Volume 17, No 2 | International Journal of Radiation Research, April 2019

Sperm DNA damage in mice irradiated with various doses of X-rays alone or in combination with actinomycin D or bleomycin sulfate: an *in vivo* study

H. Saghaei[1], H. Mozdarani[2,*], A. Mahmoudzadeh[3]

[1]*Department of Radiology, School of Paramedical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[2]*Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
[3]*Department of Bioscience and Biotechnology, Malek-Ashtar University of Technology, Tehran, Iran*

► ***Original article***

Corresponding authors:
Prof. Hossein Mozdarani,
Fax: *+ 98 21 8800 6544*
E-mail:
mozdarah@modares.ac.ir

Revised: April 2018
Accepted: July 2018

Int. J. Radiat. Res., April 2019; 17(2): 317-323

DOI: 10.18869/acadpub.ijrr.17.2.317

ABSTRACT

Background*:** DNA damage in male germ cells due to exposure to environmental and manmade physico-chemical genotoxic agents is considered as the main cause of male infertility. The aim of this study was to evaluate the effects of combined modalities (radiotherapy and chemotherapy) routinely used for cancer treatment on mouse sperm chromatin *in vivo*. ***Materials and Methods*:** Forty-eight mice were divided into 12 groups: 3 irradiation (1, 2, and 4 Gy), 2 drug [Actinomycin-D (ACTD) and Bleomycin (BLM)], 3 ACTD/irradiation, 3 BLM/irradiation, and a control. Mice received intratesticular injection of 7µg/25 g of Actinomycin-D and Bleomycin before irradiation with X-rays. Forty-eight hours after irradiation, mice were sacrificed and epididymis and testes were removed. Sperm DNA damage was assessed with the use of alkaline comet assay. Moreover, morphology, and motility of sperms were investigated microscopically. ***Results: Result showed that drug alone had slight but not significant effect on sperm DNA damage, but significantly increased when combined with irradiation. There was a significant difference between the experimental and the control group in DNA sperm damage, but no significant differences were observed in sperm morphology ($p>0.05$). In the drug+4Gy group, DNA damage increased dramatically compared to the controls ($p<0.01$), morphology changes increased to about six times that of controls. ***Conclusion:*** Results indicate that X-ray induced DNA damage and morphological changes in sperms in a dose dependent manner. Low doses of drugs led to potentiation of radiation effect. This might be indicative of necessity for radiation protection of testes when are in field of radiotherapy.

Keywords: *Ionizing radiation, chemotherapeutics, mouse, sperm DNA damage, comet assay.*

INTRODUCTION

Despite substantial advancements in the field of medicine, treatment of cancer is still in its infancy. Testicular and prostate cancer are the most common cancer in men. Although radiation therapy and chemotherapy have been effective in curing testicular cancer, their side effects (either temporary or permanent) have been the subject of many studies [1]. Preservation of fertility during and after treatment is important for most men [2–4]. However, antineoplastic agents, radiation, and surgical therapies as well as cancer itself can threaten men's fertility potential. Male infertility caused by cancer treatments may be temporary or permanent and can be mild or severe. The crucial importance of germ cell DNA integrity in the maintenance and

establishment of a viable pregnancy has been widely recognized [5]. It is well known that sperm chromatin is structurally and functionally different from that of somatic cells: it is not organized in nucleosomes [6,7]. DNA in sperm is 6 -fold more compacted and has 40-fold less volume than somatic cell DNA and has lost most, if not all, the repair capability [8]. Despite the compact packing and anti-oxidant defense provided by seminal fluid, DNA damage does occur in both developing and mature sperm, high levels of which has been reported in infertile men [9-12]. In the adult mouse testis, the mitotically active spermatogonia are the most radiosensitive, whereas spermatocytes, which undergo meiotic cell divisions, and spermatids, which develop into spermatozoa, are more resistant to ionizing radiation [8,13]. One of the major factors resulting in defective sperm function is oxidative stress (OS). OS occurs when production of reactive oxygen species (ROS) by leukocytes or spermatozoa is excessive, and/or the antioxidant capacity of semen decreases [14].

The variety of experiments has shown that irradiation is one of the exogenous sources of ROS production and DNA damage in sperm and causes temporary and permanent infertility [14,15]. Irradiation induces sperm aneuploidy, structural chromosome aberrations, chromatin structure anomalies, DNA breaks, and higher frequency of mutations. High levels of induced DNA damage in human sperm by in vitro or in vivo (radiotherapy in cancer patient) irradiation is also reported [16,17]. The varieties of studies have shown the impact of sperm DNA damage on fertilization rate and outcomes after using assisted reproduction techniques (ART).

Bleomycin-Etoposide-Cisplatin (BEP) is used in chemotherapy of testicular cancer. In this study, Bleomycin was used along with Actinomycin-D, which is also used in the treatment of a variety of cancers. Bleomycin and Actinomycin-D are antibiotics that are shown to have anti-cancer activity by inducing DNA strand breaks and inhibiting DNA repair [18]. Reports have shown the presence of an abnormally high percentage of DNA damaged sperm in samples from men after BEP chemotherapy [16].

The aim of this study was to evaluate potentiation effects of two commonly used chemotherapeutics, bleomycin sulfate and actinomycin D on radiation induced DNA damage in sperms in vivo.

MATERIALS AND METHODS

Animals

Male Balb/c mice aged 9 weeks and weighting about 26 ± 3 g (n= 48) was purchased from Pasteur Institute (Tehran, Iran). All animals were kept under controlled environmental conditions at room temperature (22 ± 2°C) with 50 ± 10% humidity and an automatically controlled cycle of 12 h light and 12 h dark in Novin Radiotherapy Institute. Standard laboratory animal feed (purchased from commercial supplier) and water were provided ad libitum. Animals were acclimatized to the experimental conditions for a period of one week prior to the commencement of the experiment. The ethical committee of Shahid Beheshti University, Tehran, Iran, and Institutional Animal Care approved the study.

Irradiation

Mice were irradiated whole body with various doses (1, 2 and 4 Gy) of X-rays. Mice were placed in an acrylic holding rack for exposure to X-ray in a linear accelerator (Electa Compact) at a dose rate of 1.6 Gy per minute, with source sample distance (SSD)= 98 cm, field size (20×20) cm and at room temperature (23 ± 2 ºC).

Chemotherapy drugs

Actinomycin-D (ACTD) and Bleomycin (BLM) (Nani Co; India)were used. Drugs were dissolved in 1 ml normal saline and were injected with single doses of 7 µg/25 g body weight intra-testicular with Hamilton micro-syringe. Two hours before irradiation, mice received same dose of drugs. Twenty-four hours after irradiation, mice were sacrificed and testes were removed and homogenized in 1.5 ml normal saline. One testis of each mouse for comet assay and others for sperm morphology was used.

Sperm collection

Both cauda vas deferens were dissected free. Sperm were retrieved from the isolated vas deferens into 1 ml of PBS (1 mmol/L KH_2PO_4, 10 mmol/L $Na2HPO_4$, 137 mmol/L NaCl, 2.7 mmol/ L KCl) pH 7.4 using a watchmakers forceps. All chemicals used from were Merck, Germany.

Morphology evaluation

To assess sperm morphology, sperm tails defects (double, multiple, short or long) were considered as H factor and sperms with incomplete or without heads or heads without tails affecting sperm motility were considered as M factor that were counted under a light microscope (×400, Karl Zeiss, Germany) and were compared within treatment groups.

Sperm comet assay

The sperm comet assay was performed as described by Haines *et al*. with some modification [19, 20]. Sperm sample (7 µl) containing 1-4 × 104 sperms per ml was suspended in 100 µl of 0.5% (w/v) low melting point agarose (LMP, Sigma-Aldrich). From this suspension, 80 µl was applied to the surface of a microscope slide (pre-coated with 1% normal melting point agarose (NMP, Sigma- Aldrich)) to form a microgel and allowed to set at 7°C for 3 min. After added second layer of 1% LMP, allowed to set for 10 min. Slides were immersed in cell lysis solution (2.5 M NaCl, 100 mM EDTA -2Na, 10 mM Tris (pH=10.0) containing 1% Triton X-100 and 50 mM Dithiothreitol) (Merck, Germany) for overnight at room temperature and dark place. The next day, proteinase K was added (0.5 mg/ml) then stayed at room out of light. For remove salt and detergent from the microgels, samples were washed twice. Following this, electrophoresis slides were coded and carried out for 30 min at a voltage of 30 V and a current of 300 mA. Electrophoresis tank (Model, CSLCOM20, Cleaver Scientific Ltd., UK) and DNA was allowed to unwind for 30 min in an alkaline solution containing (100 mM Tris-base, 500 mM NaCl and 1 mM EDTA, pH=9) (Merck, Germany). Condition electrophoresis was 28 V, 250 mA for 30 min. Ethidium bromide was applied for 1 min and Slides were rinsed. The fluorescent labelled cells was visualized (×400) using an AXIO Imager M1 fluorescence microscope (Karl Zeiss, Germany) and the resulting images (figure 1) were captured on a computer and processed with image analysis software (Comet Imager V.2.0.0). Duplicate slides were prepared for each treatment and were independently coded and scored without the knowledge of the codes by the scorer. Thousand cells were scored visually or on captured images for each sample.

Statistical analysis

The obtained data was statistically analyzed with the use of SPSS software (version 18). Analysis of variance (ANOVA) and Mann Whitney U-tests were used to compare the differences between groups. P value <0.05 was considered as significant level.

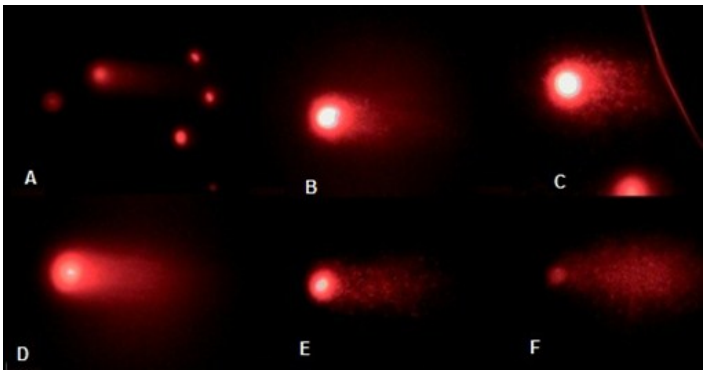

Figure 1. Representative images of various types of comets seen for different treatment groups. **A**; four normal cells and a cell with comet grade 1. **B**; comet with grade 2. **C**; comet whit grade 3. **D**; comet with grade 4. **E**: comet with grade 4. **F**: apoptosis. Magnification ×400

RESULTS

Dose-response effects of testicular X-rays

Results are summarized in table 1 and shown in figures 2 and 3. As seen radiation induced DNA damage in sperms in a dose dependent manner, i.e. the extent of damage increased with increasing radiation dose (table 1). In the 1 Gy group, DNA damage (DD) was 93% compared to 1.4% in the control group. Similar results were obtained after treatment of either BLM or ACTD, which significantly increased DD to 1.8 times compare to 1 Gy-irradiated group. In the group receiving drugs and 2 Gy radiation DD increased by 274%, which was 1.5 times that of the group receiving only 2 Gy radiation (figures 2 and 3). Moreover, DD increased by 365% in the group receiving drugs and 4 Gy radiation. However, this was not significantly different from the group receiving only 4 Gy radiation. The number of tailless sperms was not significantly different across groups. Even in the 4Gy group (highest radiation dose in the study), the number of sperms was only 5 times that of the control group, which was not considerable given the total number of sperms. Higher doses are required to separate sperm heads from tails. Sperm motility gradually decreases with higher radiation dosages. In the 4Gy group, sperm motility (M factor) decreased to half of that of the controls (39%) (table 1).

Table 1. Effects of increasing doses of testicular X-rays on DNA damage, H factor (sperms with tail defects), M factor (sperms with motility defects.

Group	Mean DD (%)	Mean H factor (%)	Mean M factor (%)
Control	1.43 ± 0.1	0.62 ± 0.07	77 ± 1.4
1 Gy	93.1 ± 1.7	1.58 ± 0.18	67 ± 0.9
2 Gy	175.5 ± 2.5	2.91 ± 0.36	52 ± 2
4 Gy	355.3 ± 4.9	4.81 ± 0.7	39 ± 2.8

Data shown represent group means ±SEM (n =4; 1000 cells scored per animal). All variable were significantly different from each other with ($p < 0.001$) (Control versus other radiation groups and within radiation groups).

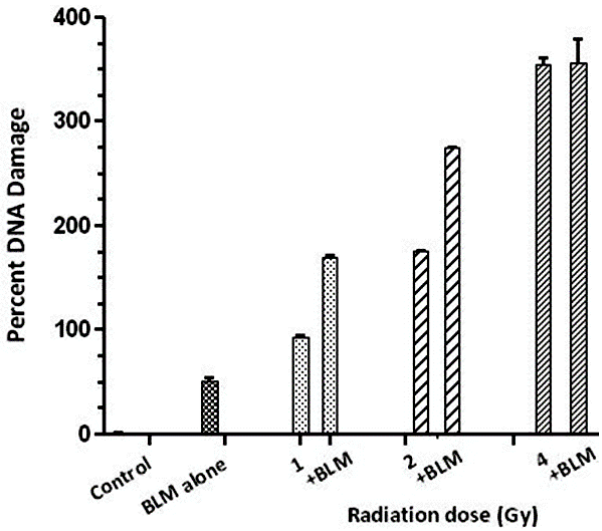

Figure 2. Percent DNA damage induced by various doses of ionizing radiation alone and in presence of BLM. Error bars indicate standard error of mean values.

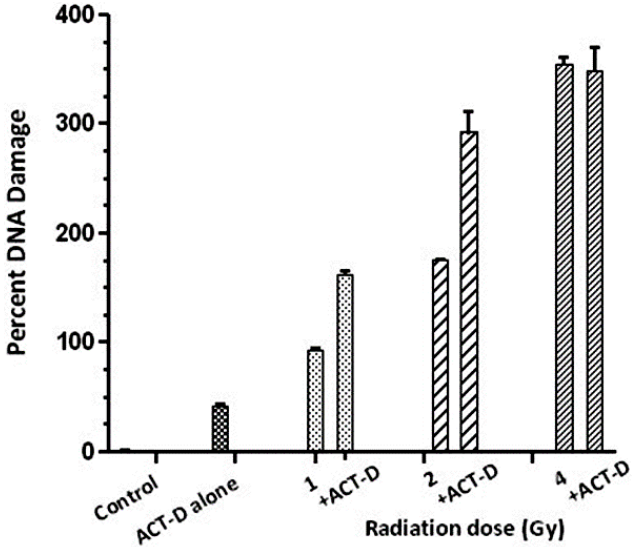

Figure 3. Percent DNA damage induced by various doses of ionizing radiation alone and in presence of ACT-D. Error bars indicate standard error of mean values.

DISCUSSION

Sperm cryopreservation before therapy is critical to fertility preservation, as it is difficult or even impossible to predict the exact impact of cancer therapy on a man's ability to father a biological child [21,22]. Unfortunately, sperm cryopreservation is underutilized in the majority of cases [23]. Later-stage germ cells such as spermatids are less sensitive to chemotherapy and irradiation since they are not dividing [24]. This explains the slow decline in the number of sperms over the ensuing months after chemotherapy.

New chemotherapy procedures have reduced the rate of infertility, although post-treatment azoospermia is still a major concern. Evidence shows only 20-50% of men recover from total azoospermia after treatment [25], while there are reports of up to 80% recovery rate depending on the type of cancer and the chemotherapy procedure [17].

Radiation doses as little as 0.15 Gy irradiation can impair sperm production [26], and doses over 0.5 Gy usually cause reversible azoospermia [27]. Semen parameters often reach their basal levels 4 to 6 months after treatment. Doses over 2.5 Gy may result in prolonged or permanent azoospermia [28]. Spermatogenesis often recovers in post-treatment azoospermic men, but the time of recovery (which could take months or years) and the quality of recovered sperm may vary [16,17]. As seen in figures 2 and 3 high percentage of DNA damage were observed after various doses of radiation alone or when combined with chemotherapeutic agents. Sperm DNA damage is attributed to male infertility through induction of chromosomal abnormalities or chromosomal microdeletions [11,13,20,29]. High-dose testicular radiation usually results in infertility [30]. Table 1 shows the morphology and motility changes induced by various doses of X-rays. The changes were observed to be dose dependent. This observation is in line with other published results regarding animal studies [31, 32] as well as human studies [33, 34]. However, since samples were evaluated relatively short time after irradiation, the changes were not so considerable because the studied cells were irradiated at latest stages of their differentiation.

Study on samples of Chinese hamster cells shows that the AD enhancement of X-ray killing is mainly due to reduced capacity for sublethal damage [35]. Augmented radiation affects the skin in patients receiving ACTD and radiation simultaneously [36,37]. Erythema that normally requires about 1200 R was reported in patients receiving ACTD after a skin dose of 350 R [38,39]. Provided evidence of the synergistic effect of

ACTD and radiation on dysplastic changes in growing hairs in mice [40] showed that radiation followed by relatively small doses of ACTD increases damage to the testis. In the present study, comet assay data showed that the mean percentage of DNA fragmented sperms in the irradiation plus drug groups was significantly higher than that in the irradiation alone or drug alone group. However, no potentiation effect was seen with the dose of 4 Gy. The reason might be the amount of damage induced by radiation alone was so high that presence of chemotherapeutics could not influence on the induced damage.

In conclusion, this study indicates that ionizing radiation induces morphological changes and DNA damage in sperm cells when irradiated in vivo. Moreover, potentiation effects of actinomycin D and bleomycin sulphates on radiation induced DNA damage in sperms was observed in a dose dependent manner.

ACKNOWLEDGEMENT

The authors would like to thank radiotherapy technologists in Tajrish Shohada Hospital (Tehran) for their kind cooperation for irradiation of animals. This work was partly supported by School of Paramedical Sciences, Shahid Beheshti University of Medical Sciences. HM is supported by a Grant number IG-39711 from Tarbiat Modares University.

***Conflicts of interest*:** *Declared none.*

REFERENCES

1. Hermann BP, Sukhwani M, Salati J, Sheng Y, Chu T, Orwig KE (2011) Separating spermatogonia from cancer cells in contaminated prepubertal primate testis cell suspensions. *Hum Reprod,* ***26(12)****: 3222–31*.
2. Coward RM, Kovac JR, Smith RP, Lipshultz LI (2013) Fertility preservation in young men treated for malignancies: Options for precancer treatment. *Sex Med Rev,* ***1(3)****: 123–34*.
3. Dzyuba B, Boryshpolets S, Cosson J, Dzyuba V, Fedorov P, Saito T, *et al.* (2014) Motility and fertilization ability of sterlet Acipenser ruthenus testicular sperm after cryopreservation. *Cryobiology,* ***69(2)****: 339–41*.
4. Tamate M, Matsuura M, Habata S, Akashi Y, Tanaka R, Ishioka S, *et al.* (2015) Preservation of fertility and subsequent childbirth after methotrexate treatment of placenta percreta: a case report. *J Med Case Rep,* ***9****: 232*.
5. Sakkas D and Alvarez JG (2010) Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril,* ***93(4)****: 1027–36*.
6. Ward WS and Coffey DS (1991) DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biol Reprod,* ***44(4)****: 569–74*.
7. Oliveira JBA, Petersen CG, Mauri AL, Vagnini LD, Renzi A, Petersen B, *et al.* (2018) Association between body mass index and sperm quality and sperm DNA integrity. A large population study. *Andrologia, 50(3).*
8. Aitken RJ and De Iuliis GN (2010) On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod,* ***16 (1)****: 3–13.*
9. Nili HA, Mozdarani H, Pellestor F (2011) Impact of DNA damage on the frequency of sperm chromosomal aneuploidy in normal and subfertile men. *Iran Biomed J,* ***15(4)****: 122–9*.
10. Shokri S, Soltani A, Kazemi M, Sardari D, Mofrad FB (2015) Effects of Wi-Fi (2.45 GHz) exposure on apoptosis, sperm parameters and testicular histomorphometry in rats: A time course study. *Cell J,* ***17(2)****: 322–31*.
11. Singh S, Joshi S, Khole V (2001) Immunochemical and functional characterization of a polyclonal antibody to human sperm antigen. *Indian J Exp Biol,* ***39(3)****: 209–17*.
12. Nili HA, Mozdarani H, Aleyasin A (2009) Correlation of sperm DNA damage with protamine deficiency in Iranian subfertile men. *Reprod Biomed Online,* ***18(4)****: 479–85*.
13. Beumer TL, Roepers-Gajadien HL, Gademan LS, Rutgers DH, de Rooij DG (1997) P21(Cip1/WAF1) expression in the mouse testis before and after X irradiation. *Mol Reprod Dev,* ***47(3)****: 240–7*.
14. Makker K, Agarwal A, Sharma R (2009) Oxidativ stress & male infertility. *Indian J Med Res, 129(4):357–67*.
15. Wang X, Sharma RK, Sikka SC, Thomas AJJ, Falcone T, Agarwal A (2003) Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. *Fertil Steril,* ***80(3)****: 531 –5*.
16. Spermon JR, Ramos L, Wetzels AMM, Sweep CGJ, Braat DDM, Kiemeney LALM, *et al.* (2006) Sperm integrity pre- and post-chemotherapy in men with testicular germ cell cancer. *Hum Reprod,* ***21(7)****: 1781–6*.
17. Howell SJ and Shalet SM (2005) Spermatogenesis after cancer treatment: damage and recovery. *J Natl Cancer Inst Monogr,* ***(34)****: 12–7*.
18. Bieber AM, Marcon L, Hales BF, Robaire B (2006) Effects of chemotherapeutic agents for testicular cancer on the male rat reproductive system, spermatozoa, and fertility. *J Androl,* ***27(2)****: 189–200*.
19. Tripathi DN, Pawar AA, Vikram A, Ramarao P, Jena GB (2008) Use of the alkaline comet assay for the detec-

tion of transplacental genotoxins in newborn mice. *Mutat Res,* ***653(1-2):*** *134-9*.
20. Mozdarani H and Mozdarani S (2016) De novo cytogenetic alterations in spermatozoa of subfertile males might be due to genome instability associated with idiopathic male infertility: Experimental evidences and Review of the literature. *AIMS Genetics,* ***3(4):*** *219- 238*.
21. Schrader M, Muller M, Straub B, Miller K (2002) Testicular sperm extraction in azoospermic patients with gonadal germ cell tumors prior to chemotherapy--a new therapy option. *Asian J Androl,* ***4(1)****: 9–15*.
22. Bahadur G, Ling KLE, Hart R, Ralph D, Wafa R, Ashraf A, et al. (2002) Semen quality and cryopreservation in adolescent cancer patients. *Hum Reprod,* ***17(12)****: 3157–61*.
23. Hartmann JT, Albrecht C, Schmoll HJ, Kuczyk MA, Kollmannsberger C, Bokemeyer C (1999) Long-term effects on sexual function and fertility after treatment of testicular cancer. *Br J Cancer,* ***80(5–6)****: 801–7*.
24. Mozdarani H and Nazari E (2009) Cytogenetic damage in preimplantation mouse embryos generated after paternal and parental gamma-irradiation and the influence of vitamin C. *Reproduction,* ***137(1)****: 35–43*.
25. Carson SA, Gentry WL, Smith AL, Buster JE (1991) Feasibility of semen collection and cryopreservation during chemotherapy. *Hum Reprod,* ***6(7)****: 992–4*.
26. Leiper AD, Grant DB, Chessells JM (1986) Gonadal function after testicular radiation for acute lymphoblastic leukaemia. *Arch Dis Child,* ***61(1)****: 53–6*.
27. Apperley JF and Reddy N (1995) Mechanism and management of treatment-related gonadal failure in recipients of high dose chemoradiotherapy. *Blood Rev,* ***9(2)****: 93–116*.
28. Trottmann M, Becker AJ, Stadler T, Straub J, Soljanik I, Schlenker B, *et al.* (2007) Semen quality in men with malignant diseases before and after therapy and the role of cryopreservation. *Eur Urol,* ***52(2)****: 355–67*.
29. Moghbeli-Nejad S, Mozdarani H, Behmanesh M, Rezaiean Z, Fallahi P (2012) Induction of genome instability in AZFc region of Y chromosome in peripheral blood lymphocytes of normal and infertile men following exposure to gamma irradiation. *Journal Assisted Reprod Genet,* ***29:*** *53–61*.
30. Classen J, Dieckmann K, Bamberg M, Souchon R, Kliesch S, Kuehn M, *et al.* (2003) Radiotherapy with 16 Gy may fail to eradicate testicular intraepithelial neoplasia: preliminary communication of a dose-reduction trial of the German Testicular Cancer Study Group. *Br J Cancer,* ***88(6)****: 828–31*.
31. Mahdavi M and Mozdarani H (2011) Protective effects of famotidine and vitamin C against radiation induced cellular damage in mouse spermatogenesis process. *Int J Radiat Res,* ***8(4)****: 223-230.*
32. Dobrzyńska MM (2011) Combined action of X-rays and nonylphenol on mouse sperm. *Cent Eur J Biol, 6(3): 320-329.*
33. Kumar D, Salian SR, Kalthur G, Uppangala S, Kumari S, Challapalli S, *et al*. (2013) Semen Abnormalities, Sperm DNA Damage and Global Hypermethylation in Health Workers Occupationally Exposed to Ionizing Radiation. *PLoS One,* ***8(7):*** *e69927*.
34. Zhou DD, Hao JL, Guo KM, Lu CW, Liu XD (2016) Sperm quality and DNA damage in men from Jilin Province, China, who are occupationally exposed to ionizing radiation. *Genet Mol Res,* ***15(1)****:* doi: 10.4238/gmr.15018078.
35. Koch CJ, Meneses JJ, Harris JW (1977) The effect of extreme hypoxia and glucose on the repair of potentially lethal and sublethal radiation damage by mammalian cells. *Radiat Res,* ***70(3)****: 542–51*.
36. Mozdarani H and Saberi AH (1994) Induction of cytogenetic adaptive response of mouse bone marrow cells to radiation by therapeutic doses of bleomycin sulfate and actinomycin D as assayed by the micronucleus test. *Cancer Lett,* ***78(1–3)****: 141–50*.
37. Bases RE (1959) Modification of the radiation response determined by single-cell technics: actinomycin D. *Cancer Res,* ***19****: 1223–9*.
38. D'Angio GJ, Farber S, Maddock CL (1959) Potentiation of X-ray effects by actinomycin D. *Radiology,* ***73****: 175–7*.
39. Griem ML and Ranniger K (1962) Modification of the radiation effect on hair roots of the mouse by actinomycin. *Radiat Res,* ***17****: 92–100*.
40. Maddock CL, Rev-Kury L, Brown BL (1965) Testicular damage resulting from actinomycin D, x-radiation, and their combination in CAF mice. *Radiat Res,* ***26(4)****: 584–92*.