RESEARCH ARTICLE

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Adverse Effects of Vincristine Chemotherapy on Cell Changes in Seminiferous Tubules and Cetrorelix GnRH Antagonist Inhibitory Effects in Mice

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Abstract

The present study was designed to determine changes in spermatogenesis in adult mice after an intraperitoneal injection of vincristine. The effect of a GnRH antagonist synchronous to chemotherapy, which might protect spermatogenesis by halting cell division in spermatogenic cells, was also investigated. **Method and Materials:** A total of 30 adult male mice were studied in three equal groups of ten. In the V group, a single dose of the chemotherapy drug vincristine was injected intraperitonally at 1.5 mg/kg. In the V+C, group, the injection of Cetrorelix was started, and one week before to one week after vincristine injection continued (for 3 weeks). Controls received no treatment. Samples were taken from the testicles, and fixed in Boueins fixative for light microscopy. **Results:** Comparing the mean number of Sertoli and spermatogony cells and the rate of spermatogenesis index (SI) in the V group with controls showed significant differences,which were not evident in the V+C group. **Conclusion:** According to the results, the cetrorelix antagonist (GnRH) could largely prevent side effects of vincristine administration regarding seminiferous tubules.

Keywords: Chemotherapy drug- vincristine- GnRH- cetrorelix- spermatogony- seminiferous tubules

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Introduction

Young patients with different kinds of cancer need to the specialized and sensitive treatments, in addition to the therapeutic aspects, those treatments lead to some deficiencies in some sensitive organs of the body, these types of treatments are done by the harmful medication and radiation which are designed as the chemotherapy and radiotherapy. One of the side effects of this treatment, especially the testicles in males, is reduced gonadal function (dysfunction testicular long-term effects of chemotherapy in treating many tumours). This treatment can cause damage to the germinal epithelium seminiferous tubules of the testes, leading to azoospermia and oligospermia and will eventually lead to sterility. (Nudell et al., 2002; Mitchell et al., 2009; de Haas et al., 2008; Krawczuk-Rybak et al., 2009; Delbès et al., 2010 ; van Beek et al., 2007; Jahnukainen et al., 2011). In 2001, high-dose chemotherapy with bone marrow transplanted to treat before the irreversible failure of germ cells was accompanied (Howell et al., 2009). karboplatin effected on spermatogenesis, resulting in oligospermia and in some cases permanent infertility (van Beek et al., 2007). Considering the high proliferation rate of the cancer cells and chemotherapy drugs on were rapidly dividing cells, but the effects of these drugs were not intelligent and could not distinguish cancer cells from normal. So all rapidly-dividing cells were the target, and the cells were being rapidly divided in the process of spermatogenesis and be targeted in chemotherapy and damaged. (Delbès et al., 2010 ; van Beek et al., 2007; El-Awady et al., 2016).

One of the most common side effects of chemotherapy is the impaired spermatogenesis in the testes in men. Damage of the germinal epithelium due to the oligo or azoospermia for a long time is a result of treatment with anti-cancer drugs. Tumours are diagnosed with the uncontrolled division of the normal tissue and cancer cells Lose limit cell division and division are not controlled. One of the chemotherapy drugs which are used in a variety of malignancies is vincristine (Chotsampancharoen et al., 2016; Dobrzyńska et al., 2015). Vincristine affects on all types of Spermatogenic cells which are dividing cells which were investigated in both the Invitro, Invivo environments (Dobrzyńska et al., 2005). for the first time, "Glode (1981)" stated that disrupting in the Hypothalamo-hypopheseal axis during the chemotherapy may reduce (FSH) and (LH) hormones and prevent the normal spermatogenesis; therefore, the Spermatogenic cells were preserved during the chemotherapy. Finally, the process of spermatogenesis

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was observed after discontinuation of the course of treatment (Glode et al., 1981). So with regard to the protection of spermatogenesis during the chemotherapy, hormone levels of the Hypothalamo – hypopheseal axis can be reduced, and the normal spermatogenesis can be preserved and cause to the spermatogenesis recovery after the chemotherapy. In a study which was done by "Wang (2016)", they first reported that hormone manipulation improved the androgenic spermatogenesis followed by mouse fertilization that was treated with anticancer agents (Wang and Shao, 2016). There were various methods for manipulating the hypothalamus-pituitary hormone, including the use of analogues and antagonists (GnRH) (Vaisheva et al., 2007; Johnson et al., 1985; Harel et al., 2011). As it was mentioned before, vincristine is used as an anti-cancer drug for the treatment of various cancers. Many studies have been done about cancer and its effects on spermatogenesis, but there is no sufficient study about the inhibitory effect antagonist Cetrorelix as vincristine. Through the present research, the protective effect of GnRH antagonists was done to inhibit the proliferation of germ cells and avoid the stem cells destruction of spermatogenesis. The aim of the present study was to study the performance of the vincristine on the germinal epithelium and Germinal Cell Population Seminiferous Tubules, to research on the protective effect of GnRH on spermatogenesis process in treated with vincristine and to study the quantitative changes and spermatogenesis cell proliferation after the treatment with vincristine and antagonist GnRH.

Materials and Methods

Animals: in the present study, 30 adult male mice (6-8 weeks) were used according to the guide care. The mentioned animals were placed under the controlled conditions in a laboratory setting Tabriz University of Medical Sciences (12-h light/12 and 12- h dark) with free access to water and food. Mice were divided into three equal groups of ten, including: control group, (V) group and (V+C) group. In (V) group, a single dose of the chemotherapy drug vincristine was injected intraperitonally at 1.5 mg/kg.

So, in the (V+C) group, the injection of Cetrorelix was started, and one week before and after the injection, the vincristine continued (for 3 weeks). The dosage was selected according to the previous studies (Dobrzyńska et al., 2005). After 35 days from beginning of treatment, all 3 groups of mice were sacrificed, testicles were removed from the abdominal cavity and carefully separated from the epididymis with the knife and then samples were taken from the testicles and fixed in the boueins fixative (Mixture of picric acid saturated in distilled water 75 cc + 37% solution of commercial formaldehyde 25 cc +Glucose Acid 5cc) for 48 hours. Then, samples were molded in paraffin blocks and microscopic sections with a thickness of 4 microns which were prepared with rotating microtome (Rotary).The prepared sections spread on the lamella and stained with Haematoxylin-Eosin. (Haematoxylin: 2.5 grams of haematoxylin + 50 cc of absolute alcohol + 50 grams of aluminium + 500 cc distilled water and then add 1.5 grams of mercuric acid and cool it very quickly, and finally add 20 cc glucose and Eosin: 10 grams of eosin + 1000 cc of water, stained with filter paper, glossy glue). Finally, they were found for light microscope. For the quantitative studies and statistical analysis, the prepared specimens were provided by (Simple Uniform Random). For this purpose, samples of 4 microns were prepared at the intervals of 150 microns. Approximately 30 samples were taken to measure the diameters of seminiferous tubules used on the Motic Image plus 20 Software. 20 fields of seminiferous tubules in each mouse were prepared, with lens 10, the diameters were measured perpendicular to the seminiferous tubules that were cross-sectioned. Then, the average of the diameters was calculated, and the obtained data were analysed by the descriptive statistics (mean ± standard deviation) and with Kruskal-Wallis test by using SPSS 13Software, and the statistical analysis was performed. In the current study, the amount of (P) less than 0.05 was considered significantly. In the transverse sections of the Seminiferous tubules, the number of Spermatogonium and Sertoli cells were counted and thickness of the epithelium was investigated in 20 fields with 40 lens(17). Spermates in mice were divided into 3 groups. 1) Round spermatid which was seen in step 1-8, 2) Extending spermatid that was seen in step 9-14, 3) Longer spermatozoa located in step 15-16. In SI, the number of sperm in the seminiferous tubulescontaining15-16 compared to other seminiferous tubules in 20 microscopic fields for each sample and the average SI of the three groups were studiedwithlens40. All data were calculated by the descriptive statistics method (The average Standard deviation), the obtained data was evaluated with Kruskal-Wallis test by using SPSS.13 software.

Results

Light microscopy

The findings of Light microscopy

Light microscope showed that the seminiferous tubules had a thick epithelium and interstitial spaces and containing mature sperms and interstitial spaces contained connective tissue with lydig cells (Figure 1A). The thickness of the germinal epithelium in the seminiferous tubules in the group (V) was reduced compared with the control group, and epithelium was damaged in some of the tubules. And the numerous empty spaces inside these miniferous tubules were shown (arrow head), (Figure 1B). In the (V) group, Sertoli cells just could be seen in the number of the seminiferous tubules (arrow head) (Figure 1C). Germinal epithelium in (V+C) group was similar to the control group, and detachment was observed between cells in several tubules (Figure 1D).

Histomorphometric evaluation

Histomorphometrical of seminiferous tubules was indicated the significant changes spermatogonia, average spermatogonia in the control group = (47/42 ± 1/96) and (V) group = (16/53 ± 2/37) and (V+C) group was (36/28 ± 7/98), respectively. The number of spermatogonial cells in the group (V) significantly decreased in comparison

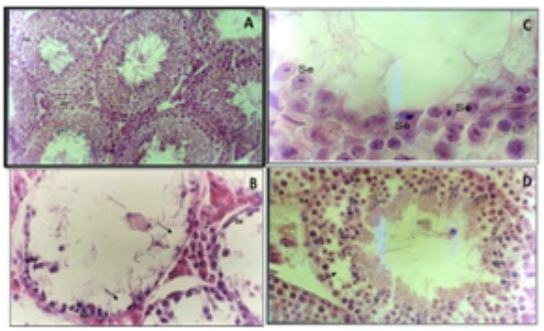

Figure 1. Light Microscope Observations of the Seminiferous Tubules of Mice in Three Experimental Groups; A, Seminiferous tubules in the control group have a thick germinal epithelium; B, The Spermatogenic epitheliums in the vincristine (V) group was reduced in thickness; C, Sertoli cells just were seen in the number of the seminiferous tubule. D, Germinal epithelium of the group (vincristine + Cetrorelix) was little changed and very similar to the control group.

with (V+C). And Group (V+C) was very similar to the control group (C).the statistical analysis of the number of spermatogonial cells in the (V) and(V+C) group as well as the statistical analysis of the number of spermatogonial cells in the (V) and (V+C) groups along with the control group showed the significant differences.(P=0.001) (Figure 2A).

Histomorphic study of the seminiferous tubules revealed the average number of Sertoli cells. In the seminiferous cross-section of 20 tubule per sample/ tubules in control, (V) and (V+C) group was (13/20 2/20), (16/85 1/14) and (10/20 1/13), respectively. The number of Sertoli cell in the group (V) significantly decreased in comparison with (C), and the Sertoli cells in Group (V+C)

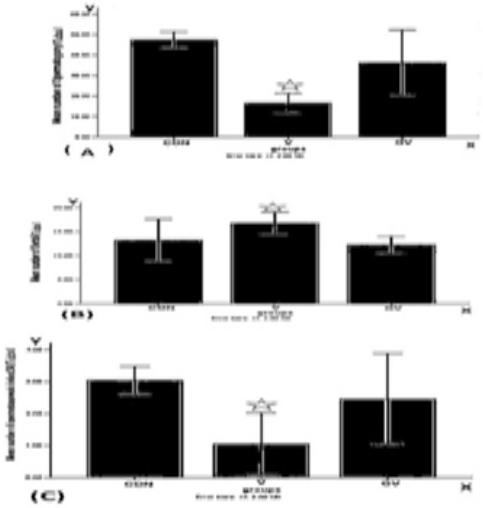

Figure 2. Histomorphometric Evaluation of the Seminiferous Tubules in Three Experimental Groups; A, Comparison the number of Spermatogony cells and B, Sertoli cells and C, spermatogenesis index in the three Experimental groups. (Transverse Axis represents the three groups participating in the Experimental and the longitudinal axis indicates the average number of Spermatogony cells(A) and Sertoli cells (B) and spermatogenesis index (C) in the seminiferous tubules in three groups),(Error bars = 0.002 Standard deviation).

Table 1. Number Cells

Groups	Control groups	(V) group	(V±C) group
Histo-Pharmonetric Evaluation			
Spermatogony Cell	(47.42±1.96)	(16.53±2.37)	(36.28±7.98)
Sertoli Cell	(13.20±2.20)	(16.85±1.14)	(10.20±1.13)
Spermatogenesis Index (SI)	(3.03±0.21)	(1.04±48.0)	(1.90±0.40)

were very similar to the control group (C).

Statistical analysis of the number of Sertoli cells in (V) and (V+C) groups, as well as the statistical analysis of the number of Sertoli cells between (V) and (V+C) groups and between control group and (V) group (P=0.001) showed the significant differences (Figure 2B).

Histomorphometric evaluation of SI in 20 microscopic field for each sample: The number of the seminiferous tubules, which were Containing the sperm 16-17,were counted and compared with other seminiferous tubules and marked the average SI in the control, (V) and (V+C) groups: (3/03 ± 0/21) , (1/04 ± 48) and (1/90 ± 0/40). Statistical analysisSI in the control and (V) groupsshowed the significant differences (P=0.001), as well as comparing group (V+C) with the control group showed a significant differences (P=0.001) (Figure 2C).

Disscusion

The purpose of the present research was to study the effects of vincristine as an anti- cancer drug to the little changes of testicular germinal epithelium, and the ability Cetrorelix (the antagonist GNRH) was to inhibit these effects. Studies show that the effect of vincristine in the germinal epithelium prevented the proliferation of spermatogonia, causing serious injury of germinal epithelium. Also, the current study illustrated that Cetrorelix was able to prevent the adverse effects of vincristine. The present investigation was the first research to the adverse effects of vincristine on the cellular population of seminiferous tubules and improved by Cetrorelix effects. In group I, the group receiving vincristine, germinal epithelium showed a reduction in the group receiving vincristine. This finding is consistent with the findings of other researchers that the chemotherapy drug has adverse effects on spermatogenesis (Mitchell et al., 2009; de Haas et al., 2008; Krawczuk-Rybak et al., 2009; Delbès et al., 2010; van Beek et al., 2007; Jahnukainen et al., 2011; Dobrzyńska et al., 2005; Vaisheva et al., 2007; Khachatryan et al., 2015). Optical microscopy study showed the appearance of the empty spaces within the confines of spermatogonia which represented the loss of cells and ultimately reduced the number of spermatogonia population (van Beek et al., 2007; Howell et al., 2009). The study showed that the number of Sertoli cells in the group treated with vincristine were increased.

Just Sertoli cells are in the seminiferous tubules which create in the ideal environment for the propagation and preservation of sperm cells (Gundersen and Solberg, 2007), so any changes in these cells can lead to infertility

(Shinoda et al., 1999).

In rat, the Sertoli cell proliferation continued at day 16 of embryonic life and two weeks after birth (O'donnell et al., 2001). After the proliferation, the cells stopped and differentiation began and continued over the life (Johnston et al., 2004). There are androgen receptor - estrogen and FSH In the Sertoli cell (O'donnell et al., 2001), and the activity of the pituitary hormone depend on the factors within the testicular factors (Johnston et al., 2004). Therefore, any changes in the levels of pituitary hormones and testicular factor affected on the cell function. After that the chemotherapy has been shown that Sertoli cells produced more value C-K18 (there is not the marker in the adult Sertoli cells). However marker in normal cells inactivated the Sertoli which could eventually affected on the quality of spermatogenesis (Maymon et al., 2004). Reports on the number of Sertoli cells to chemotherapy were contradictory. The number of Sertoli cells to chemotherapy did not change (Aich and Manna, 2001), the number of Sertoli cells reduced after chemotherapy (Jansz and Pomerantz, 1985; Boujrad et al., 1995). Other studies have shown that there was a proliferation of Sertoli cells after radiation therapy and Cryptorchidism (Ghosh et al., 1992).

Increase the number of Sertoli cells is significant in the recent study in the group receiving vincristine. Since the diameter of seminiferous tubules significantly reduced, the argument was that with the loss of testicular germ cells and reducing the diameter of seminiferous tubules, Sertoli cells appeared clearer and enhanced. In the Group II, GnRH antagonist had a protective effect on the side effects of vincristine, and vincristine reduced the toxicity effects. In light microscopy study of the seminiferous tubules in group II, the thick epithelium containing spermatogonia and Sertoli and finally the results of the quantitative and qualitative approached to the control group. In support of our results, Udagawa's study showed an improvement of spermatogenesis in supporting the treatment along with (GnRH), following the treatment with vincristine as the chemotherapy drug (Udagawa et al., 2001). According to other studies, the researchers found the increases of hormones (FSH) and (LH) after chemotherapy and germinal epithelium (Udagawa et al., 2001; Shetty and Meistrich, 2005). In addition, the increases of the secretion of testosterone inhibited the expression binding stem cells factor(SCF) to the basement membrane which is essential for the spermatogenesis process(Shetty and Meistrich, 2005).

Apparently, reducing the intra testicular testosterone to protect the spermatogenesis is due to the reduced FSH, LH and the testosterone hormones in the use of GnRH antagonist before the treatment with chemotherapy, and because the non-dividing cells are less susceptible to the toxic effects of chemotherapeutic agents, Spermatogenic cells in non-dividing stage are less affected and protected.

In general, chemotherapy drugs, in addition to the direct degradation effected on spermatogenesis, were potentially harmful to spermatogenesis by increasing testosterone, such as estradiol. Therefore, spermatogenesis could be maintained by reducing the testosterone in the testicle. Treatment with antagonists GnRH, before, during and after the administration of toxic substances had the ability to maintain spermatogenesis, in order to decrease FSH, LH and testosterone in the use of these toxic substances, before treatment, in habited spermatogonial proliferation directly and protected these cells from the harmful effects of chemotherapy. The use of analogues and antagonists GnRH after the use of toxic substances was also effective in the improvement of spermatogenesis. Since by reducing FSH and testosterone, it protected the remaining spermatogonia from the effects of malignant hormones.

According to the current hypothesis, "Shetty et al., (2002)" and "Xiong et al., (2014)", curing by testosterone therapy caused to improve the process of spermatogenesis followed by radiation therapy. Also, Mitchell et al., (2009) showed that after exposure to toxic substances, by reducing testosterone, spermatogenesis can be improved after Shetty et al., (2016). The results of the study showed that using of vincristine as the anti-cancer drug in mice damaged the testicular germinal epithelium, and applying the antagonist before and in chemotherapy could partially protect the effects of hypothalamic gonadal axis on the germinal cells.

In conclusion, according to the accessed results, Cetrorelix antagonists (GnRH) can partially prevent the side effects of the chemotherapy drugs before and during the cancer treatment.

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