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Protection Role of GnRH Antagonist on Cisplatin Infertility Side Effects

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ABSTRACT

Bachground: One of the main factors which could have side effects in spermatogenesis is used in chemotherapy to treat cancer patients. It is proved that the agents affect on cells especially with high proliferation such as spermatocytes and kill them through apoptosis induction. Since gonadotropin hormones (FSH, LH) affect spermatogenesis and are suppressed by chemotherapy agents, so administration of GnRH Antagonist to protect the cells against its side effects is in researchers' interest. Present study tried to investigate protective effects of cetrorelix in suppression of side effects of cisplatin as chemotherapy agent on the germinal epithelium. ***Methods:*** In the present study 30 male mice were divided randomly into 3 groups: Control, Experimental 1 received 2.5 mg/kg cisplatin daily intraperitonealy and Expermental 2 received cisplatin and subcutaneously injection of 0.25 mg/kg cetrorelix. Cetrorelix injection initiated 1 week before cisplatin treatment and continued at second and third weeks. After 35 days of last injection, the testes were removed and processed for histological and apoptotic analysis according to routine protocols. ***Results:*** Histological findings of seminiferous tubules showed that in cisplatin receiving group, number of spermatogonia cells, thickness of germinal epithelium, seminiferous tubules diameter and spermatogenesis index rate (SI) were decreased. Also in some of the tubules only sertoli cells were observed. In addition, our study showed that number of apoptotic cells was increased in this group. Cetrorelix had prohibitive potential against side effects of cisplatin via morphology of seminiferous tubules and decline apoptotic cells. ***Conclusion:*** Cisplatin has adverse side effects on germinal epithelium by induction apoptosis and cetrorelix can protect spermatogenesis process from cisplatin adverse side effects.

Introduction

Disturbance in spermatogenesis process and subsequently abnormal sperms are most common reasons in male infertility. Chemotherapy drugs are agents that affect spermatogenesis and result to infertility in men who suffer diseases such as cancer. The drugs are effective in killing cancer cells but unfortunately, don't have potency to determine division in normal and cancer cells thus while using they have effect in addition to cancer cells, on normal ones which have high division rate such as spermatogenesis.[1] Cisplatin as an effective chemotherapy drug used in treatment of various tumor types as testis, ovarian, lung, urinary bladder and lymphoma. This agent isn't allcylate but has allcylation property and cause to biochemical and tissue change in testis such as germinal epithelium.[2-10] Turk et al (2007) showed that cisplatin decreased testicular weight, epididium and seminal vesicle in treated rats.[11] Various mechanisms were proposed on how the agent damage to cells.[12] Apoptosis is programmed cell death which occur in various cells and tissues, while fetal life and also after birth.[13] The various researches showed that depletion of cells following chemotherapy is done through apoptosis induction.[14-18] Koberle B and his colleague (2010) showed that inter strand cross linked is one of most important factors which inducted by cisplatin.[19] Seaman (2003) showed that toxicity of cisplatin is resulted from sertoli cells damage which causes to pathogenesis of germinal cells.[7] Findings new prohibitive strategy against cytotoxic effects of cisplatin on spermatogenesis following chemotherapy in male and female particularly in fertility ages is necessary. Glode and his colleagues first (1981) indicated that while chemotherapy could decrease LH and FSH by disturbance in hypophysis – hypothalamus axis and cause to recovery of spermatogenesis

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following interruption of chemotherapy.[20] There are different ways to suppress hypophysis – hypothalamus axis such as using of GnRH analogues and /or antagonists.[4,10,21-24] Cetrorelix is a GnRH antagonist which has extensive use. It is a decapeptide which causes spermiogenesis inhibition by suppression of LH and FSH secretion through hypophysis and finally protect testicular stem cell against side effects of chemotherapy drugs which damage cells with high proliferation rate such as sprematogonia.[25-28] It is noteworthy that, there are little knowledge about cell protective effects of anticancer properties of antagonists agents against damages occured by cisplatin, hence, present study tried to find mechanism of destructive effects of cisplatin in germinal epithelium of seminiferous tubules and protective effects of cetrorelix as a GnRH antagonist in suppression of proliferation and prohibition of testicular stem cells destruction after cisplatin treatment.

Materials and Methods

Animal treatments

In this study 30 male balb/c mice with 6-8 weeks were used. All the experiments were approved by the ethical committee of the Tabriz University of Medical Sciences. They kept under controlled light and temperature conditions with free access to water and food. They had 12 hour light and 12 hour dark condition. Cisplatin was purchased as fresh solution from Eber, cetrorelix from Serono and Tunnel kit from Roche Company. The mice randomly divided into 3 groups. Control group with no treatment (n=10), cisplatin group received 2.5mg/kg for 5 days cisplatin as IP (n=10). Cisplatin+ Cetrorelix group received cisplatin along with GnRH antagonist (cetrorelix). 0.25mg/kg Cetrorelix injected subcutaneously three times weekly, which started a week before cisplatin injection and proceeded in second and third weeks.

Histological study

After 35 days of last injection the mice were sacrificed by cervical dislocation and right testis was cut in 2 parts, one immersed in bouine fixative for 48 hours for histological study and other part put in formaldehyde solution for immunohistochemical (Tunnel) study. The 5 µ sections were provided after tissue passage, followed by PAS staining. In order to measure of seminiferous tubules diameter and thickness of germinal epithelium, the motic image plus 2.0 software was used according to routine protcols. Also, seminiferous tubules epithelium cells including spermatogonia and sertoli cells was counted and SI (number of seminiferous tubules which have long sperm) was numbered with lens 20.

Detection of Apoptosis

Apoptosis induction of testis tissue followed by cisplatin identified by TUNEL assay with an *in situ* cell death detection kit (Roche Applied Science). Briefly the 3 µ sections deparafinized with xylol and autoclaved with sodium citrate buffer in 121°C for 5 minute. The slides incubated in BSA solution for 15 minutes and washed 3 timed with PBS. Then the slides incubated with tunnel solution for 1.5 hours in 37 °C and finally developed with DAB solution for detection of apoptotic cells. The apoptotic cells seen as brown cells.

Statistical analysis

The all data presentd as mean ± SD. Differences were determined using ANOVA with the Tukey–Kramer multiple comparisons test and the value lower than 0.05 was considered significantly**.**

Results

Histological findings

The results obtained in light microscope indicate thick germinal epithelium and active spermatogenesis in seminiferous tubules in control group. Based on morphological characteristics the various stages of spermatogenesis (12 stages) were identifiable. The germinal layer and setoli cells were recognizable inside somniferous tubules. Histological findings in cisplatin receiving group showed decrease in thickness of germinal epithelium, presence of only sertoli cells in some seminiferous tubules and absence of various steps in spermatogenesis because of germinal epithelium destruction. Also nuclei of spermatogonia in most tubes were hyperchromic than control group. In group received cisplatin along with cetrorelix, seminiferous tubules had thick epithelium and cell layers in various steps were observable which showing active spermatogenesis (Figure 1).

Mean of Seminiferous tubules diameter in control group was 97.79±7.48µm, in cisplatin group 74.91±10.22 µm and in group received cisplatin along with cetrorelix was 97.61±5.33. According to results, the tubules diameter decreased in cisplatin group than control significantly ($p<0.05$) but differences between third group and control was not significant ($p>0.05$) (Diagram 1). Mean of Germinal epithelium thickness in control group was 57.33±4.1 µm, 20.90±3.84 µm in cisplatin group and 50.09±4.08 µm in group received cisplatin along with cetrorelix. As is it indicated the thickness in cisplatin group decreased significantly in compare to control group ($p<0.01$) but in it is not significant in third group ($p>0.05$) (Diagram 1).

Germinal epithelium cells were counted and its results presented as average in Diagram 1. It is noted that number of spermatogonia in cisplatin group (21.42±2.44) declined significantly than control group (43.22±1.55) ($p<0.05$) but in third group (40.53±2.38) the number of spermatogonia cells was similar to control group ($p>0.05$). Interestingly, germinal epithelium supporting cells (sertoli cells) in cisplatin (8.61±0.80) group increased significantly in compare to

control (5.50± 0.52) (P<0.05) but decreased significantly in group received cisplatin along with cetrorelix (1, 5.09±0.52) (P<0.05). Presence of seminiferous tubules containing sperms stages 15 and 16 (SI mean) was 24.43±9.22 in control group, 1.52±1.39 in cisplatin group and 22.00±4.43 in group received cisplatin along with cetrorelix. SI mean in cisplatin group was lower than control significantly (P<0.001) (Diagram 2).

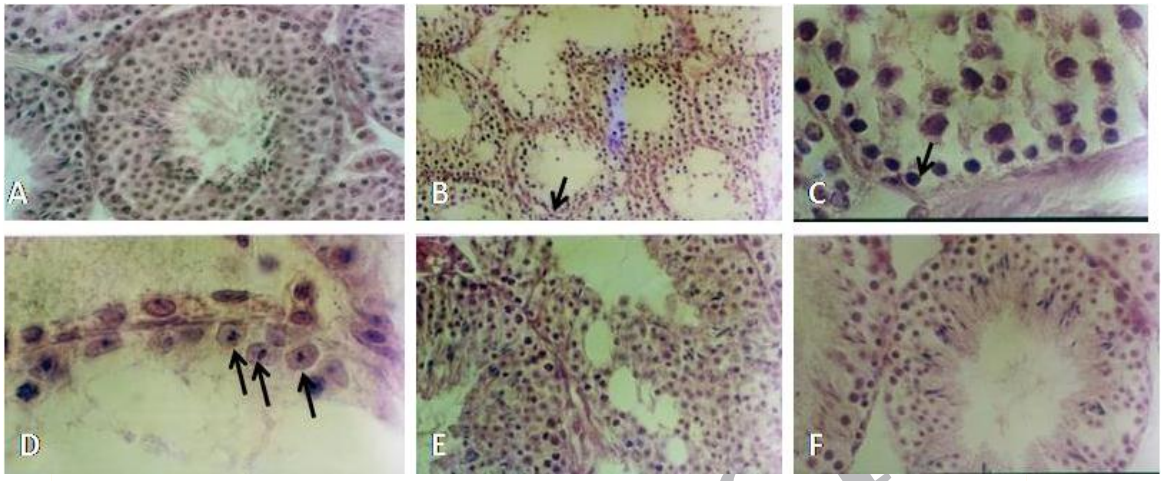

Figure 1. (A) seminiferous tubules in control group with active spermatogenesis inside tubes. PAS staining, magnification 330. (B and C): seminiferous tubules in group received cisplatin. Decrease germinal epithelium and hyper chroming nuclei (arrow). PAS staining, magnification 330 and 660 respectively. (D) seminiferous tubules which received cisplatin, sertoli cells increased inside tubules (arrows).PAS staining, magnification 660. (E-F) seminiferous tubules received ctrorelix along with cisplatin. Notice to normal germinal epithelium thickness and active spermatogenesis. PAS staining, magnification 330.

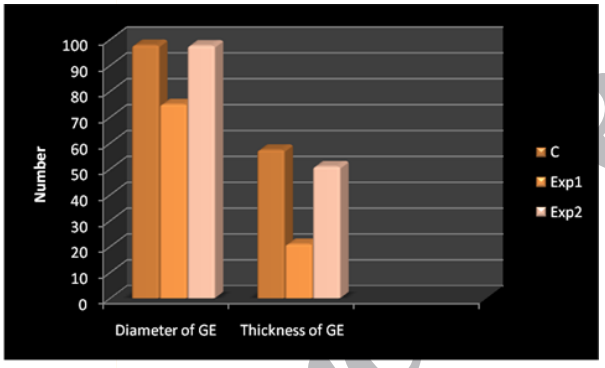

Diagram 1. Shows germinal epithelium thickness and diameter of seminiferous tubules in control (c), Cisplatin (Exp1) and cislatin and cetrorelix (Exp 2) groups.

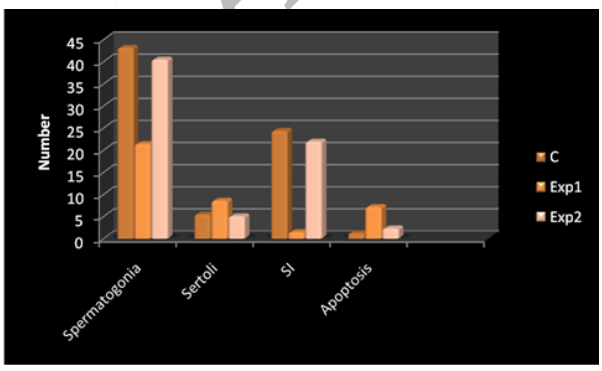

Diagram 2. Indicates number of spermatogonia, sertoli and apoptotic cells in control (c), Cisplatin (Exp1) and cislatin and cetrorelix (Exp 2) groups. SI shows spermatogenesis index in different groups.

Apoptosis in seminiferous tubules

The apoptotic cells in cisplatin group were remarkable high in compare to other two groups (p<0.05). The apoptotic cell number in control group was 1.27±0.20, 7.21±0.90 in cisplatin group, 2.37±0.32 in group received cisplatin along with cetrorelix (Figure 2 and Diagram 2).

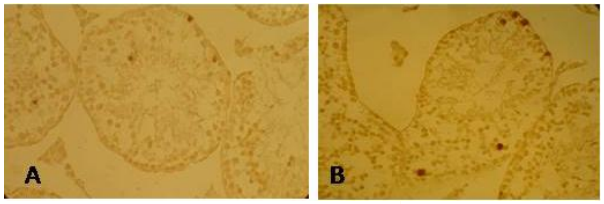

Figure 2. Apoptosis in control (A) and group received cisplatin (B). Brown cell as apoptotic cells. Tunnel staining, magnification 330.

Discussion

Present study reviewed side effects of cisplatin as anticancer agent on testis germinal epithelium and also investigated protective effects of cetrorelix as GnRH antagonist. Our study showed that cisplatin has side effects in germinal epithelium in particular through apoptosis induction and decline thickness of germinal epithelium and also it showed that cetrorelix has potency to prohibit side effects of cisplatin. The present study corresponded with findings of other researches which showed side effects of agents using in chemotherapy on spermatogenesis.[1-10,29-36]

Apoptotic cells increased in cisplatin receiving group and it corresponded with the findings of other researchers who indicated increased apoptotic cells number following using of chemotherapy agents.[5,16-17,37] As Print (2000) and Fraccavilla (2002) reported, apoptosis was found in normal spermatogenesis finitely.[38,39] The role of apoptosis in normal spermatogenesis is remove of defective spermatogonia and prohibition of defective sperms production and transmission of genetic defects to infant[39,40] or creating balance in sertoli and germinal cells number. There are researches on side effects of cisplatin on spermatogenesis through apoptosis induction which approve our findings. Giri and his colleagues (1998) showed that prescription of cisplatin strengthens ROS production inside cell and over production of ROS fortifies DNA fragmentation and has destructive effects on mitochondria and cytoplamic temperance of sperm. In addition, because of high concentration of saturate fatty acids and weak antioxidant system sperms are in exposure to oxidative damage.[41,42] Bieber AM and his colleagues (2006) showed that in treated mice with cisplatin and Etoposid, apoptosis increased at least 3 times in germinal cells.[43] It is in congruent with our findings about apoptosis induction on germinal epithelium and subsequently decreased thickness of germinal epithelium and SI.

Our other finding was decline of seminifrous tubules diameter in cisplatin receiving group. It is suggested that there are other factors rather than apoptosis resulted to tubules damaging following chemotherapy. It has been determined that following chemotherapy, LH, FSH and testosterone levels increased[43-46] and increased testosterone in testis tissue affects spermatogenesis.[47] Testosterone overproduction cause to change of membrane bound stem cell factor (SCF) expression which is necessary for spermatogenesis. Thining of seminifrous tubules diameter could be related to contraction of myoid cells. It has been revealed that myoid cells containing estrogen and androgen receptors during fetal life and after birth.[48-50]These cells also contain actin, myosin, Desmin and actinin filaments. On the other hand these cells are similar to smooth muscle[51] and with regard to being androgen receptor in myoid cells, the testosterone caused to their contraction. In other suggestion, decreased diameter would be related to drain spermatogenic cells from seminifreous tubules and finally shrinkage of them.[52]

Sertoli cells number following cisplatin incresed in our study that is in conflicting with Aich and his colleagues (2001) and Veccinop and his colleagues (2001) who reported that sertoli cells number didn't change following chemotherapy.[53,54] Also Boujard and his colleagues (1995) and janes and his colleagues (1985) reported that number of sertoli cells decreased following chemotherapy.[55,56] As researchers reported, after radiotherapy and in cryptorchidism sertoli cells proliferate.[57] Sertoli cells proliferation in present study could be explain with two hypotheses. While in cisplatin receiving group the seminifreous tubules diameter decreased significantly, thus because of spermatogenic cells digestion the sertoli cells is observed compact and distinct which seems their number has been increased. Second argumentation is that apoptosis maintains balance between sertoli and spermatogonic cells number under normal conditions.[58] Because, treatment with cisplatin causes to increased apoptosis and in result, depletion of spermatogenic cells thus, the number of sertoli cells increase (remedial proliferation). In the group received cisplatin along with cetrorelix as GnRH antagonist the probe of spermatogenic epithelium showed that cetrorelix partly suppresses side effects of cisplatin. There are conflicting reports about agonist or antagonists GnRH on recovery of damaged spermatogenesis and also reports refer failure in treatment of spermatogenesis after the damage.[59] It is suggested that the differences back to different responces of various species animal to similar treatment.[60]

In our study seminifreous tubules in group received cisplatin along with cetrorelix comparatively had close morphology and morphometric characterization to normal ones, while, some vacuoles observed inside their epithelium. In concruent with those findings, Udagawa (2001) showed that treatment with GnRH analogue following chemotherapy causes to recovery of spermatogenesis[44] and Meistrich and his colleagues (2001) showed protection of spermatogenesis with agonists and antagonists GnRH mechanism following radiotherapy.[22] Simply, chemotherapy agents such as cisplatim not only affect directly on dividing cells (particularly spermatogonia) and apoptosis induction but also impose their effect partly through change in hormones. As we know following chemotherapy and destruction of spermatogenesis increase in level of LH, FSH and finally testosterone hormones has negative effect on the process.[44,47,61] Thus, through decreasing testosterone and FSH could protect spermatogenesis against side effects of chemotherapy agents.[15,44] Shetty and his colleagues (2002) showed that testosterone suppresses recovery of spermatogenesis following irradiation[62] and also Meistrich and his colleagues (2003) showed that after using agents which decrease testosterone level causes to recovery of spermatogenesis.[62]

In addition to testosterone in irradiated rats, increasing FSH also causes to suppression of spermatogonia differentiation[62] and another study revealed that suppression of gonadotropines and testosterone cause to recovery of damaged spermatogenesis.[47]

Conclusion

cisplatin has adverse side effects on germinal epithelium by induction apoptosis and cetrorelix can protect spermatogenesis process from cisplatin adverse side effects.

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