Effects of testosterone treatment on recovery of rat spermatogenesis after irradiation

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Abstract

Objective: To determine the effects of two different radiation doses on sperm parameters and the role of testosterone treatment on rat spermatogenesis.

Methods: The experimental animal study was conducted at Marmara University, Istanbul, Turkey, from September 2012 to January 2013. Male Sprague Dawley 4-6 months old rats weighing 300-350g were randomly divided into 5 equal groups as control, low dose irradiation, testosterone administration following low dose irradiation, high dose irradiation, and testosterone administration following high dose irradiation. The animals were kept at a constant temperature in a room with 12h light and dark cycles. After the group-wise intervention, sperm concentration, testicular size, and histopathological examination of seminiferous tubules were noted. SPSS 10 was used for statistical analysis.

Results: The 40 rats in the study were divided in 5 groups of 8(20%) each. In low dose radiation, adverse effects were only temporarily observed with the return of almost normal testicular function at the end of two months with or without testosterone supplementation. In contrast, in high dose radiation, hormonal treatment effect was controversial.

Conclusion: Testosterone treatment had no significant effect upon recovery after irradiation. In order to prevent the untoward effects of radiation, shielding of the remaining testis in a proper manner is crucial to avoid the harmful effects of the scattered radiation.

Keywords: Testosterone, Radiotherapy, Rats, Spermatogenesis. (JPMA 65: 300; 2015)

Introduction

Testicular cancers are among the most common malignant tumours in males between the ages of 15 and 34[1] and approximately half of them are seminomas. Testicular seminoma is one of the most curable cancers with over 90% five-year survival rate in numerous reporting series.[2] External beam radiation therapy (EBRT) is rarely used in the management of non-seminomatous testicular germ cell tumours whereas it is used as an adjuvant treatment in favourable early stage seminoma. It is used in eradication of subclinical metastases to retroperitoneal and pelvic lymphatic regions with the conventional doses of 25-30 Gy, and achieves a relapse-free survival rate of 98% to 100%.[2,3] However, a major drawback is the "scattered irradiation". Despite the use of gonadal shielding, patients irradiated from infradiaphragmatic portals still receive almost 0.5 Gy scattered irradiation on the remaining testicle while the non-shielded doses were calculated between 1.7 to 5 Gy.[3]

Moreover, the impairment of spermatogenesis was seen in these patients and both the probability of impairment and time to recovery are dose-dependent.[4] Thus fertility and genetic implications have assumed the major importance in terms of life quality which may not be as promising as disease control.[5] Therefore, the long survival expectations of the early-stage disease sets the course towards maximising the benefit and minimising the morbidity of radiation therapy.[3] There are several studies designed to reduce the detrimental alterations in testis and they assessed the combinations of radiation and hormonal treatment modalities like gonadotropin-releasing hormone (GnRH) analogues, testosterone and estradiol.[6-8]

The current study was planned to demonstrate the effects of two different radiation doses on sperm parameters and corresponding histopathological changes as well as to define the protective role of testosterone treatment.

Material and Methods

The experimental animal study was conducted at Marmara University, Istanbul, Turkey, from September 2012 to January 2013, after obtaining approval from the Animal Ethics Committee of Marmara University, Turkey. Male Sprague Dawley 4-6 month old rats weighing 300-

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350g were kept at a mean constant temperature of 22±1ºC in a room with 12 hour light and dark cycles.

The rats were randomly divided into 5 equal groups as controls, low dose irradiation alone (IR0.5), testosterone administration following low dose irradiation (IR 0.5+T), high dose irradiation alone (IR5), and testosterone administration following high dose irradiation (IR 5+T).

The animals were anaesthetised with 100mg/kg ketamine and 3mg/kg chlorpromazine. Each animal was irradiated with linear accelerator (Saturne 42, GE) producing 6MV photons at a focus to skin distance 100cm to 4x4cm field. Each irradiation field covered both testes and each testis received the given dose equally. In addition, a bolus material was used to cover the testis in order to obtain proper dose distribution. Radiation dose was 0.5Gy for IR0.5 and IR0.5+T groups, whereas IR5 and IR5+T groups were irradiated with the dose of 5Gy.

Intramuscular (IM) testosterone, which was a mixture of testosterone propionate 30 mg, testosterone phenylpropionate 60 mg, testosterone isocaproate 60mg, and testosterone decanoate 100mg (Organon, Turkey) was administered immediately after radiotherapy to IR0.5+T and IR5+T groups and repeated every 3 weeks for 2 months.

At the end of 2-months treatment, each rat was anaesthetised with 100mg/kg ketamin and 3mg/kg chlorpromazine. After exposure via midline vertical incision of scrotum, dimensions of each testis (length, width, depth) were measured and a testicular biopsy was performed. Volume of each testis was calculated by using the empiric formula of Lambert: length x width x depth x 0.71.[9,10] Immediately after obtaining the biopsy, samples were placed in tissue culture tubes containing modified human tubule fluid (modified HTF, Irvine Scientific®, USA). Each biopsy specimen was kept at 37ºC and spermatogenesis was evaluated within one hour. Also, a piece of the biopsy specimen was placed in a tube which contained Bouin's solution to fix the specimen for pathological investigation

Each of the biopsy specimens was placed in modified human tubal fluid (mHTF) (Irvine Scientific®, USA). The biopsy specimens were disintegrated in a petri box with the tip of a needle subsequently vortexed for 30 seconds, and were centrifuged at 2000rpm for 20 minutes. A drop of this homogenous mixture was placed on Makler Chamber. Sperm concentration was calculated by the count of sperm cells in a total of 100 squares.

Testicular specimens were fixed in Bouin's solution,

embedded in paraffin and cut into 4µm thick sections, which were stained with iron-haematoxylin, and Masson-trichrome stain. Histopathological evaluations were performed under the light microscope (Olympus BX 50) and for each animal, 6 different areas were examined under X100 objective. Seminiferous tubules were labeled as type A, B, C according to the spermatogenetic activity variations between tubules; A: Tubule consisting of sertoli cells and spermatogonia, no spermatocytes or spermatids; B: Tubule consisting of sertoli cells, spermatogonia and spermatocytes, no spermatids; C: Tubule consisting of sertoli cells, spermatogonia, spermatocytes and spermatids.

The number of seminiferous tubules labelled as A, B, C in each microscopic area were counted. After examining 6 different areas, total number of labelled seminiferous tubules was calculated and proportion of the seminiferous tubules that consisted of sertoli cells with only spermatogonia; spermatogonia + spermatocyte; spermatogonia + spermatocyte + spermatid was obtained.

SPSS 10 was used for statistical analysis. One-way Analysis of Variance (ANOVA) was also performed. For multiple comparisons to determine the significance of the difference between the treated groups and the control group, Tukey post-hoc test was used. P less than 0.05 was taken as statistical significance.

Results

The 40 rats in the study were divided into 5 groups of 8(20%) each and pathological findings and sperm parameters were analysed separately for each group.
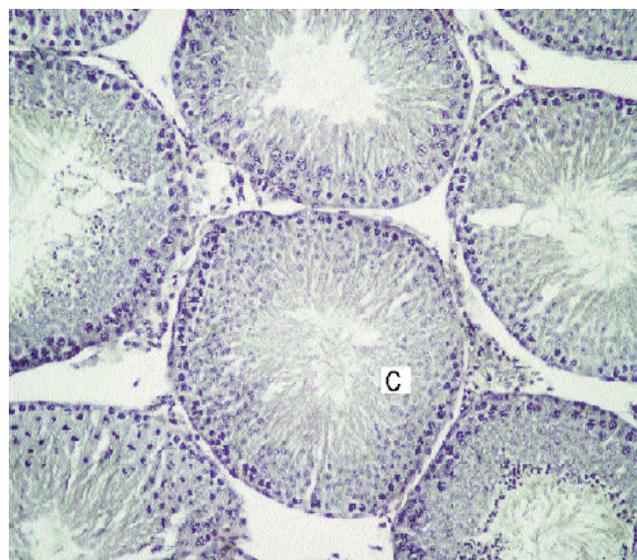

Figure-1: Microscopic appearance of control group testis; composed of mixture of seminiferous tubules labeled as A, B, C, (iron-haematoxylin, x 200).

Table: Concentrations of sperm cells, volumes of testicles and histology of seminiferous tubules in each group is given with their means and standard deviations.

Groups	n	Sperm concentration (1.000.000/cc)	Volume (cc)	Type A Spermatogenetic activity %	Type B spermatogenetic activity %	Type C spermatogenetic activity %
I Control	8	19.37 ± 1.79	2.17 ± 0.19	23.12±5.34	27.5±3.6	49.37±7.5
II (IR0.5)	8	12.62 ± 1.7	1.52 ± 0.08[a]	1.25± 0.81[d,e]	19.8±6.8	78,88±18,27[a,d,e]
III (IR0.5bT)	8	29.00 ±6.04[b,d,e]	1.42 ± 0.04[a,d,e]	8.6± 8.6[e]	11.1±2.7	80,15±29,69[a]
IV (IR5)	8	0.92 ± 0.01[a,c]	0.46 ± 0.04[a,b,c]	61.3± 8.1[a,b,c]	36.48±8.7	0,73±0,44[a,b,c]
V (IR5bT)	8	3.18 ± 1.76[a,c]	0.28 ± 0.03[a,b,c]	51.88±11.8 [b,c]	39.53±8.3[c]	8,5±18,0[a,b,c]

Note: Values are means ± SD.

[a] p<0.05, compared with control group

[b] p<0.05, compared with second group

[c] p<0.05, compared with third group

[d] p<0.05, compared with fourth group

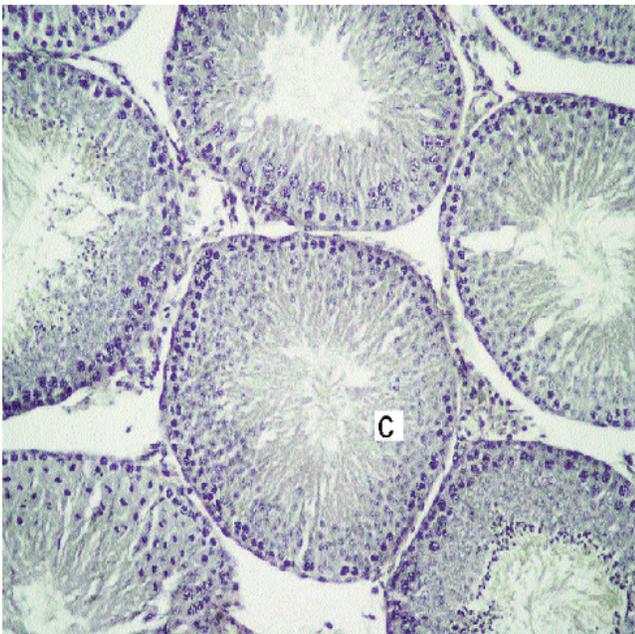[e] p<0.05, compared with fifth group.

Figure-2: Microscopic appearance of 0.5Gy radiotherapy received group testis; composed of seminiferous tubules mostly labeled as C (iron-haematoxylin, x 200).

The mean sperm concentrations of the groups were 19.375±1.79 x106/ml (range: 17-21x106/ml) in the control group, 12.625±1.7 x106/ml (range: 11-15x106/ml) in IR0.5, 29±6.04 x106/ml (range: 26-44x106/ml) in IR0.5+T, 0.925±0.01 x106/ml (range: 0.8-1.2x106/ml) in IR5, and 3.188±1.76 x106/ml (range: 3-7x106/ml) in IR5+T.

Type A spermatogenetic activity, consisting of sertoli cells and spermatogonia, no spermatocytes or spermatids, was 23.12%, 1.25%, 8.6%, 61.3%, and 51.88%, respectively. Tubules consisting of type B spermatogenetic activity in the groups were also noted (Table). Type C
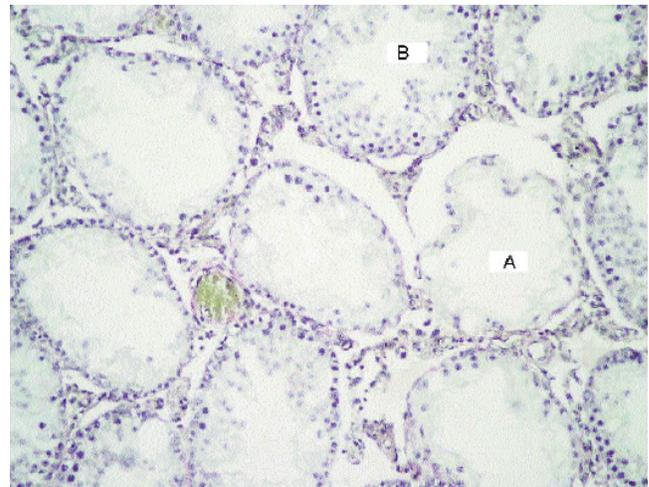

Figure-3: Microscopic appearance of 5Gy radiotherapy received group testis composed of seminiferous tubules mostly labelled as A, a few tubules labelled as B also seen (iron-haematoxylin, x 200).

spermatogenetic activity, consisting of sertoli cells, spermatogonia, spermatocytes and spermatids, was 49.37%, 78.88%, 80.15%, 0.73%, 8.5%, respectively.

The mean volume of testes for each group was 2.17±0.19 cc (range: 2-3cc), 1.52±0.08cc (range: 1-2cc), 1.42±0.04cc (range: 1-2 cc), 0.46±0.04cc (range: 0.2-05cc), and 0.28±0.03cc (range: 0.1-0.5cc), respectively.

The mean sperm concentration of IR0.5+T was statistically higher than the other groups, except for the control group (p<0.05). The mean sperm concentration of IR0.5 was lower than the control group, but the difference was not statistically significant (p>0.05). Also, there was no significant difference between high IR5 and IR5+T groups.

The mean testes volume of the control group was

statistically greater than the other groups (p<0.05). Testosterone administration resulted in a decrease in testicular volumes both for IR0.5 and IR5 groups.

Immature spermatogonia percentages in the peripheral zones of seminiferous tubules of low dose groups were lower than the control (p>0.05) and significantly lower than the high dose groups (p<0.05). Testosterone administration resulted in an increase in the number of mature spermatogonia for both high and low dose groups but this was not statistically significant from IR0.5 (p>0.05).

The analysis of spermatid, spermatocyte and spermatogonia in the central zones of seminiferous tubules revealed that mature cell percentages in low dose groups were higher than both the high doses and the control groups (p <0.05) (Figures 1-3).

Discussion

Many features of spermatogenesis are very similar in man and animals used in the laboratory, mainly mice and rats.[11] The length of the spermatogenic process in the rats is about 12.9 days.[12]

Radiation is one of the cytotoxic factors that kills testicular germ cells and results in sterility. The effects of irradiation on spermatogenesis in healthy male population are well known.[13] Irreversible azospermia can be achieved when the testicular dose exceeds 6-8Gy.[13] The stem spermatogonia are relatively radioresistant and they immediately repopulate the seminiferous epithelium in mice.[14] Of all spermatogenetic cells, the differentiating spermatogonia are the most sensitive to radiation and chemotherapy.[15,16] After the cessation of the chemotherapeutic treatment, provided that there is minimal killing of stem spermatogonia, sperm production recovers to normal levels within 6-8 weeks and in 12 weeks in rats and humans, respectively.[17,18] The duration was planned as 2 months in our study due to the fact that the spermatic cycle of the rats is only 12.9 days and, as mentioned above, recovery time varies between 6-8 weeks. The dose of the testosterone therapy might be increased, but this may be investigated in further studies.

Our purpose was to evaluate the alterations of sperm parameters and histopathological findings in rats with or without hormonal treatment following testicular irradiation. We used two different radiation doses. The sperm concentration was lower than the control in the group of 0.5Gy but, this decrease was not statistically significant, whereas testosterone administration made a significant improvement in sperm concentration in 0.5Gy irradiation (IR) group (p=0.04). We interpreted this

increase in sperm concentration as the bounce effect of testosterone treatment. However, the use of testosterone treatment was not conclusive. Sperm concentration in the group that received 5Gy was found to be significantly lower than the control group and the group that received both 0.5Gy radiation and testosterone (p=0.05, p=0.0001). Irradiation with 5Gy almost completely eliminated the sperm cells (0.92x106/cc). It can be postulated that applying radiation doses higher than 5Gy may result in an irreversible damage.

Our results were consistent with a study.[13] Testosterone treatment increased the sperm concentration by three times in the group that received 5Gy irradiation, but this result was not statistically significant. Nevertheless, testosterone treatment resulted in some improvement. It can be concluded that the higher the radiotherapy dose, the lower is the efficiency of testosterone treatment. Therefore, testosterone therapy may have additional benefits only in patients exposed to low dose scattered radiation. The statistical significance of 3-time increase might be shown in further studies which can include more rats.

According to our results, the analysis of each group's testis volume revealed that it decreased significantly in the low and high dose radiation groups compared to the controls, and the application of testosterone did not improve the volume during our study period.

High dose radiation therapy caused maturation arrest. But this maturation arrest was not observed in low dose radiation group. A possible explanation can be the "repopulation phenomenon". At low doses of irradiation, sertoli and Leydig cells do not show any histological changes because they are fairly radioresistant.[19] At dosages above 10Gy, no morphological changes are seen in these cells, but they start to shrink.[19] The differentiating spermatogonial cells are radiosensitive while spermatocytes, spermatids and spermatozoa are relatively radioresistant.[20] Non-differentiating spermatogonial cells, also referred to as stem cells, are the most radioresistant of all spermatogenic cells.[21,23] It is the division and differentiation of these surviving cells that is responsible for the repopulation of the seminiferous epithelium following exposure to radiation.[23] In our study, highest percentage of type C spermatogenic activity was observed in low dose radiation groups independent of testosterone effect and this was similar to a study.[22,23] It found that at doses below 3Gy, the surviving stem cells can repopulate the seminiferous tubules without hormone or drug intervention, although the period of recovery is dependent on the radiation dose.[22,23] In our model, lowest percentage of type C spermatogenic activity with the highest percentage of type A

spermatogenic activity was observed in high dose of radiation groups (Figure-3). These results can be explained with the help of a couple of studies.[23,24] One found that dose levels between 3.3Gy and 4Gy of irradiation and above destroys all the classes of differentiating spermatogenic cells.[23] The other reported that following exposure of the testes to 3.5Gy, repopulation of spermatogenic cells was not observed for 60 weeks post irradiation.[24]

High dose radiation resulted in an increase in immature sperm cell number compared to low dose radiation. Also, positive effect of testosterone on maturation is prominent in high dose radiation when it is compared to low dose. Mature sperm percentage was almost 10 times greater with the administration of testosterone. However, the effect of high dose radiation on sperm concentration was so striking that the overall improvement of sperm maturation was negligible. Testosterone administration can help to improve the maturation process to some extent, but the final outcome depends on radiation damage.

A study demonstrated in 1983 that spermatogenic cell was the radiosensitive tissue in testis and constituted 70% of the normal testicular weight.[25] Therefore, it seems logical that germ cell loss would lead to a decrease in testicular mass, and it explains the volume loss with radiation in our model.[25] Testosterone had no statistically significant effect on testicular volume within the two-month study period. This might be explained by hypogenesis.

Further, 5Gy IR impairs all kinds of sperm cells, but since mature forms are more radiosensitive, thus they are affected more. This results in a relative increase in the immature sperm concentration. But testosterone treatment did not improve the differentiation of immature sperm cells from spermatogonia to spermatids. This finding was similar to a study which investigated the effects of chemotherapy and radiotherapy on spermatogenesis. It found that surviving stem cells may remain as spermatogonia without initiating differentiation.[23] Another study found that in rats who previously received cytotoxic therapy, the administration of a GnRH agonist restored the ability of spermatogonia to differentiate. As a result, normal spermatogenesis returns.[23] Result of this study concerning high dose radiation group supports the previous findings of the above-mentioned study where radiotherapy blocked further differentiation of spermatogonia to more mature forms.

Additional testosterone treatment seems to restore the maturation of spermatogonia in both low dose and high dose radiation groups. However, this was only marginal in high dose radiation exposure, resulting in much lower levels of mature spermatogonia compared to low dose radiation exposure. The reason why spermatogenesis fails to recover after radiation and the mechanisms by which testosterone treatment partially restores the spermatogenesis is not completely understood.

The small sample size was a limitation of the study, but only 8 rats per group were allowed by the ethics committee. Further studies are needed with a larger sample size.

Conclusion

Spermatogenesis can be compromised at dose levels between 0.5Gy to 5Gy. Over 5Gy radiation dose leads to significant and possibly permanent injury. In low dose radiation (mimicking shielding of testis during radiation therapy), adverse effects were only temporarily observed with the return of almost normal testicular function at the end of two months with or without testosterone supplementation. In contrast, in high dose radiation, hormonal treatment effect was controversial. Thus, in order to prevent the untoward effects of radiation, shielding of the remaining testis in a proper manner which is crucial for avoiding harmful effects of the scattered radiation.

Acknowledgements

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