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Protective Role of GnRH Antagonist on Chemotherapy-induced Spermatogenesis Disorder: A Morphological Study

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ABSTRACT

Purpose: Anti cancer drugs is one of the most important chemotherapeutic factors which can influence spermatogenesis process and germinal epithelium. Since dividing cells are mainly affected by anticancer drugs, the aim of the present study is to investigate the preventive effect of GnRH antagonist on spermatogenic defect produced by anticancer drugs. ***Methods:*** In the present study thirty adult male mice aging 6-8 weeks were divided into 3 groups as: Control, Experimental 1 and Experimental 2. Experimental 1 group received Cisplatin for 5 days as 2.5 mg/kg intraperitoneally and Experimental 2 group received 0.25 mg/kg cetrorelix (GnRH antagonist) one week before cisplatin treatment and continued for 3 weeks. The mice in all groups were sacrificed 35 days after the last injection and testis specimens were fixed in boueins, formaldehyde fixative and 2.5% Glutaraldehide then prepared for light and electron microscopic examination. ***Results:*** Light microscopy (LM) study showed that the number of spermatogonial cells, thickness of germinal epithelium, was decreased in Experimental 1group. Electron microscopy revealed that in this group several intercellular spaces appeared between spermatogenic cells and secretory granules in interstitial cells was increased. There were several vacuolated mitochondria and destroyed organelles in spermatogonial cells but in Experimental 2 group condition was similar to control group. ***Conclusion:*** These results indicate that the cetrorelix administration before cancer treatment may protect germinal epithelium against side effects of cisplatin.

Introduction

Up to 20% of young couples are suffering from infertility problem. One of the major causes of infertility is the use of anticancer drugs.[1] The effects of anticancer drugs are based on their antimitotic action such as spermatogenic cells.

Spermatogenesis is influenced by radiotherapy and cancer cytotoxic drugs.[2-5] Cisplatin is used for treatment of various malignancies including cancer of testis, ovary, lung, bladder and Hodgkin and non-Hodgkin lymphoma.[6-8] The previous studies showed the side effects of chemotherapeutic drugs such as azoospermia after treatment.[3,9] The side effects of Cisplatin on the spermatogenesis when used as anticancer drugs are known.[10] In comparison to normal tissue, tumors are characterized by uncontrolled division. The process of cell division- normal or cancerous cell- is through the cell cycle. The cell cycle goes from the resting phase, through active growing phases and then to mitosis (division). The ability of chemotherapy to kill cancer cells depends on its ability to halt cell division. Therefore chemotherapy is most effective at killing cells that are rapidly dividing. Unfortunately, chemotherapeutic agents can not differentiate between the cancerous and the normal cells. It has been shown that apoptosis is one of the mechanisms in cell destruction following chemotherapy.[10-14] Some agents could increase the rate of germ cell apoptosis in testis such as genes factors, testicular ischemia, heat stress, exposure to irradiation and toxic substances.[5,10-15] On the other hand, it is known that spermatogenesis is effected by FSH, LH and testosterone and suppression of them could suppress spermatogenesis. Thus it is logical to conclude that suppression of gonadotropins during chemotherapy would protect spermatogenesis by inhibition of their proliferation. For the first time, Glode *et al*. showed that disturbance of hypothalamo – hypopheseal axis and reduction the FSH and LH secretion during chemotherapy resulted in promotion of spermatogenesis after chemotherapy.[16] There are different methods for inhibition of hypothalamo – hypopheseal axis including usage of agonist and antagonist of GnRH.[16-19] As mentioned earlier, cisplatin is commonly used for treatment of different

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malignancies and its effect on spermatogenesis is well studied[6-8] but there is not enough study about ultrastructural alterations. Since ultrastructural studies are useful for evaluation of intercellular junction and status of organelles could help in understanding of the mechanism of action of cisplatin therefore we decided to study the ultrastructural changes induced by cisplatin. Similarly the inhibitory effect of Cetrorelix, as an antagonist of GnRH, on spermatogenesis following treatment with cisplatin has poorly been studied and there is no ultrastructural study about this matter. Therefore, the aim of present study is 1) to investigate the action mechanism of cisplatin on germinal epithelium based on morphological criteria. 2) To investigate the protective effect of cetrorelix on spermatogenesis following treatment with cisplatin, using ultrastructural criteria.

Materials and Methods

Male balb/c mice aging 6-8 weeks (n = 30), were cared for according to the *Guide for the Care and Use of Laboratory Animals* of Tabriz University of Medical Sciences and housed under controlled conditions (12-h light/12-h dark) with free access to water and food. The mice were divided into three groups equally as follows: control (n=10), experimental 1 (n=10) and experimental 2 (n=10). In experimental 1 group, mice received 2.5 mg/kg daily cisplatin intraperitonally for five days. In experimental 2 group the mice in addition to cisplatin received cetrorelix subcutaneously 0.25 mg/kg 3 times a week. Cetrorelix application started first week prior to cisplatin treatment and continued for three weeks. Since spermatogenic cycle in mice is 35 days, the mice in all three groups were sacrificed by cervical dislocation 35 days after last injection. Then, testes were removed from the abdominal cavity and separated from the epididymis carefully by using a surgical blade. Right testis was fixed in Bouins fixative for 48 h for quantitative studies and then processed for light microscopy. The paraffin sections were stained with H & E and examined with light microscope. For quantitative analyses, we used Motic Image plus 20 Software. 20 microscopic fields, in seminiferous tubules from each mouse, the number of spermatogonia was counted and thickness of germinal epithelium was determined.[4] The obtained data were analysed with Kruskal-Wallis test by using SPSS.13 software.

The specimens from the left testis were fixed in 2.5% Glutaraldehyde (Pro. Sci. Tech. Au) for 12 h and washed with 0.2 M phosphate buffer and post fixed with 2% osmium tetroxide (TAAB, UK) for 2 h. After dehydration, clearing and Infiltration they embedded in resin (Pro. Sci. Tech. Au) and sectioned using ultramicrotome (Richert-Jung, Au). Semithin section stained with toluidine blue and studied with light microscope. The thin sections (80 nm thickness) were stained with uranyl acetate and lead citrate and were studied with Leo 906 transmission electron microscope (Leo, Germany).

Results

Light microscopy

In control group, Light microscopy revealed that seminiferous tubules have a thick germinal epithelium and their lumina contained numerous mature sperms and interstitial spaces contained connective tissue and lydig cells (Figure 1A).

The spermatogenic epitheliums in the experimental 1 group compare to the control group were reduced in thickness and in some tubules germinal epitheliums was destroyed (Figure 1B). The condition of germinal epithelium in experimental 2 group was similar to control group and detachment between spermatogenic cells were still present in few tubules (Figure 1C).

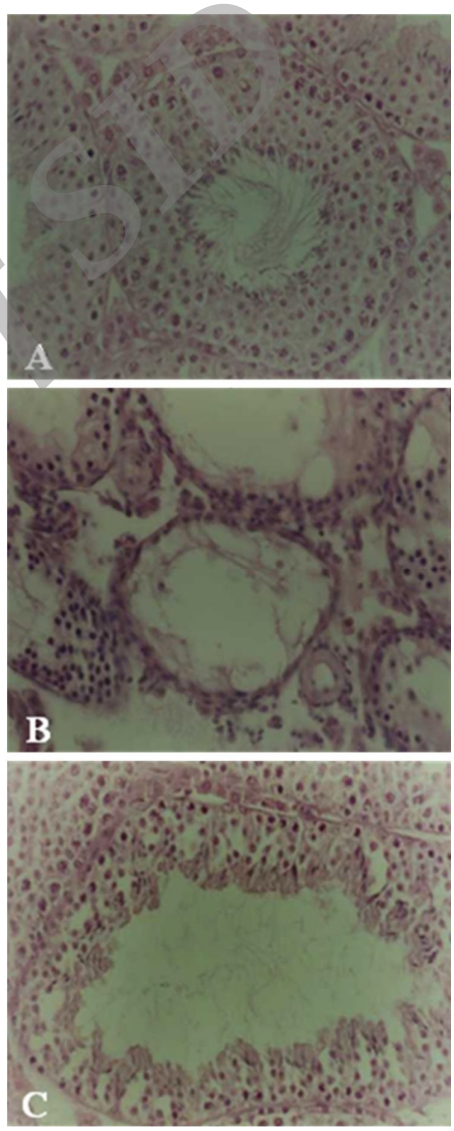

Figure 1. Photomicrograph of a seminiferous tubule: A) from a control mice testis. Note the regular arrangement of spermatogenic cells. B) from experimental 1 group received cisplatin. Note tubular destruction and their depletion from spermatogenic cells. C) from experimental 2 group received both cisplatin and GnRH antagonist. Note relatively complex recovery of spermatogenic epithelium. H & E staining.660 X.

Histomorphometric evaluation

Histomorphometric evaluation of seminiferous tubules showed that the mean number of spermatogonia/ tubule in control group, experimental 1 and 2 group was 43.22±1.55, 21.42 ±2.44 and 40.53±2.38 respectively. The number of spermatogonia was significantly reduced in experimental 1 group in comparison to control group ($P<0.05$) but the spermatogonial number in experimental 2 group was similar to control group ($P>0.05$) (Table 1). Thickness of germinal epithelium in control group was 57.33±4.1 µm but it decreased to 20.90±3.84 µm in experimental 1 group and reached to 50.09±4.08 µm in experimental 2 groups. Statistical analysis showed that the difference between control and experimental 1 group was significant ($P<0.05$) but experimental 2 group was not significant ($P>0.05$) (Table 1).

Table 1. Comparison of different parameters in testis from three groups.

Groups	No. of Spermatogonia cells/tubule	Thickness of germinal Epi (µm)
Control	43.22±1.55	57.33±4.1
Cisplatin	21.42±2.44*	20.90±3.84*
Cis+GnRH	40.53±2.38	50.09±4.08

*: Significantly different from control group

Electron microscopy

EM study was specially focused on spermatogonia cells because these cells are germ cells and have prime role in normal spermatogenesis.

Study with EM in control group showed that in the seminiferous tubules the spermatogonia cells located on basal lamina and had oval nuclei (Figure 2A). High magnification showed some mitochondria scattered in the cytoplasm. The cells were tightly attached to each other or basal lamina (Figure 2B).

There were also interstitial cells between seminiferous tubules containing secretory granules and extensive smooth endoplasmic reticulum (Figure 2C).

In experimental 1 group, spermatogenic cells were separated from each other or basal lamina by large spaces (Figure 3A). Spermatogenic cells had several damaged organelles such as vacuolated mitochondria as well (Figure 3B). Furthermore in this group intracellular secretory granules were dramatically increased in interstitial cells (Figure 3C).

In experimental 2 group, the structure of tubules was similar to control group but in some tubules small spaces were still present between spermatogenic cells.

Discussion

In the present study the protective role of cetrorelix on adverse effects induced by cisplatin has been investigated. The study revealed that in experimental 1 group which received only cisplatin, the thickness of germinal epithelium and the number of spermatogonia in somniferous tubules were reduced which is considered as common side effect of chemotherapy[3,4,20,21] and or indicates that cellular proliferation was reduced in the somniferous tubules. In accordance with our finding a reduction in mitosis and DNA changes has been reported following treatment with chemotherapic agents.[22,23]

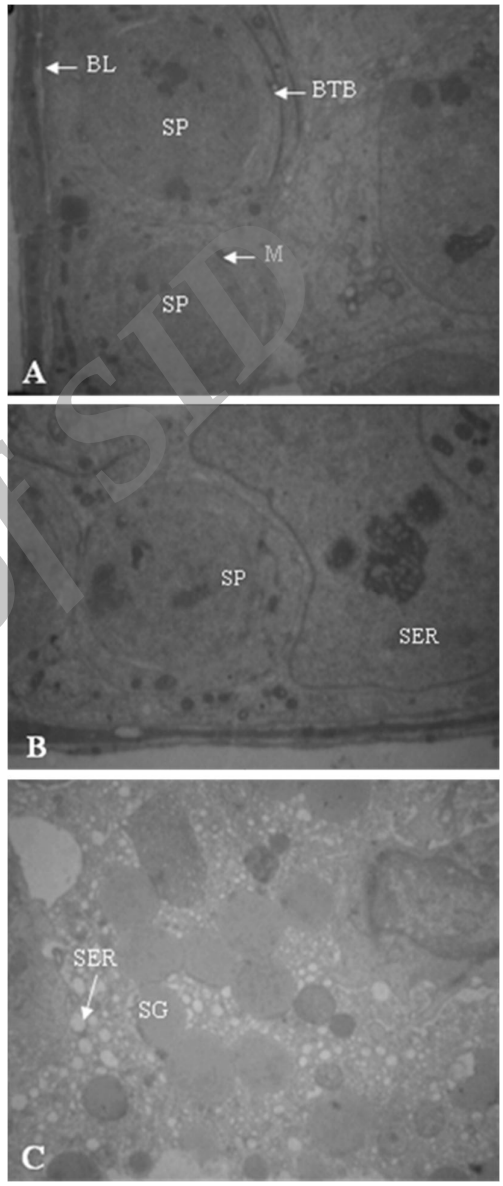

Figure 2. An electronmicrograph from control mice seminiferous tubule: A) Spermatogonial cells (SP) rested on basal lamina (BL), 21560 X; B) Sertoli cell 2784 X; C) Interstitial cell 6000 X. Note: mitochondria (M), Blood - Testes - Barrier (BTB), secretory granules (SG) and smooth endoplasmic reticulum (SER).

It has shown that alkilating agents that are used in chemotherapy affect actively dividing cells such as spermatogonia and block their division.[2,3,24]

In addition, there are some reports showing that anticancer drugs could induce apoptosis in germinal

epithelium.[1-10,25] Our findings such as mitochondrial vacuolization are evidence of apoptosis in germinal epithelium. Other changes, such as separation of spermatogenic cells from each other could also be considered as preapoptotic sign which supports hypothesis that cisplatin as a chemotherapic agent induces apoptosis on spermatogenic cells and cause to reduction of germinal epithelium.

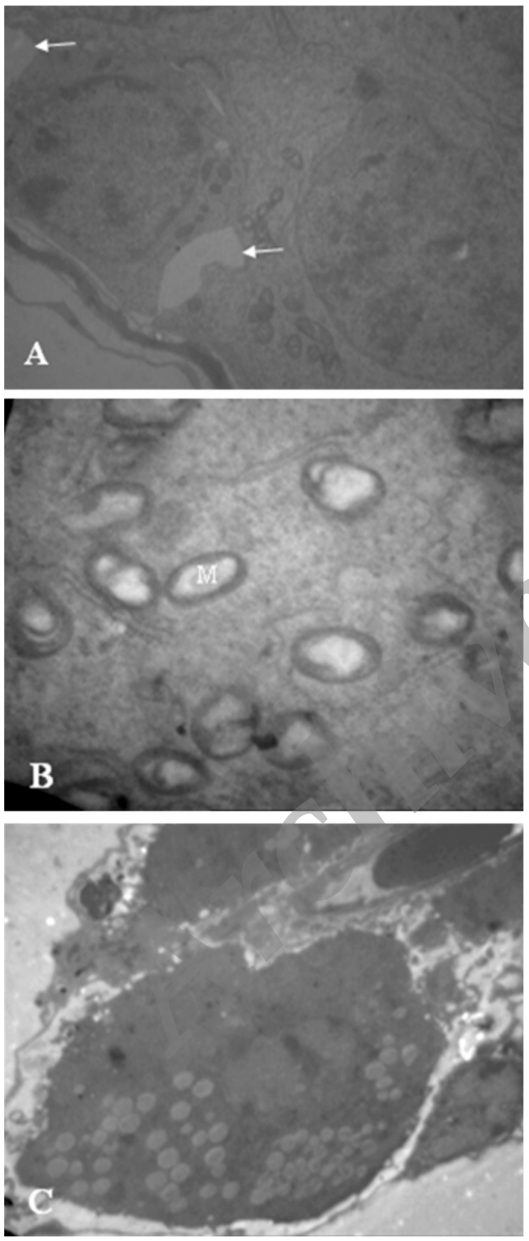

Figure 3. A, B: An electronmicrograph from seminiferous tubule of a mouse from experimental 1 group; A) Spermatogonial cells in the figure are separated from each other by a large space (arrow). 2784 X and B) Note, vacuolated mithochondria from spermatogonial cells (M). 12930 X, C) Electronmicrograph showing an interstitial cell from testis of a mice experimental 1 group. Note numerous secretory granules in the cytoplasm. 1670 X.

In vitro study of Newton *et al* showed that cellular junction mediated by cadherin molecules; result in longevity of germ cells.[26] Cadherins are molecules that control the interaction between sertoli and germ cells and provide vitality of germ cells. It is believed that cadherin- mediated junctions are involved in transmission of intercellular signals and consequently in differentiation controls, migration and vitality of cells.[27,28]

In cisplatin treated mice, separation of cells from each other, probably deprive cells from mitotic factors secreted by adjacent cells and cause reduced mitotic activity in spermatogenic cells.

According to our results, intracellular secretary granules in interstitial cells were increased in experimental 1 group. In this regard it is appears that, interstitial cells are the only cells that have LH receptors and in response to LH secretes estrogen and testosterone. It is shown that levels of FSH and LH increases following chemotherapy.[3,25,29] Therefore it can be concluded that the increased granules in interstitial cells are a response to increased LH following cisplatin treatment.

In experimental 2 group, cetrorelix relatively neutralized the adverse effects of cisplatin which indicates that cetrorelix has a protective effect against induced spermatogenesis toxicity of cisplatin.

In support of our findings, Udagawa has shown that treatment with GnRH analogs improve spermatogenesis recovery following chemotherapy[30] and Meistrich *et al*. have shown that following radiotherapy, the administration of GnRH agonists and antagonists before and after spermatogenesis disorder have a protective effect.[19]

It is known that the secretion of FSH and LH increases after chemotherapy and destruction of germinal epithelium.[30,31] Besides, secretion of testosterone increases after exposure to reprotoxic substance.[32] Increased testosterone result in suppression of membrane bound stem cell factor (SCF) expression which is necessary for spermatogenesis.[30] Thus it appears that reduction of intratesticular testosterone would protect spermatogenesis. This explains the action mechanisim of GnRH antagonists, i.e. using GnRH antagonist before treatment leads to reduction of FSH, LH and testosterone. Consequently, reduction of these hormones suppresses spermatogonial proliferation. Since non-dividing cells are less prone to toxic effects of chemotherapeutic agents, spermatogenic cells would not be affected during their non-proliferating period. In agreement with this postulation, Shetly *et al*. have demonstrated that testosterone-therapy following radiotherapy, will inhibit spermatogenesis improvement.[33] Meistrich *et al* also demonstrated that with reduction of testosterone can improve spermatogenesis after exposure to reprotoxic substestrone.[34]

It is also shown that in rats undergone radiotherapy protocol elevation of FSH level result in inhibition of

spermatogonial differentiation and steradial therapy following radiotherapy could improve spermatogenesis, after radio therapy, by suppressing the inhibitory effect of testosterone.[33,35] Suppression of gonadotropins and testosterone has also been demonstrated to improve the damaged spermatogenesis.[32]

Our results indicate that administration of cisplatin, as an anticancer drug in mice, destroys testicular germinal epithelium and Administration of cetrorelix prior and along with chemotherapy could partially protect germinal epithelium by acting on hypothalamic gonadal axis.

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Conflict of Interests

Authors have no conflict of interests.

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