effects of cyclophosphamide on testis were chiefly attributed to oxidative stress on seminiferous tubules and Sertoli cells, impairing spermatogenesis and androgenesis, and inducing apoptosis of germ cells [10, 11]. In addition, the treatment of animals with cyclophosphamide resulted in a reduction of the testis, and epididymis weight, decrease the number of spermatogonia inside the seminiferous tubules, decrease the levels of testosterone and in last infertility [10]. Researches have shown that the use of cyclophosphamide in an adult male animal leads to a reduction in the reproductive organs weight and also reduces reproductive capacity in them [12,13]. In fact, testicular weight depends on the number of germ cells produced, and weight loss may indicate a decrease in the production of these cells [14]. In the study of Tripathi and Jena, 2008 [15], it was shown that cyclophosphamide administration induces the abnormality in the morphology of sperm head. The studies of Selvakumar et al. 2006 [16] and Ilbey et al. 2009[17], revealed the treatment of male rats with cyclophosphamide induces the abnormal sperm and dead. Çeribaşi et al. 2010 [18] who had reported the cyclophosphamide induces tail sperm abnormality in adult male rats, Administration of cyclophosphamide to adult male mice causes abnormality in the sperm head morphology [19].

The aim of the current work was to analysis the sperm morphology of NMRI adult male mice exposed to single dose of cyclophosphamide during the embryonic period.

Materials and methods

The NMRI healthy female and male mice 8-10 weeks of old, weighing 30 ± 32 were purchased from the mouse breeding center of Pasteur Institute of Iran, based on work license with the Razi University Animal Ethics Committee. Males housed individually and females housed as colony 5 per plastic cage for 2 weeks for adaptation to environmental conditions in the animal house. The mice were kept under standard conditions of temperature (22 ± 2°C), humidity (30-40%), light (12: 12 h of light: dark), with free access to food and water ad libitum. Every 2 females through the proestrous phase mated with 1 male, then the females were examined for evidence a vaginal copulatory plug. Vaginal plug manifestation at the following morning established pregnancy start and the day was considered as the zero pregnancy day. Pregnant mice removed from the males, weighted and randomly divided into 2 groups: the control group was received normal saline, and treatment group was given 10 mg/ kg of cyclophosphamide intraperitoneal (i.p.) (Baxter company, Germany) at the 11^{th} day of pregnancy. The weight of males was measured every day from (1^{st} to 60^{th} day after birth) by using balance (Japan).

Sperm Collection

60 days after the birth of mice. After weighing of animals, adult offspring male mice were sacrificed by cervical dislocation, and the testes were weighted by using sensitive balance (GR-120 A & D, Japan). The epididymis removed, and the cauda of epididymis placed in 0.5 ml of T6 medium (Sigma Company) containing 10% bovine serum albumin (BSA, Sigma Chemicals). For uniformity, spermatozoal suspension incubated in a CO2-containing incubator at 37°C for 10 minutes [20].

Assessment of Sperm Viability

For investigation of the sperm viability, one drop of the suspension was putted on a glass slide and trypan blue % 0.4 dye (Thermo. fisher) was introduced. In this technique if the sperm damaged or dead at the time of staining the vital colors such as trypan blue dye % 0.4, can penetrate into head and trunk of sperm, thus appears as blue color, while if the sperm is viable at the staining time [21], the trypan blue dye cannot enter into cell, therefore seems as a light (white) color. To evaluate the survival rate of sperms, the spermatic suspension with trypan blue was mixed to 4: 1 ratio, 40 µl of spermatic suspension with 10 µl of trypan blue color was mixed and after 5 minutes examined by using 40X magnification under the light microscope [20]. The viable sperm percentage is calculated by calculating the ratio of unstained sperm into the total count of sperm [22].

Assessment of Sperm Motility

About 10 µl of sperm suspension placed on a glass slide and covered with a lamina. In 10 random fields, sperm motility was evaluated based on World Health Organization (WHO), [21] expressed as percent motile sperms, which criteria by using an inverted microscope (Olympus, Japan) and equipped with a digital camera (D-D72.Olympus, Japan).

Assessment of Sperm Count

The sperm analysis performed according to the (WHO) criteria [21]. 10 µl of sperm suspension placed on the hemocytometer and observed under light microscope 40X –magnification). The total number of sperm present in 100 squares and expressed in million per ml of semen.

Sperm Morphology

A thin smear of sperm suspension was placed on a glass slide. The air-dried slides fixed in 70% ethanol for 2 minutes and stained by Papanicolaou staining. This technique carried out as in the following briefly steps: Alcohol 70% 10 beats, wash in water 10 beats, hematoxylin 5minutes, wash in water 10 beats, alcohol-acid 1-2 beats, alcohol 96% 10 beats, OG6 color 5minutes, alcohol 96% 10 beats, EA 50 5minutes, alcohol 96% 10 beats and alcohol 100% 10 beats [20].

Statistical analysis

The data of current study was determined by using the SPSS, version 21. Statistical significance among control and tested groups was calculated by using the One-way ANOVA. The data are expressed as mean ± Standard Error Mean (SEM) and the differences were considered significant at $P \leq 0.05$.

Results

Animal and Testis Weight

The animal weight was 23.66 ± 0.33 g in control group, but in the group that exposed to cyclophosphamide during their embryonic period was 18.00 ± 0.57 g, which was lower than the control group ($P \leq 0.05$) (Figure-1a). Also, our results showed that the testicular weight was 0.09±0.002 g in the control group, while in the cyclophosphamide group was 0.06±0.001 g which was lower than the control group ($P \leq 0.05$) (Figure-1b).

Sperm Viability

The average of sperm viability was 75.54 ± 1.13% in the control group, while in the cyclophosphamide group was 56.74 ± 1.23%, which reduced in compared to the control group ($P \leq 0.05$) (Figure-2).

Sperm Motility

The sperm motility average was 60.00 ± 0.81% and 29.00 ± 1.16% in the control and cyclophosphamide groups respectively, in the cyclophosphamide group when which was reduced in comparison to control group ($P \leq 0.05$) (Figure- 2).

Sperm Count

Our results revealed that the sperm count was $15.40 \pm 0.24 \times 10^6$ and $7.80 \pm 0.26 \times 10^6$ in the control and cyclophosphamide groups respectively, which showed a reduction in the cyclophosphamide group when compared with the control group ($P \leq 0.05$) (Figure- 3).

Sperm Morphology

The results of this work showed the percentage of sperm head abnormality in control group was % 0.56±0.25, but, in cyclophosphamide-treated mice was 8.40±2.04 %, which revealed a significant increase in comparison to control group. The defective neck sperm percentage was % 10.04±0.49, in control group, while in cyclophosphamide group was % 16.33±2.80, which showed a significant increase in comparison to control group. On another hand, the results of present study showed that the

percentage of tail sperm abnormality was %24.11±0.71 and %55.67±2.76 in control and cyclophosphamide groups respectively, which increased significantly in mice that received cyclophosphamide throughout their embryonic period (Figure-4).

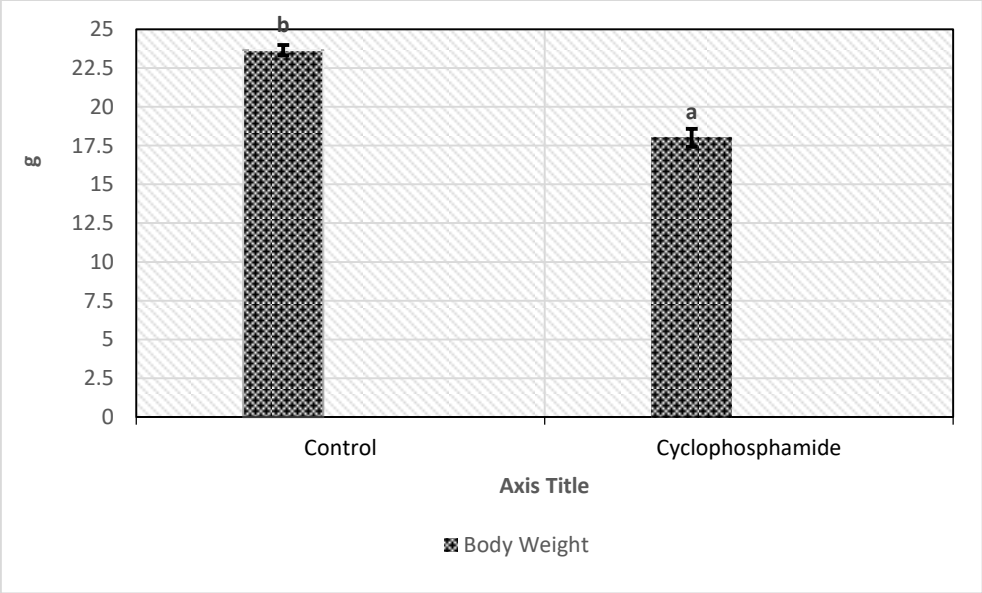


Figure 1 (a)-Mean of body weight in adult male mice that received 10 mg/kg of cyclophosphamide during embryonic development period.

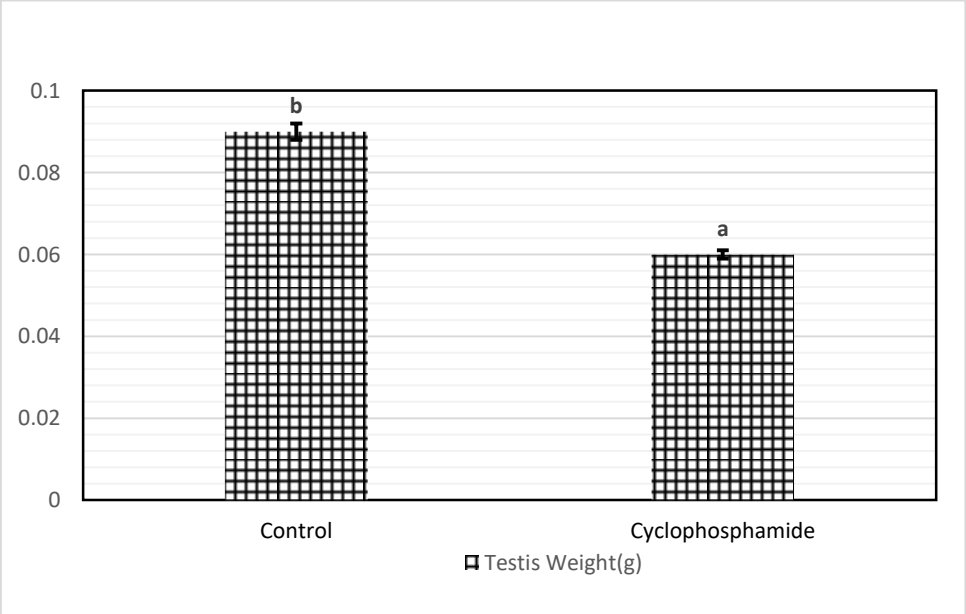


Figure 1 (b)- Mean of testis weight in adult male mice that received 10 mg/kg of cyclophosphamide during embryonic development period.
a Significant difference from control group
b Significant difference from cyclophosphamide group
Values are expressed as mean ± Standard Error Mean (SEM). (ANOVA: P≤0.05)

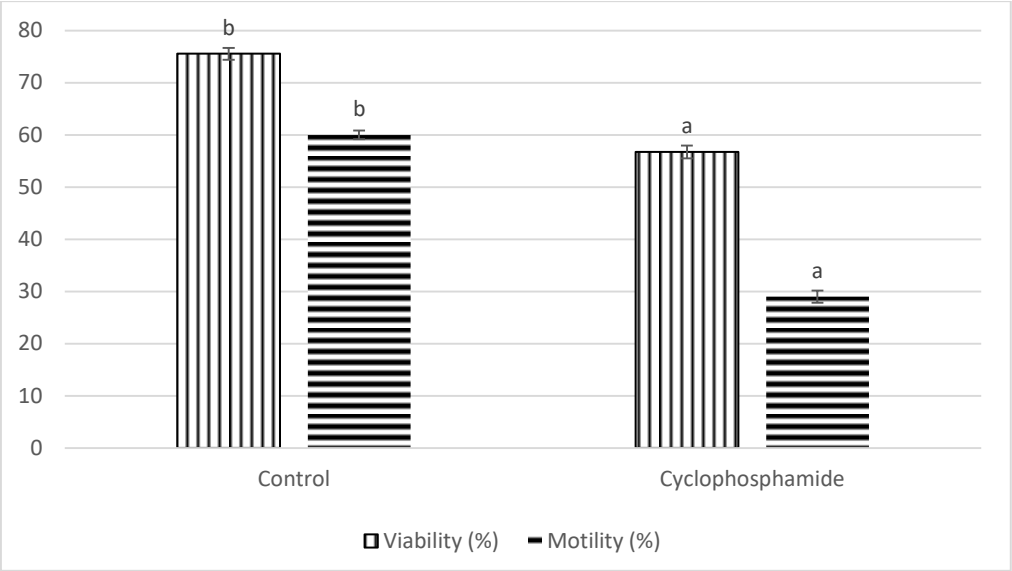


Figure 2-Mean of percentage of viability and motility of sperm in adult male mice that received 10 mg/kg of cyclophosphamide during embryonic development period
a Significant difference from control group
b Significant difference from cyclophosphamide group
Values are expressed as mean ± Standard Error Mean (SEM). (ANOVA: P≤0.05)

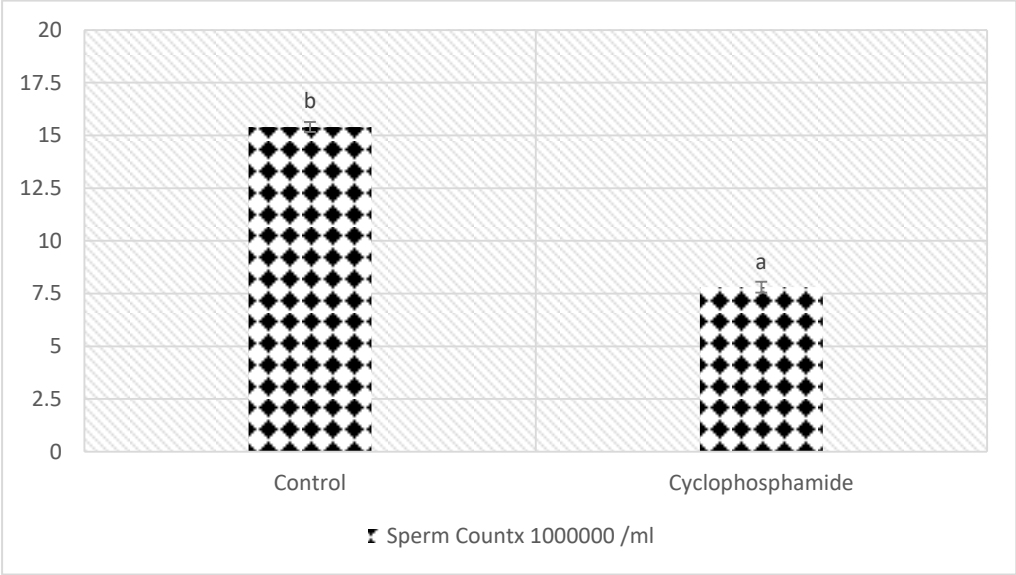


Figure 3-Mean of sperm count/ ml in adult male mice that received 10 mg/kg of cyclophosphamide during embryonic development period.
a Significant difference from control group
b Significant difference from cyclophosphamide group
Values are expressed as mean ± Standard Error Mean (SEM). (ANOVA: P≤0.05)

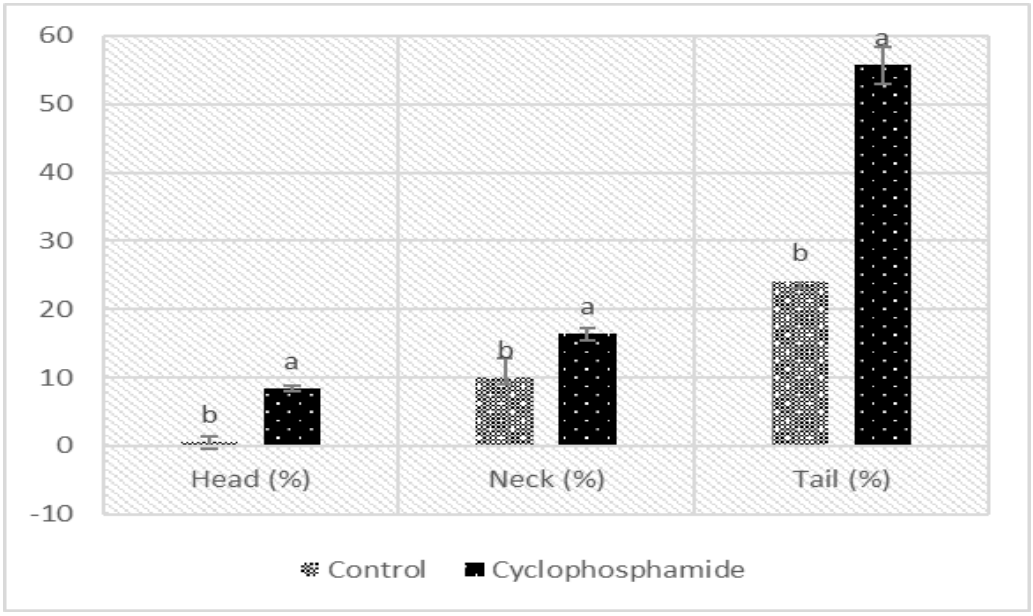
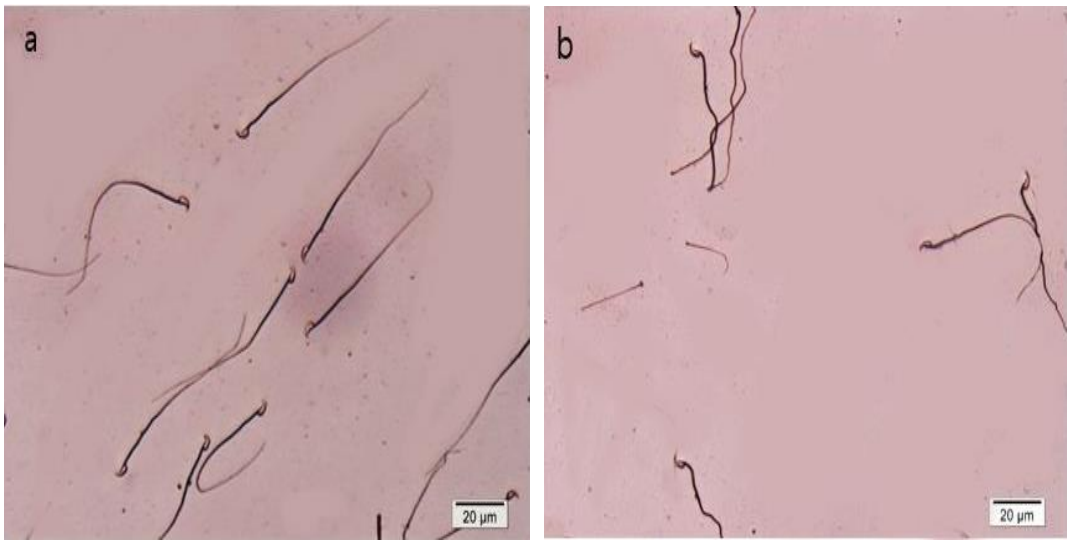


Figure 4-Mean of percentage of sperm abnormalities in adult male mice that received 10 mg/kg of cyclophosphamide during embryonic development period.
a Significant difference from control group
b Significant difference from cyclophosphamide group
Values are expressed as mean ± Standard Error Mean (SEM). (ANOVA: P≤0.05)



Picture 1-Sperm Morphology (Scale Bar 20µm) in: a) Control group b) Cyclophosphamide group

Discussion

The findings of current work showed that the adult male mice treated by 10mg/kg of cyclophosphamide during the 11th embryonic day had a lower weight than the control group. Previous studies have revealed that the administration of cyclophosphamide at doses 50– 100 mg kg induces loss weight of testes [23, 24], male Fi-mice (C3H/HexC57Bl/6J) treated with 40 or 80 mg/kg of cyclophosphamide had lost weight in body and testes [25]. The study by Ramose *et al*., [26], proved that cyclophosphamide treatment in adult male rats decreases the body weight significantly. Other studies also showed that the treatment with cyclophosphamide reduces the body and reproductive organs weight in male rats [27- 28]. These results are consistent with our results, this due to induction of atrophy in testes and epididymis by cyclophosphamide, and leads to infertility [29]. Cyclophosphamide also causes the reduction in germ cells production and finally decreases the weight of testes [30].

Our findings demonstrated that the cyclophosphamide-exposed adult male mice on the 11th embryonic day had decline in sperm viability and motility. These results agree with previous studies by Lu *et al*. [29]; Nayak et al. [19]; Oyagbemi et al. [28] and Shabanian et al. [30]. The decrease of viability and motility of sperm may be explained by ability cyclophosphamide to decrease the concentration of testosterone, follicle luteinizing hormone(LH) and stimulating hormone (FSH) in serum [31]. In addition, the cyclophosphamide administration into male rats rises the abnormalities incidence of sperm flagellum [12]. The reduction in the sperm motility may also be due to the capability of cyclophosphamide to modify the permeability of the mitochondrial membrane to calcium ions in sperm which has adverse impacts on motility of sperm because of the calcium is a vital regulator for cell motility [19].

The results of the current investigation showed that the adult male mice received that 10 mg/kg of cyclophosphamide on their 11th day of gestation had to decrease in sperm count. Previous studies administered that cyclophosphamide injection to mice induces the reduction in sperm count [32-33], the results of these studies are in agreement with our results. The sperm count reduction may have explained by the ability of cyclophosphamide to decrease the serum level of testosterone and changes the germ cells count and finally the spermatogenesis disrupted [34]. Cyclophosphamide also causes the epithelial cells loss, which damages the somatic cells in the testes and destroys cytoplasmic bridges, and so, can decrease the count of sperm [35]. It has been observed that the exposure to cyclophosphamide causes apoptosis of germ cells and resulting in the decline of sperm count [33].

Our findings showed that the injection of cyclophosphamide at a dose 10 mg/kg to mice during embryonic phase could cause abnormality in sperm morphology at puberty, which includes a defect in head, neck, and tail of the sperm. It has been also reported that the administration of the single l dose of cyclophosphamide into adult rats induces abnormality in sperm morphology, which includes amorphous head, small head, two heads, folded and short tail [36]. Çeribaşi *et al*. [18] have revealed that the cyclophosphamide administration at a dose of 15 mg⁄ kg increases a tail sperm abnormality in adult male rats. This result is in-line with our results. This may be due to the ROS effect on DNA of sperm, which can induce changes in the structure of nitrogen bases, complement nitrogen bases deletion, morphological and cross junction changes of DNA and variations in chromosomes reorganization [35, 36].

Conclusion

Based on our results using using of cyclophosphamide during embryonic development period can influence the body and testicular weight of male offspring at puberty, reduced, viability, motility, and count of sperm and also increased sperm abnormality. Therefore, it was suggested that the adverse effects of cyclophosphamide chemotherapy continue from the embryonic phase into the adult phase.

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