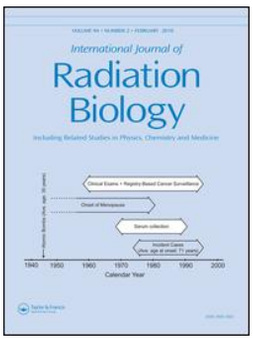

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Abstract

Propose- CatSper protein channels are responsible for the entry of Ca2+ into sperm cells. These proteins play an important role in motility and male fertility. So it is important to find out whether or not environmental factors, such as gamma radiation, have an effect on the expression of Catsper genes. In this study, we investigated the effects of gamma radiation on the expression of CatSper1 and CatSper2 genes.

Materials and Methods- Twenty-one male NMRI mice were divided into three groups: a control group without gamma radiation, and two experimental groups; Group 1 treated with 1Gy of gamma radiation, and Group 2 treated with a higher dose of 2Gy gamma radiation. Testes were removed from all groups of animals 35 days following irradiation and the testicular tissue, processed and embedded in paraffin blocks for sectioning and histological examination. Sperm samples were also taken from the epididymis for microscopic. Sperm parameters such as sperm count, morphology, motility and viability rates were analysed. Expression of CatSper genes was evaluated using Real-time PCR. Data were analysed using the SPSS software and ANOVA test.

Results- Our results showed that after treatment with gamma radiation, testes morphology was changed. Epididymal sperm count, motility, and morphology rates were significantly affected in both experimental groups compared to the control group. The relative expressions of CatSper 1 and 2 genes were significantly reduced in the irradiated mice (1 Gy and 2 Gy) than non-irradiated ones.

conclusions- Gamma radiations not only change testes histology and sperm parameters, but also decrease the expression of CatSper 1 & 2 genes in male mice.

Key words: Gamma Radiation; Catsper; Gene expression; Sperm

Introduction

Factors that may affect the reproductive systems include lead and formaldehyde exposure, radiation, chemicals, pesticides and warmth. These factors affect ovum or sperm and cause temporary or permanent infertility (Stefankiewicz et al., 2006, Mohammadi et al., 2016a, Mohammadi et al., 2015, Mohammadi et al., 2016b). Different types of radiation, such as energetic particles and electromagnetic waves, can penetrate cells and cause ionization in water, macromolecules such as DNA, lipid membranes, and proteins. Ionizing radiation and chemicals can cause defects in the spermatogenesis (Lett et al., 1992, Schulte-Frohlinde et al., 1991,

Somosy et al., 2000). On the other hand, calcium ions play a central role in the regulation of sperm cells behaviour (Darszon et al., 2006).

Sperm possesses several calcium channels, including voltage-dependent calcium channels (VDCCs), transient receptor potential channels (TRPCs) and cyclic nucleotide-gated (CNG) channels (Darszon et al., 2006). A novel family of four unique channel-like proteins—CatSper1 through CatSper4—and two auxiliary subunits—CatSper ß and Cat-Sper G—have been identified to be expressed only in the sperm cells (Darszon et al., 2006, Qi et al., 2007, Mohammadi et al., 2018). Studies revealed the crucial role played by this family of genes in sperm mobility and male fertility (Darszon et al., 2006, Qi et al., 2007, Mohammadi et al., 2018). Ion radiation affects cell metabolism and proliferation by mutations, apoptosis, and necrosis. Testes and spermatogenic cells are sensitive to radiation. These radiations lead to decrease in sperm count, as well as increase in abnormal sperm, and these factors lead to temporary or permanent infertility (Gong et al., 2014, Amini et al., 2014). Sharma et al. (2011) reported that 7.5 Gy of gamma irradiation for 5 days decreased body weight with increasing levels of lipid peroxidation (Sharma et al., 2011). Histopathologic results showed a decrease in diameter, degradation, and shrinkage of the tubules, tissue oedema and necrosis. In another study, rats were exposed to a single dose of 6 Gy of gamma rays. Gamma radiation caused up-regulation of somatic cell genes in testis tissue (Zhou et al., 2010). Previous studies have focused on the effects of radiation on morphological changes of seminiferous tubules and histology of testis and spermatozoa (Koruji et al., 2008, Sharma et al., 2011, Zhou et al., 2010, Kumar et al., 2007, Eissa et al., 2007). A number of studies evaluated the expression of somatic cells (Zhou et al., 2010, Lee et al., 2002) and apoptotic genes in the testis (Gobé et al., 1999). None of these studies have examined the changes in gene expression of CatSper genes after receiving a gamma ray yet. Hence, the aim of the present study was to evaluate the effect of gamma rays on the expression of CatSper 1, 2 genes, sperm quality, and histopathology of testis in NMRI male mice.

Materials and methods

Animals

Twenty-one 2–3-month-old male NMRI mice were obtained from the Razi Institute (Mashhad, Iran). The animals were housed in a room under standard laboratory conditions (12-h light/12-h dark cycle at 22°C); drinking water and food pellets were made available ad libitum. The study was approved by the Ethical Committee for Animal Research of Gonabad University of Medical Science (Ethical Code: 92/62). Twenty-one male NMRI mice were divided into three groups: control group without gamma radiation, experimental 1 with 1Gy gamma radiation, experimental 2 with 2Gy gamma radiation. Whole body radiation was given to the mice by cobalt 60 (Theratron, phoenix model) with an average dose of 0.7 Gy / min. Doses of 1 Gy and 2 Gy were administered for 1 minute in the radiation department of Omid Hospital with a 70 cm distance from the source of radiation. The animal died 35 days after gamma-irradiation.

Sperm analysis

Sperms were analysed in accordance with the WHO guidelines given for human sperm examination after 35 days. Two epididymis was finely minced in phosphate buffer saline and incubated at 37° C for 15–30 minutes. The count and motility of the sperm were evaluated using a Neubaur haemocytometer under a 400× magnification light microscopy (Mohammadi et al., 2018)

Histological examination of testis tissue

After fixation of the left testis, the samples were gradually dehydrated through graded ethanol, immersed in xylene, and mounted with Entellan (Merck, Germany). The results were observed with a light microscope and evaluated by two separate observers.

Total RNA isolation and cDNA synthesis

RNA extraction was isolated from right testis by homogenization in RNX plus solution (CinnaGene, Iran) in accordance with the manufacturer's instructions. Briefly, 1 ml of RNX solution was added to each sample and incubated at room temperature for 5 min, followed by the addition of 200 µl of chloroform. Sample was centrifuged at 12000 g, at 4°C, for 15 min. The upper phase was precipitated with an equal volume of isopropanol, followed by centrifugation at 12000 g for 10 min. The pellet was washed with 75% ethanol (made with diethyl pyrocarbonate-treated water), and dissolved in DEPC-treated water. The purity of RNA was determined by 260/280 nm ratio measurement. The integrity of the RNA was evaluated by visualization of ribosomal bands on a 1% agarose gel electrophoresis. Complementary DNA was synthesized using the cDNA Synthesis Kit Revert Aid (Fermentas Corporation, Germany), in accordance with the manufacturer's protocol. Total RNA (1 µg) from each sample was transformed into cDNA by reverse transcription in a total volume of 20 µl of containing oligo (dt)18 prime, MMLV reverse transcriptase, reaction buffer, Ribolock RNase inhibitor and dNTP (Fermentas Corporation, Germany) (Mohammadi et al., 2018)

Real-time polymerase chain reaction (PCR)

The primer sets are listed in our previous paper (Eissa et al., 2007). In order to determine the efficiency of primers, standard cure was obtained using serial dilutions of complementary DNA. The efficiency measurements were close to 100%. PCRs were performed by the M3000P Real-Time PCR system in a total volume of 25 µl containing 12.5 µl SYBR Green PCR Master Mix, 0.6 µM of each primer, 9.3 µl

water and 2 µl of synthesized CDNA. The PCR conditions were 95 °C for 10 s, followed by 40 cycles of 95 °C for 25 s, 60 °C for 30 s, and 72 °C for 30 s. The expression level of each target gene was compared to that of ß-Actin. The relative expressions levels were calculated using the $2^{-\Delta\Delta Ct}$ formula (Mohammadi et al., 2018)

Statistical analysis

The data was analysed using SPSS 21.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical analysis of data was performed by ANOA and followed by LSD-test. Due to normality of data, the correlations were assessed using the Pearson correlation coefficients. A P-value less than 0.05 was accepted as statistically significant.

Results

Effects of Gamma radiation on sperm parameters in the adult male mice

Gamma radiation caused a significant decrease in sperm count and motility (Table 1). Besides, gamma-radiated mice had significantly higher abnormal morphology rate of sperm than the control group ($P \leq 0.001$; Table 1). The most common types of morphologic abnormalities were bent tail in Experimental 1 group with 1Gy gamma radiation, while head defects were the most in Experimental 2 group with 2 Gy gamma radiation (Figure 1).

Histological examination

The histological changes of testicles in low dose irradiated (1G) rats were small. Most cases showed mild degrees of edema and congestion. Some of them showed disintegration of germ cells from their basal membrane. In addition, focal and mild amounts of maturation arrest, was identified in some tubules. Histological examination of testicles from the high dose irradiated (2G) group, revealed more pronounced

changes. Most had edema and congestion. There were areas of coagulative necrosis in some cases as well as remarkable decrease in the thickness of germinal epithelium in many of them. Additionally, there were some degrees of disintegration of germ cells from their basement membrane in most cases. Leydig cells hyperplasia, probably as a result of maturation arrest and decrease in spermatogenesis, were seen in most of the rats (Figure 2). .

Effects of 1Gy gamma radiation on CatSper 1 and CatSper 2 genes expression in the adult male mice (Figure 3)

The relative intensity of CatSper genes expression is presented in Figure 2. The relative expression of CatSper 1 in the 1 Gy was lower than that of the control group but was not statistically significant (-0.20 ± 1.58 vs. 1 ± 0.50, P = 0.29). The relative expression of CatSper 2 in the 1 Gy was lower than that in the control mice (-0.37 ± 2.32 vs. 1 ± 0.50, P = 0.25).

Effects of 2 Gy gamma radiation on CatSper 1 and CatSper 2 genes expression in the adult male mice (Figure 3)

Data on the changes of the gene expression from the control and the gamma-exposed mice are presented in Figure 3. As it is shown in Figure 3, the relative expression of CatSper 1 was significantly lower in the 2 Gy compared to the control ones (-1.13 ± 1.94 vs. 1 ± 0.50, P = 0.02). Besides, 2 Gy gamma radiation caused a significant down-regulation in the expression of CatSper2 gene (-3.24 ± 1.39 vs. 1 ± 0.50, p ≤0.001). The relative expression of CatSper 2 in the 2 Gy was lower than that in the 1 Gy mice (-3.2 ± 1.39 vs. 0.37 ± 2.32, P = 0.023).

A correlation in sperm count, motility and viability was found in the 1G y and 2 Gy (p≤0.0001). The sperm count, motility and viability in the 2Gy was correlated statistically with CatSper gene expression in the 2 Gy (p≤0.0001).

Discussion

The present study investigated the effects of two doses of gamma radiation 1 Gy and 2 Gy on testes histology, sperm parameters, and gene expression of CatSper 1 & 2 in sperms. The radiation caused

oedema in seminiferous tubules, the rupture of germ cells from the basal membrane, and arrest in spermatogenesis. The effects became more severe in higher doses of gamma rays. Several studies have demonstrated the effects of gamma radiation at different times on testis tissue that were similar when compared to the results of this study (Sharma et al., 2011, Kumar et al., 2007, Eissa et al., 2007). For example, exposure of 7.5 Gy of gamma radiation in mice for 5 days increased levels of lipid peroxidation, reduced the diameter seminiferous tubules, degradation and shrinkage of the tubules, tissue oedema, vacuolization, and necrosis (Sharma et al., 2011).

In another study in gamma-irradiated mice with 8 Gy of gamma irradiation for 15 consecutive days, disorganized tubules and shrinkage were observed (Kumar et al., 2007). Eissa et al. (2007) reported tubular atrophy with reduced interstitial cells after 3 and 6 Gy of gamma rays. The basement membrane was irregular. In addition, testicular vacuoles, congestion and reduction in sperm count, motility and viability were found (Eissa et al., 2007). These changes were more intensive in dose (2Gy). Koruji et al. (2008) showed that the gamma rays decreased sperm parameters, especially in higher doses of gamma (Koruji et al., 2008). Also, in the study by Gong et al (Gong et al., 2014) after a dose of 2 Gy of gamma rays, the number and motility of sperm reduced in mice.

Eissa et al. (2007) reported that after a week of receiving 3 and 6 Gy doses of gamma radiation, spermatogonia, primary spermatocytes and spermatids decreased. This effect was more intense at a dose of 6 Gy (Eissa et al., 2007). The present findings confirmed these results, considering that divisible cells can be sensitive to the radiation and gamma radiation produces free radicals and breakdown in the DNA and causes an arrest in spermatogenesis (Subbotina et al., 2006, Shah et al., 2009, Erickson et al., 1978). Germinal epithelium changes and reduction in sperm count is thereby justified. Sharma et al. (2011) reported exposure of 7.5 Gy of gamma radiation reduced the germinal epithelium thickness (Sharma et al., 2011). Eissa et al. (2007) reported tubular atrophy and reduction in sperm parameters after 3 and 6 Gy of gamma rays (Eissa et al., 2007).

Previous studies showed that environment damage e.g drug abuse (Tajaddini et al., 2016) or formalin (Tajaddini et al., 2014) can be decreased motility-relate CatSper genes. Expression of CatSper 1 & 2 decreased after gamma radiation. This reduction was more intense in higher gamma doses. In line with these results, Taki et al. (2009) reported a direct relationship between the reduction in expression of genes in testis and increasing doses of gamma radiation using microarray technique (Taki et al., 2009). Zhou et al. (2010) showed that a single dose of 6 Gy of gamma rays increases the expression of somatic cells in the testes of rats (Zhou et al., 2010). In another study, 1 Gy and 1Gy × 2 gamma rays changed the expression of Dazl- Tnp2- vps26a genes in testis. These changes were more severe in the higher dose of radiation (Shah et al., 2009). It seems that the mechanism of the effect of gamma radiation on CatSper gene expression is via changes in the flow of calcium, especially in the unique calcium channels, CatSpers channels. Another study showed increases in the survival rate in gamma-irradiated mice after administration of calcium blockers (Goyal et al., 2001). In another study, the flow of calcium in voltage-dependent calcium channels in brain reduced after exposure to gamma rays (Kandasamy et al., 1991). The first study investigated the effects of gamma radiation on gene expression CatSper that is research strengths.

Conclusion

Overall, it can be concluded that gamma radiation causes damage to the germinal epithelium in seminiferous tubules, and causes arrest of spermatogenesis. By considering the important role of the expression of CatSper 1 & 2 genes in sperm motility and fertility, infertility after gamma radiation is justified. In this study, effects of gamma radiation evaluated on CatSper genes expression for the first time comprised one of the strengths of this study. The weaknesses of this study included lack of evaluation of the effects of different doses of gamma radiation over the short and long terms.

Declaration of Interest statement

The authors declare no conflicts of interest.

Acknowledgments

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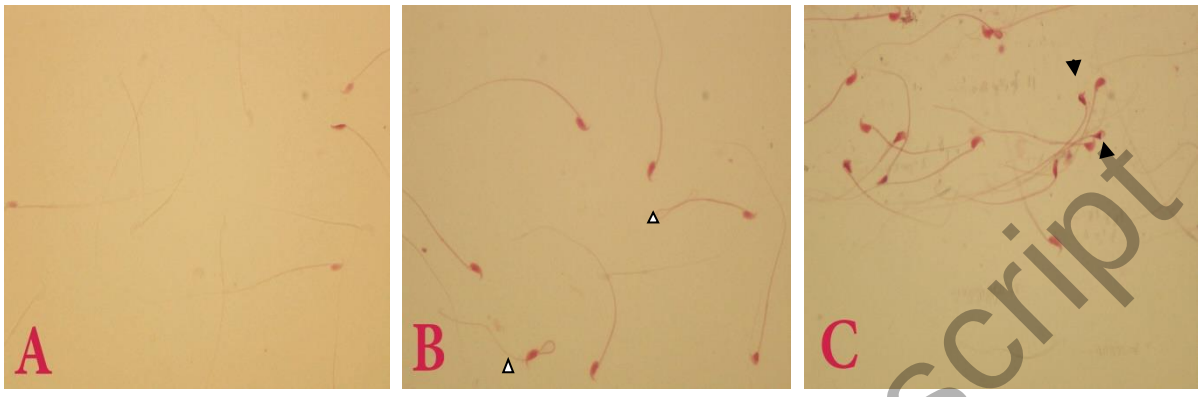

Figure 1. Mice Sperm cells after low and high dose irradiated rates. A. Control group, B. 1 Gy group, C. 2 Gy group (eosin B, X400). Arrow heads shows bent tail in 1 Gy group. Arrow shows head defects in 2Gy group.

Table 1- Effect of gamma radiation on sperm count, sperm motility, and normal morphology in mice

Sperm parameters	Control group	1 Gray group	2 Gray group
Sperm Count (million/mL)	4.5 ± 2.1	3.7 ±1.5*	2.4 ±1.9*
Sperm motility (%)	78 ± 3.5	61 ±4.6*	39 ±5.3*
Normal Morphology (%)	80 ± 4.2	68 ±5.4*	49 ±3.7*

Values are presented as Mean± SD of seven mice in each group. Data were analyzed using ANOVA test.
* P≤0.001 compared to the control group

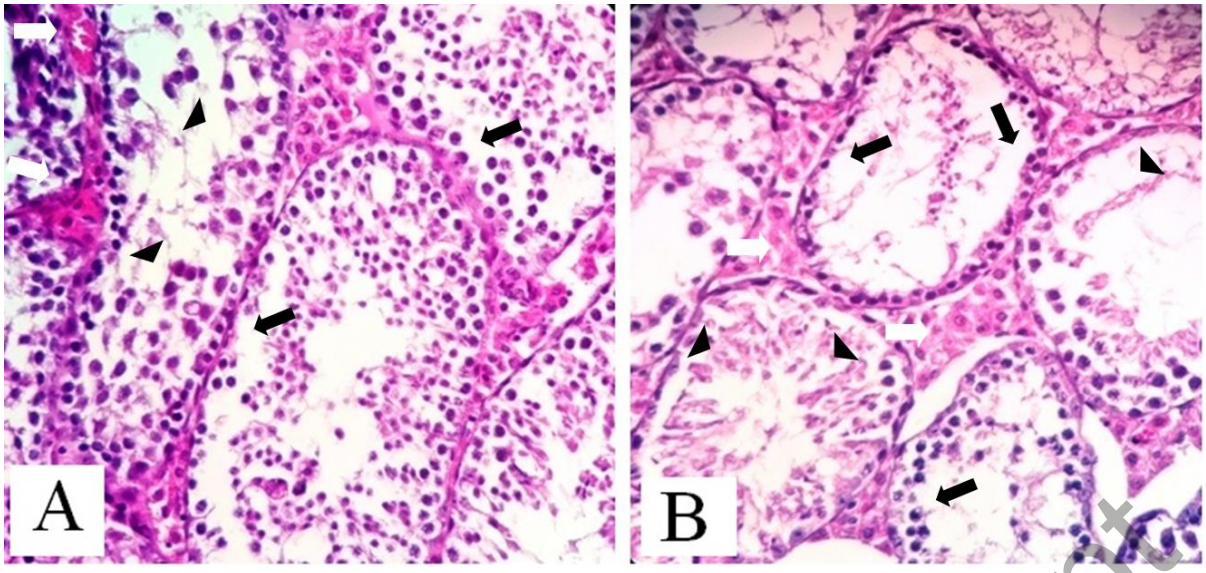

Figure 2. Testis cross section from low and high dose irradiated rates. **A**. In this case of low dose irradiated mice there is mild congestion (white arrows), maturation arrest (arrow heads) as well as disintegration of germ cells from basal membrane (black arrows) (H&E, X400). **B**. Necrosis (arrow heads), leydig cell hyperplasia (white arrows) and notable decrease in the thickness of germinal epithelium (black arrows) is evident in this high dose irradiated mice (H&E, X400).

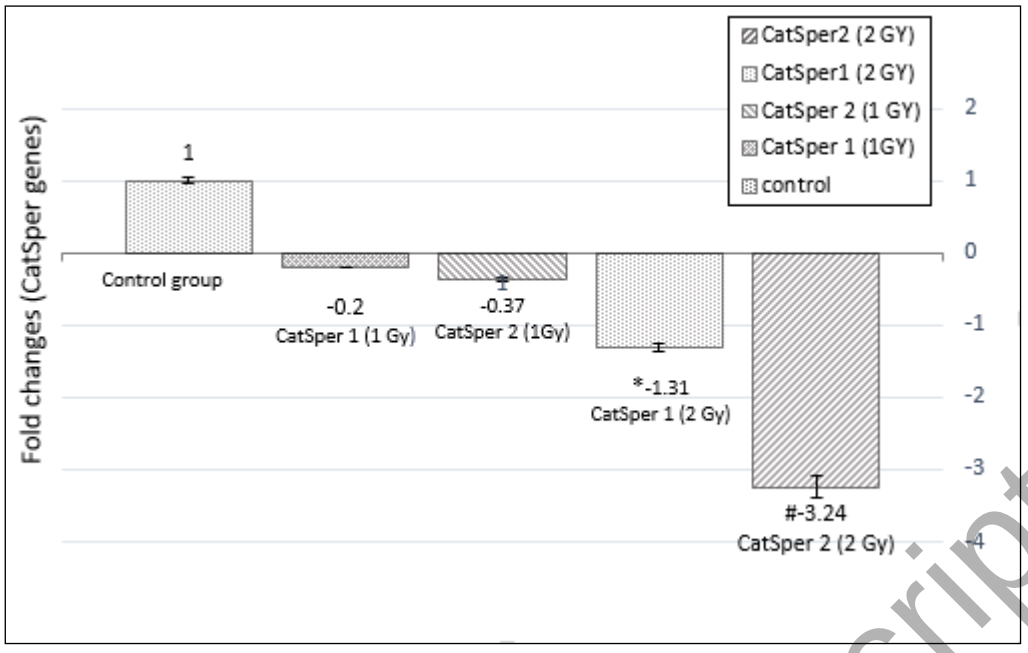

Figure 3. Comparing the relative expression of CatSper 1, 2 gene to ß-actin gene expression in control, 1 GY and 2 GY groups: (n =7 mice in each group).
Statistical analysis showed the down-regulation of CatSper 2 gene expression compared to control group; # $P \leq 0.001$. A significant down-regulation of CatSper 1 gene expression compared to control group; * $P = 0.02$. The negative values mean a reduction in gene expression.