Radioprotective effects of Silymarin on the sperm parameters of NMRI mice irradiated with γ-rays

Daryoush Fatehi[a], Mohsen Mohammadi[b,c], Babak Shekarchi[d], Arash Shabani[e], Mohammad Seify[f], Ayoob Rostamzadeh[g,h,*]

[a] Department of Medical Physics, Faculty of Medicine, Shahrekord University of Medical Sciences, Shahrekord, Iran
[b] Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Lorestan University of Medical Sciences, Khorramabad, Iran
[c] Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran
[d] Department of Radiology, 501 Hospital, AJA University of Medical Sciences, Tehran, Iran
[e] Department of Radiology Technology, Faculty of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[f] Department of Anatomical Sciences, Faculty of Medicine, Tarbiat Modares University, Tehran, Iran
[g] Department of Anatomy and Neuroscience, Faculty of Medicine, Shahrekord University of Medical Sciences, Shahrekord, Iran
[h] Medical Plants Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran

ARTICLE INFO

ABSTRACT

Free radicals and reactive oxygen species (ROS) are generated using various endogenous systems or from external sources such as exposure to different physiochemicals. Ionizing radiation damage to the cell can be caused by the direct or indirect effects of radiotherapy processes. Silymarin (SM), a flavanolignan compound, has been identified as a natural potent antioxidant with cytoprotection activities due to scavenging free radicals. The aim of the present study was to evaluate the radioprotective effect of SM on sperm parameters of mice induced by γ-rays. A total number of 40 adult, male NMRI mice were randomly divided into four equal groups. The control group was neither treated with SM nor irradiated by γ-rays. The second group was only irradiated with 2 Gy of γ-rays. The third group was firstly treated with 50 mg/kg of SM for 7 consecutive days, and one day later, last injections were irradiated by 2 Gy of γ-rays. The fourth groups received only 50 mg/kg of SM for 7 consecutive days. All the animals were treated intraperitoneally. Histopathological and morphometrical examinations were performed. The data were analyzed using ANOVA and Tukey post hoc test. A value of $p < 0.05$ was considered significant. The results showed that in the radiation-only group when compared with those treated with SM and irradiated, a significant different was observed in testicular parameters and DNA damage ($p < 0.05$). In conclusion, SM can be considered as a promising herbal radioprotective agent in complementary medicine which may play an important role to protect normal spermatocytes against possible effects of γ-radiation-induced cellular damage.

1. Introduction

Spermatogenesis is a highly synchronized, regular, long and complex process that takes place in the germinal epithelium of the testes [1]. The seminiferous tubule within the testes is sensitive to endogenous or exogenous stresses. Exposure of testes to such stressors may affect somatic testicular cells or germinal cells at different stages of differentiation, leading to temporary or permanent irreversible infertility [2–4]. Among potential reproductive toxic agents, ionizing and non-ionizing radiation have been extensively studied that can affect the fertility and reproduction. Among potential reproductive toxic agents, ionizing and non-ionizing radiation have been extensively studied that

can affect the fertility and reproduction [5–9]. Payne CM. et al. showed that 24 h after the mice were exposed to 1–4 Gy γ radiation, increases the frequency of apoptosis in the germ line cells was found [10]. It has also been found, morphological changes and azoospermia were observed following the mice testes irradiated with γ-ray [11]. Ionizing radiation, e.g. X- and γ-ray, may induce DNA damages resulting in mutation in gametes; thus, this could produce congenital diseases and malignancies in the next generation(s) [12]. X- and γ-ray can affect the cells directly/indirectly and produce free radicals, oxidative stress and reactive oxygen species (ROS) [13]. Furthermore, ionizing radiation can damage intra/extra cellular signaling, and induce DNA damages, mutation and cancer [13]. Radioprotector agents can protect normal

cells against the destructive effect of radiation in radiotherapy. Normal cells that surround a tumor in the pelvic area may be reproductive cells with a high mitotic index. This shows the effect of the radioprotectors and the radiation [14]. Prostate is one of the organs near the reproductive system. Prostate cancer is usually treated using X- or γ- ray of accelerators, ^{125}I or ^{60}Co. X- and γ-ray are categorized as low LET radiation (LET: linear energy transfer). Radioprotectors can save normal tissues due to scavenging of free radicals [15]. Radioprotectors decrease bystander effects of ionizing radiation, consequently, reducing the side effects of radiotherapy [14]. Application of radioprotectors, which are mainly chemical drugs as well as antioxidants, has a long history. Radioprotectors play their roles via the following processes: free radical scavenger, hydrogen atom detonation and electron transfer, detoxification of metabolic elements, DNA repair, and activation of cytokines or melatonin hormone [14,16]. Silymarin (SM), a hydro-alcoholic extract from the *Silybum marianum* (milk thistle) plant, consisting of a mixture of the six flavonolignans (silychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B) and one flavonoid. These compounds represented together about 65–80% of the total extract composition [17–20]. In recent years, SM is widely prescribed as a complementary and alternative treatment in preventing and reversing various diseases/disorders [18,19,21]. Moreover, SM has been shown to be more potent in antioxidant capacity as compared to vitamin E [20,22]. Based on some studies, SM act as a radioprotective agent against radiation induced hepatotoxicity in rat [23]. In an in-vitro study, Adhikari et al. showed that specific dose (25 μg/ml) of SM can ameliorate the deleterious effects of γ–radiation on human embryonic kidney cells. They revealed that nano-Silymarin was radioprotective, supporting the possibility of developing new approaches to radiation protection via nanotechnology [24,25]. Deep and Agarwal showed that SM, with targeting signaling molecules, could adjust the process of epithelial mesenchymal transition, protease activation, adhesion and prevention of metastasis [26]. SM is a non-toxic bioactive component that has radioprotective and detoxification capabilities. As *in vitro* and *in vivo* studies indicated that silibinin, one of the structural isomers of silymarin, has a strong radiopotective to prevent gamma radiation-induced DNA damage [27], and also reduced some of the toxic effects of methotrexate (MTX) in mice testicular tissue [28]. Meanwhile chen et al have reported that silymarin, as a hepatoprotection agent, has the potential antifibrotic activity against thioacetamide (TAA)-induced liver fibrosis in mice [29]. In the present study, the effects of two doses of SM on NMRI mice irradiated with a total dose of 2 Gy γ-rays was determined to evaluate sperms parameters.

2. Methods and Materials

2.1. Animals and Ethics

In this study, 40 Adult male NMRI (The Naval Medical Research Institute) mice, weighing 36–41 g, pathogen free and purchased from the Pasteur Institute, Tehran, Iran, were used. They were kept in an animal house with good conditions, given standard mice clear Plexiglas box, water ad libitum, controlled artificial lighting of light/dark (12:12 h, light from 8 to 20), and temperature of 23 ± 2 °C. All procedures were approved by the Institutional Animal Care of the Shahrekord University of Medical Sciences (SKUMS), Shahrekord, Iran (Ethical code: IR.SKUMS.REC.1396.20).

2.2. Gamma Irradiation

Gamma irradiation was performed by applying a Cobalt-60 source (Theratron 780C, Atomic Energy of Canada Limited, Kanata, ON, Canada), at the Shohadaye Hafte Tir Hospital, Tehran, Iran. The characteristics of the source were: half life: 5.27 years, dose rate: 0.5 Gy min^{-1}, medium energy: 1.25 MeV. The source had a diameter of 20 mm. The source to skin distance (SSD) was 80 cm and the collimator

to isocenter distance (CID) was 30 cm. The mice were put in a ventilated Plexiglas cage (with size of $10 \times 10 \times 8$ cm), and then all mice were irradiated with a total dose of 2 Gy γ-rays, at room temperature of 23 ± 2 °C. The reason for applying this dose of radiation is because each fractionation dose in radiotherapy for human prostate cancer treatment is 2 Gy.

2.3. Animal Grouping and SM Administration

The mice were randomly and equally divided into 4 groups of 10. The first group, control group: was neither treated with SM nor irradiated by γ-rays. The second group was only irradiated with 2 Gy of γ-rays. The third group was firstly treated with 50 mg/kg of SM (registered with Herbarium Code: 172 in Medical Plant Research Center in SKUMS) and then 24 h later, last injection were irradiated with 2 Gy of γ-rays. The fourth group received only 50 mg/kg of SM for 7 consecutive days. The solution of SM was administered intraperitoneally for 7 consecutive days.

2.4. Testes Weight Measurement & Sperm Collection

The following procedures were performed in order to ascertain protective effects of SM. Twenty four hours after γ irradiation, mice skin was sterilized with ethanol, and they were sacrificed by cervical spine dislocation method under anesthesia using chloroform. In the next step, to show the abdominal cavity of the mice, the skin and peritoneum of each mouse were removed. Then, epididymis tubule of the mice was transferred to a Petri dish including Ham's F-10 culture media (Gibco Labs, Grand Island, NY; #450-1200). Temperature of the Petri dish was already balanced to the room temperature using an incubator (Memmert, Germany). After that, the tail of epididymis was dissected, and vasodefrens duct divided into eight smaller pieces. The cauda epididymal sperm was transferred to the culture medium for sperm preparation, and for sperm capacitating, the cells were kept in a 37 °C incubator with 5% CO_2 for 1 h. Finally, the weight of the testes (excluding epididymis) was measured using a digital scale (Sartorius; model-BL210S). The accuracy of the measurement was ± 0.01 mg with a precision of ± 0.001 mg.

2.5. Sperm Parameters Evaluation

2.5.1. Sperm Count and Sperm Motility
The total sperm count and motility were calculated using the method previously described [30]. In brief, for sperm count 20 μl of the diluted sperm was transferred into a hemocytometer using a Pasteur pipette (Thoma, Assistant Sondheim/Rhön, Germany), and kept for 10 min in order for sperm to reach the counting location. Next, 1 ml of the culture media and sperm was diluted and fixed with 9 ml of formalin 2%. Using a light microscope (Olympus BX51, Germany), and under magnification of 400 ×, the sperms were counted from 5 squares of 25 and the average was calculated. The counting was repeated 3 times for each sample and the mean of the values was allocated as sperm frequency of each specimen. For sperm motility, briefly 10 μl of the prepared suspension was placed on a special slide. At least, 5 microscopic fields were assessed for sperm motility, and it was performed for 200 sperm of each sample. The motility index was classified as "immotile-", "in-situ-" (sperm flagella beating without midpiece movement) and "progressive-" movements. Sperm were counted as "motile" when they either exhibited progressive movement or spontaneous flagellar beatings if the sperm head is attached to the glass slide. Three independent experiments analysis were performed for > 100 sperm for each condition.

2.5.2. Sperm Viability and Sperm Morphometry Analysis
To evaluate the viability of sperm, Eosin-Nigrosin staining technique was used as previously described with a slight modification

Table 1
The mean ± SEM[d] values of different sperm parameters as found in the present study for different groups.

Group	testis weight (g)	Sperm count × 10^6	Sperm viability	Sperms with normal morphology	Sperm motility		
					Immotile	In-situ	Progressive
1: Control	3.93 ± 0.22	47.62 ± 0.46[b,c]	71.84 ± 2.04	83.31 ± 3.1	26.07 ± 1.2	17.83 ± 0.74	59.78 ± 0.32
2: γ irradiation alone	1.64 ± 0.07[a]	19.74 ± 0.04[a]	43.97 ± 2.31[a]	50.06 ± 2.27[a]	41.15 ± 2.84[a]	28.13 ± 1.73[a]	36.7 ± 1.68[a]
3:Silymarin (50 mg/kg) & γ-ray	3.13 ± 0.15[b]	29.8 ± 0.04[b]	52.04 ± 3.5	66.02 ± 1.29[b]	32.57 ± 0.29[b]	24.22 ± 0.82[b]	47.52 ± 1.04[b]
4:Silymarin (50 mg/kg)	4.08 ± 0.04[a,c]	53.12 ± 0.24	84.17 ± 1.66[a]	94.23 ± 1.37[a,c]	17.42 ± 0.35[a,c]	13.07 ± 1.26[c]	67.42 ± 0.09[c]

[a] p-Value ≤ 0.05 vs. control group.
[b] p-Value ≤ 0.05 vs. γ irradiation alone.
[c] p-Value ≤ 0.05 vs. Silymarin (50 mg/kg) & γ-ray.
[d] SEM: standard error of the mean.

[31,32]. Briefly, Eosin 1% (Merck, Darmstadt, Germany) and Nigrosin 10% (Merck, Darmstadt, Germany) were prepared in distilled water. Firstly, one volume of sperm suspension in culture media was mixed with two volumes of 1% eosin. Secondly, after keeping the suspension in the 37 °C incubator for 30 min, an equal volume of Nigrosin was added to the Eosin-sperm suspension. Thirdly, a thin slice of the suspension was prepared and dried in the laboratory temperature. In the prepared slice, normal live sperm appeared white in color; whereas, it was pinkish for the dead sperm (those with loss of membrane integrity because of eosin up take). Finally, the viability of sperm was determined using the light microscope with magnification of 1000 ×. For the assessment of sperm morphometry, microscope stained glass slides were prepared and considered to evolution of changes in sperm morphometric including; cytoplasmic droplet, amorphous head, as well as coiled or curled tail. For each specimen, 100 sperm were scored using the light microscope under magnification of 1000 ×. In this part of the experiments, frequency of the sperm with normal morphology was evaluated. The counting was repeated 3 times for each sample. Finally, average values were calculated.

2.5.3. Quality of Sperm Chromatin

Using the thin dried slice of the prepared suspension, the sperm spreads were stained with Acridine orange to evaluate DNA integrity using method described by Tejada et al. [31]. A fixator solution was made using methanol and acetic glacial acid (3:1) in which the sperm spreads were fixed in 4 °C for 14 h. Then, the spreads were stained applying Acridine crydin orange (0.19 mg/mL; Sigma Chemical Co, St Louis, USA) in phosphate citrate buffer with pH of 2.5 for 10 min in darkness. In the next step, the samples were slightly washed with water for 5 min, and dried in the laboratory temperature. The preparations were evaluated using a fluorescent microscope (Leitz, Germany; at the excitation wavelength of 450–490 nm) by the same examiner under magnification of 1000 ×. Two staining patterns were used for the sperm head as follows: 1) normal status of chromatin (sperm with green head affirmed as double stranded DNA) and 2) damaged chromatin (including yellow head as single stranded DNA and orange-red head as denaturized single stranded DNA). At least 100 spermatozoa were evaluated in each sample to determine percentage of the single and double strand DNA of the sperm.

2.5.4. Evaluation of Replacement Protamine by Histone

In this part of the work, for analysis of replacement of protamine by histone in nucleus chromatin during spermiogenesis, the sperm were fixed in formalin 4% for 5 min and then they were washed with distilled water. Next, the slides were stained with 5% aqueous Aniline Blue (Sigma Chemical Co. Poole, U.K.) prepared with 4% acetic acid (pH 3.5) for 5 min [33]. In the next step, the samples were washed with distilled water for 5 min. Finally, the samples were stained with Eosin 0.5% for 1 min and then dried in at laboratory temperature. The sperm were evaluated using the light microscope with magnification of 1000 ×. Under the microscope, the immature sperm with high histone

showed grayish dark blue color in their nucleus and mature sperm head were pale, as they include protamine. At least, 100 sperm were evaluated to find the mature or immature sperm.

2.6. Statistical Analysis

The ANOVA test was applied to compare the averages of the damages within the each group; i.e. SM or γ irradiation group, and Tukey's post hoc test was used to compare the four groups. SPSS software (Version 19; SPSS Inc., Chicago, USA) was used for the statistical analysis. The p-value was considered for two-sided test at statistically significance level of 0.05 or less.

3. Results

Details of different sperm parameters evaluated in the present study are summarized in Table 1. Furthermore, sperm with morphological abnormalities, DNA integrity and replacement of protamine by histone in nucleus chromatin, are presented in Figs. 1 to 3, respectively.

3.1. Testes Weight

As shown in Table 1, the weight of the mice testes significantly decreased in the 2nd group (γ irradiation alone) in comparison with the control group (p = 0.009). In contrast, comparing the 3rd group [SM (50 mg/kg) & γ-ray] with the 2nd group, a significant increase was observed in the testes' weight (p = 0.01). Furthermore, a significant increase was seen when the 4th [only SM (50 mg/kg)] and 1st groups (p = 0.017) were compared. With respect to the testes weight, a significant difference between the 4th and the 3rd groups (p = 0.036) was also found.

3.2. Sperm Count and Sperm Motility

As compared to the control group, the data demonstrated that the average frequency of sperm significantly decreased in group 2 (p = 0.009). Contrary to this, it significantly increased when the results of the 3rd group were compared with those of the 2nd group (p = 0.01). Additionally, average of the 4th group was remarkably higher than that of the 3rd group (Table 1). The sperm motility results show that the percentage immotile sperm was significantly increased in group 2 as compared control group (p = 0.007). In contrast, a significant decrease in immotile sperm was observed when the 3rd group was compared with the 2nd group (p = 0.019). Furthermore similar results were obtained for the in-situ type of motility with statistical difference (p = 0.014). Contrarily, when compared with the control group, the progressive motility type significantly decreased in the 2nd group (p = 0.005). However, this type of motility significantly increased when the 3rd (p = 0.01) group was compared with the 2nd group.
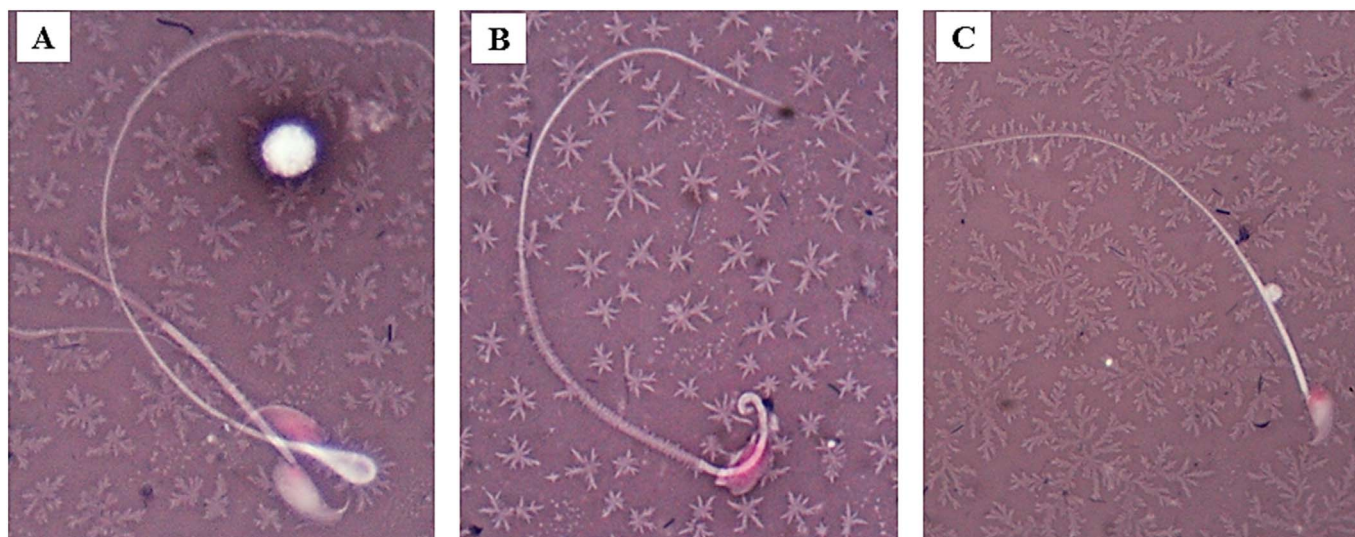
Fig. 1. Three morphological abnormalities of the NMRI mice sperm found in the 2nd group (γ irradiation alone). A) Sperm with coiled or curled tail. B) An amorphous head sperm. C) Cytoplasmic droplets along the tail of the sperm which may indicate an immature sperm. The staining was performed applying Eosin–Nigrosin with magnification of 1000 ×.

3.3. Sperm Viability and Sperm Morphometry

The sperm viability significantly decreased when the 2nd group was compared with the control group (p = 0.006). In contrast, sperm viability increased in the mice of groups 3 but was not statistically significant. A significant difference was seen for the sperm viability when the 4th group was compared with the 1st group (p = 0.001) (Table 1). Sperm morphometric results show that the average frequency of sperm with normal morphology, significantly decreased for the 2nd group when was compared with the control group (p = 0.0001), also, in comparison with the 2nd group, the average was significantly different for the 3th group (p = 0.038). As sperm with three types of morphological abnormalities shown in Fig. 1: sperm with coiled or curled tail (Fig. 1A), sperm with amorphous head (Fig. 1B) and sperm in which cytoplasmic droplets are along its tail (Fig. 1-C). In addition a significant difference was observed for the sperm morphology when the 4th group is compared with the 1st group (p = 0.024).

3.4. Quality of Sperm Chromatin

The frequency of the sperm with damaged chromatin increased significantly in the 2nd group (p = 0.035) when compared with the control group. In contrast, when the 3rd group was compared with the 2nd group, a significant decrease was seen in the sperm with damaged chromatin (p = 0.014). There was no difference between the 3rd and the 4th groups. Fig. 2-A show a normal status of sperm chromatin (1st group) and 2-B illustrate a damaged chromatin of sperm's head regarding DNA integrity in the 2nd group. Furthermore, Fig. 2-C and -D illustrates adjusting and inhibition of DNA damaged sperm in the 3rd and 4th groups, respectively.

3.5. Evaluation of Replacement Protamine by Histone

Comparing the control and the 2nd groups, the frequency of the immature sperm significantly increased (p = 0.01). However, when the 3th group was compared with the 2nd group, a significant decrease was seen in the frequency of immature sperm (p = 0.026). Abnormal replacements of the protamine by histone in sperm are demonstrated in Fig. 3. As shown, the head of the normal sperm is red-orange in color. Fig. 3-A shows mature sperm with protamine in the nucleus. Fig. 3-B shows an abnormal illustration of the mice sperm of the 2nd group with its head being dark blue in color, since it includes a lot of histone.

4. Discussion

The present research shows that SM protects sperm of the NMRI mice irradiated with 2 Gy of γ-rays since SM improved sperm parameters of the mice. Ionizing radiation including γ-rays could disrupt the endocrine system and change concentration of hormones such as follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone [34]. Additionally, ionizing radiation could destroy tissues such as seminiferous tubules, Sertoli cells (a "nurse" cell of the testicles which is a type of somatic cell around which, spermatids develop in the seminiferous tubule that can lead to progression of the process of spermatogenesis), or cause disorder in antioxidant system of epididymis' sperm via induction of oxidative stress [35]. Furthermore, ionizing radiation can damage sperm' DNA and change its instruction; consequently, it will change the capacity for conception [3,35]. Moreover, exposure to radioactive materials could induce the same effects on the sperm and will change parameters such as sperm viability and motility, resulting in defect in the conception process [36]. Despite the negative effects of ionizing radiation such as γ-rays, radiotherapy is an effective modality for cancer treatment. Studies reported that radiotherapy is the main treatment modality in 52% of cancers [37]. However, it could induce short- and long-term effects in the normal tissues around the tumor. Hence, cancer patients are compelled to be irradiated with ionizing radiation; however, they will face side effects due to the irradiation [12,13]. SM is an herbal strong non-enzyme antioxidant that can protect the reaction of lipid peroxidation in the cell membranes. This is done by limitation in behavior of free radicals; consequently, the cell membrane is protected against the injuries induced by free radicals [21,24,28]. Irrespective of the mechanism involved, SM has been shown to protect the genome from mutations and damages, also the function of cells within tissues stimulates protein synthesis (such as glutathione); an increase in the production of new cells to replace the damaged ones, in particular, play a critical role in many tumors to reduce the side effects during long-term radiotherapy and chemotherapy treatment for normal tissues adjacent tumors [14,16,23]. A wide range of antioxidant compounds were applied as radioprotective. In the study carried out by Katiyar SK. et al. reported that SM due to the antioxidant effect applied as a radioprotective agent against the skin of the mouse irradiated with UV-rays [38]. Results of the present study confirm that SM is a radioprotector, as sperm viability in group 3 [SM (50 mg/kg) & γ-ray] was significantly higher than that of group 2 (γ irradiation alone). Furthermore, the average frequency of sperm with normal morphology in group 2 was significantly lower than
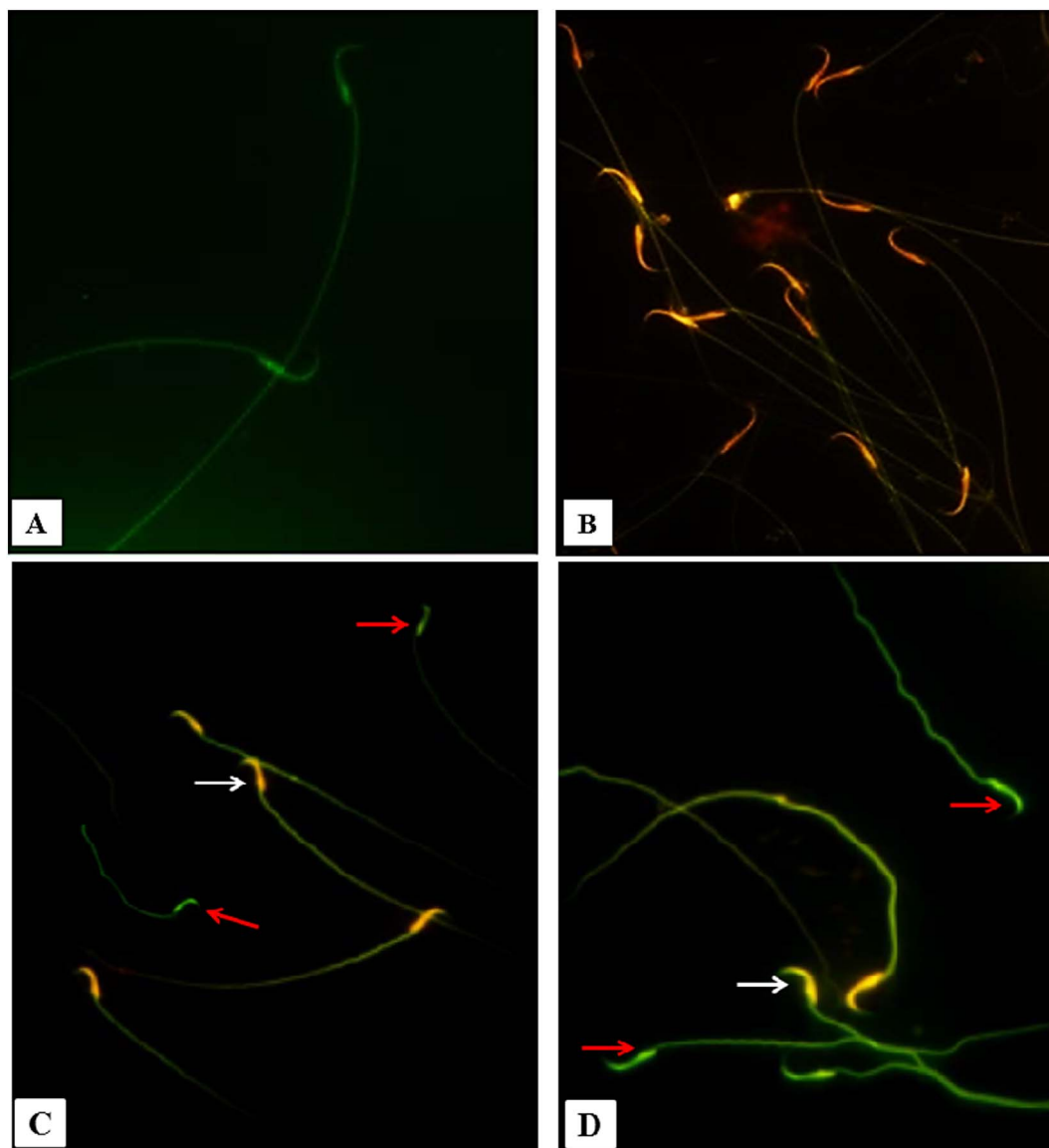
Fig. 2. DNA integrity of the NMRI mice sperm found in the present study. A) Representation of a normal status of sperm chromatin with green color in the control group. B) A damaged chromatin of sperm of the 2nd group (γ irradiation alone) with its head being orange in color. C) & D) Two illustrations of adjusting and inhibition of DNA damaged sperm (red arrows) in the 3rd (50 mg/kg SM & γ-ray) and 4th groups (100 mg/kg SM & γ-ray) of the study, respectively. The white arrows show damaged sperm. Staining was performed applying Acridine orange with magnification of 1000 ×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that of the control group, while it increased for the groups treated with SM (groups 3 and 4) and significantly different for the 4th group. Therefore, the results of the current study reveal that the mean frequency of sperm in group 2 was lower than that of group 3 and it was significantly different from that of group 4. In support of this, in the study conducted by Marzban et al. on effects of gamma rays on histological and histomorphological parameters of seminiferous tubules and the biological characteristics of Leydig cells, it was shown that SM can be used with radiation therapy to prevent male reproductive function [39]. Furthermore, a report suggested that SM decreased the toxicity of Cisplatin in the liver with scavenging free radicals and activation of antioxidant enzymes as well as reduce Glutathione (GSH) [40]. Sperm viability and motility are the most important parameters that measure sperm membrane integrity and sperm conception capability [33,36]. Sperm membrane of mammalians has a lot of non-saturated fatty acids which make it sensitive to lipid peroxidation of oxidative stress that cause decrease in intracellular ATP and sperm viability as well as sperm motility [36]. Therefore, changes in the sperm viability and motility

found in the current study are probably due to the induction of oxidative stress produced by lipid peroxide of the sperm membrane. Since SM has antioxidant property, it can increase sperm defense antioxidant system and increase sperm viability and motility [24,27]. This characteristic of SM is similar to the effects of superoxide dismutase, glutathione peroxidase and catalase [21,24]. Chromatin and flagellum of the mammalian sperm has a lot of protamine enriched with thiol and sulfhydryl groups for cross linking. Additionally, semen has various antioxidants such as taurine, vitamin C, catalase, superoxide dismutase which prevent sperm against oxidative stress. It also keeps sperm motility in conditions where sperm is not in the seminal plasma [11,27,35]. Reduction in frequency of progressive sperm shown in the present study probably has two reasons. Firstly, it could be due to connection (crosslink) between free radical and thiol-sulfhydryl groups of the protein of the sperm; secondly, it might be due to inhibited mitochondrial enzyme that is responsible for sperm motility [41]. Free radicals, also generally called oxidants, are generated from normal essential metabolic processes in the cells or from external sources such as
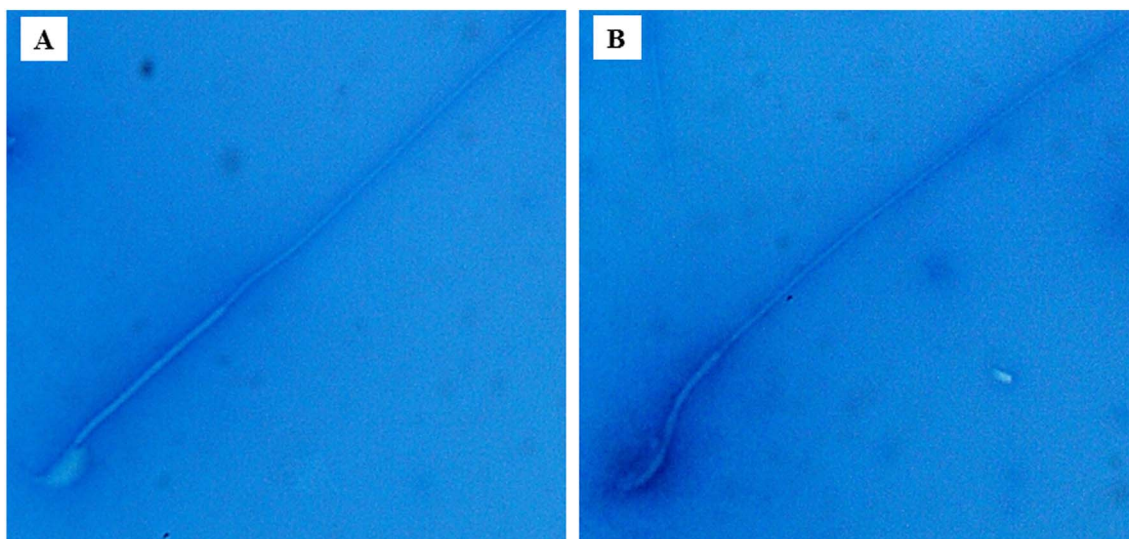
Fig. 3. Evaluation of protamine replacement by histone in the present study. A) Normal sperm of NMRI mice with pale head in the control group expressing matured sperm with protamine in the nucleus. B) An abnormal mice sperm of the 2nd group (γ irradiation alone) in which the head is grayish dark blue in color, due to the existence of a lot of histone in its head. The staining was performed by applying Aniline Blue with magnification of $1000 \times$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

exposure to physical and chemical agents. Normally, the body's ability to regulate free radicals, but if antioxidants are unavailable, or if the free-radical levels become excessive, can attack a number of macro-molecules including lipids, proteins and DNA as a consequence cell damage and even death can occur. Therefore application of external source of antioxidants, chemical and herbal compounds, can assist in coping directly with ROS preventing oxidative damage. Antioxidants play important roles in the prevention of free radical formation and help to prevent or reduce various disorders [42–46]. Silymarin, as natural active phytochemical has antioxidant properties by scavenging free radicals as well as ROS production. Moreover SM can protect organs from oxidative damages through decreasing stress oxidative resulting save the cell against apoptosis [23,24]. Furthermore, the point is that protective and noninvasive effect of SM has no considerable toxicity [27,28]. In summary, results of the mentioned studies and current research show that SM acts as a radioprotector. Although, the exact mechanism is not clear yet, SM may play main role in adjustment of the sperm cell membrane permeability, restraining 5-lipoxygenase pathway and ROS, and decreasing DNA damages, by preventing necrosis factors (NF-ƙB) [21,23,27]. All aerobic living creatures encounter a paradox named molecular oxygen (O_2). On one hand, aerobic life is completely dependent on O_2; on the other hand, cell O_2 could create products such as $OH°$, anion peroxide ($O2°$), and hydrogen peroxide (H_2O_2) during chemical reactions, after irradiation with ionizing radiation. These products have negative effect on the function and structure of the living cells and could be a reason for cell death [33,36]. It is believed that O_2 is a two-edge-blade. The derivatives of O_2 known as ROS are the main factors that induce infertility in men. ROS has a dual effect on the function and structure of the sperm cell. While it is necessary for some normal processes of acrosomic reactions in sperm cells, it (in high concentration, known as oxidative stress) could restrain motility of the sperm and change its morphology, resulting sperm infertility. Thus, basis on such data, pretreatment with SM offers protection to normal spermatocytes against possible the effects of γ-radiation-induced cellular damage. Fortunately, SM possesses wide range of in vitro and in vivo studies at various levels. Since successful development of radio-protectors needs a clear understanding of molecular radiobiology, tissues' response to ionizing radiation, and differentiation between normal cells and tumor. Therefore, this work will make for another issue of SM as a radioprotective agent in the future.

5. Conclusion

In conclusion, our finding show that the SM as an active phytochemical can be a factor influencing maintenance of sperm characteristics following gamma irradiation exposure which may act by preventing the stress oxidative and inflammatory activity as well as enhancing the antioxidant defense system or regeneration of sperm cells. Hence, it can be considered as an herbal radioprotective agent in complementary medicine to prevent or reduce adverse effects during long-term radiation therapy.

Conflicts of Interest

The authors declare no conflict of interest.

References

[1] A.-F. Holstein, W. Schulze, M. Davidoff, Understanding spermatogenesis is a pre-requisite for treatment, Reprod. Biol. Endocrinol. 1 (2003) 107.
[2] A. Rostamzadeh, M. Mohammadi, R. Ahmadi, A. Nazari, O. Ghaderi, M. Anjomshoa, Evaluation of mouse embryos produced in vitro after electromagnetic waves exposure; morphometric study, Electron. Physician 8 (2016) 1701.
[3] G. Take, D. Erdogan, F. Helvacioglu, G. Göktas, G. Ozbey, C. Uluoglu, B. Yücel, Y. Guney, A. Hicsonmez, S. Ozkan, Effect of melatonin and time of administration on irradiation-induced damage to rat testes, Braz. J. Med. Biol. Res. 42 (2009) 621–628.
[4] E. Cordelli, A. Fresegna, G. Leter, P. Eleuteri, M. Spano, P. Villani, Evaluation of DNA damage in different stages of mouse spermatogenesis after testicular X irradiation, Radiat. Res. 160 (2003) 443–451.
[5] C. Paul, D.W. Melton, P.T. Saunders, Do heat stress and deficits in DNA repair pathways have a negative impact on male fertility? Mol. Hum. Reprod. 14 (2008) 1–8.
[6] W. Sapp, D. Philpott, C. Williams, J. Williams, K. Kato, J. Miquel, L. Serova, Comparative study of spermatogonial survival after X-ray exposure, high LET (HZE) irradiation or spaceflight, Adv. Space Res. 12 (1992) 179–189.
[7] W.J. Sapp, D.E. Philpott, C.S. Williams, K. Kato, J. Stevenson, M. Vasquez, L. Serova, Effects of spaceflight on the spermatogonial population of rat seminiferous epithelium, FASEB J. 4 (1990) 101–104.
[8] R.J. Reiter, D.-X. Tan, T.S. Herman, C.R. Thomas, Melatonin as a radioprotective agent: a review, Int. J. Radiat. Oncol. Biol. Phys. 59 (2004) 639–653.

[9] D. Fatehi, M. Anjomshoa, M. Mohammadi, M. Seify, A. Rostamzadeh, Biological effects of cell-phone radiofrequency waves exposure on fertilization in mice; an in vivo and in vitro study, Middle East Fertil. Soc. J. (2017), http://dx.doi.org/10.1016/j.mefs.2017.10.002.

[10] C. Payne, C. Bjore, D. Schultz, Change in the frequency of apoptosis after low-and high-dose X-irradiation of human lymphocytes, J. Leukoc. Biol. 52 (1992) 433–440.

[11] M. Koruji, M. Movahedin, S.J. Mowla, H. Gourabi, A. Jabbary Arfaee, The morphological changes of adult mouse testes after ^{60}Co γ-radiation, Iran. Biomed. J. 12 (2008) 35–42.

[12] A. Young, R. Berry, A.F. Holloway, N.B. Blackburn, J.L. Dickinson, M. Skala, J.L. Phillips, K.H. Brettingham-Moore, RNA-seq profiling of a radiation resistant and radiation sensitive prostate cancer cell line highlights opposing regulation of DNA repair and targets for radiosensitization, BMC Cancer 14 (2014) 808.

[13] H. Paganetti, B.S. Athar, M. Moteabbed, J.A. Adams, U. Schneider, T.I. Yock, Assessment of radiation-induced second cancer risks in proton therapy and IMRT for organs inside the primary radiation field, Phys. Med. Biol. 57 (2012) 6047.

[14] E.M. Rosen, R. Day, V.K. Singh, New approaches to radiation protection, Front. Oncol. 4 (2014).

[15] R. Baskar, K.A. Lee, R. Yeo, K.-W. Yeoh, Cancer and radiation therapy: current advances and future directions, Int. J. Med. Sci. 9 (2012) 193–199.

[16] B. Hazra, S. Ghosh, A. Kumar, B. Pandey, The prospective role of plant products in radiotherapy of cancer: a current overview, Front. Pharmacol. 2 (2011) 94.

[17] G. Karimi, M. Vahabzadeh, P. Lari, M. Rashedinia, M. Moshiri, "Silymarin", a promising pharmacological agent for treatment of diseases, Iran J. Basic Med. Sci. 14 (2011) 308–317.

[18] M. Mohammadi, A. Rostamzadeh, A. Moayeri, A. Allahveisi, H.R. Mohammadi, M.J. Rezaie, Role of Silymarin in regeneration and treatment of skin disorders; progress in signaling pathways, RJPBCS 7 (2016) 1844–1849.

[19] H. Masoumi, M. Anjamshoa, M. Mohammadi, S. Fallahi, A. Rostamzadeh, Clinical and pharmacological applications of Silymarin components at cellular and molecular level: a review, Res. J. Med. Sci. 10 (2016) 102–107.

[20] P.F. Surai, Silymarin as a natural antioxidant: an overview of the current evidence and perspectives, Antioxidants 4 (2015) 204–247.

[21] H. Greenlee, K. Abascal, E. Yarnell, E. Ladas, Clinical applications of *Silybum marianum* in oncology, Integra. Cancer Ther. 6 (2007) 158–165.

[22] D. Awang, Milk thistle, Can. Pharm. J. 126 (1993) 422.

[23] L.A. Ramadan, H.M. Roushdy, G.M.A. Senna, N.E. Amin, O.A. El-Deshw, Radioprotective effect of Silymarin against radiation induced hepatotoxicity, Pharmacol. Res. 45 (2002) 447–454.

[24] M. Adhikari, A. Dhaker, J. Adhikari, V. Ivanov, V. Singh, R. Chawla, R. Kumar, R. Sharma, Y. Karamalakova, V. Gadjeva, In vitro studies on radioprotective efficacy of silymarin against γ-irradiation, Int. J. Radiat. Biol. 89 (2013) 200–211.

[25] M. Adhikari, R. Arora, Nano-silymarin provides protection against γ-radiation-induced oxidative stress in cultured human embryonic kidney cells, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 792 (2015) 1–11.

[26] G. Deep, R. Agarwal, Antimetastatic efficacy of silibinin: molecular mechanisms and therapeutic potential against cancer, Cancer Metastasis Rev. 29 (2010) 447–463.

[27] H.G. Oufi, N.N. Al-Shawi, The effects of different doses of silibinin in combination with methotrexate on testicular tissue of mice, Eur. J. Pharmacol. 730 (2014) 36–40.

[28] P. Tiwari, A. Kumar, M. Ali, K. Mishra, Radioprotection of plasmid and cellular DNA and Swiss mice by silibinin, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 695

[29] I.S. Chen, Y.C. Chen, C.H. Chou, R.F. Chuang, L.Y. Sheen, C.H. Chiu, Hepatoprotection of silymarin against thioacetamide-induced chronic liver fibrosis, J. Sci. Food Agric. 92 (2012) 1441–1447.

(2010) 55–60.

[30] T.G. Cooper, E. Noonan, S. Von Eckardstein, J. Auger, H.G. Baker, H.M. Behre, T.B. Haugen, T. Kruger, C. Wang, M.T. Mbizvo, World Health Organization reference values for human semen characteristics, Hum. Reprod. Update 16 (2010) 231–245.

[31] R. Tejada, J.C. Mitchell, A. Norman, J. Marik, S. Friedman, A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence, Fertil. Steril. 42 (1984) 87–91.

[32] S. Vega, P. Guzman, L. Garcia, J. Espinosa, C.C. de Nava, Sperm shape abnormality and urine mutagenicity in mice treated with niclosamide, Mutat. Res. Genet. Toxicol. 204 (1988) 269–276.

[33] A. Wong, S.S. Chuan, W.C. Patton, J.D. Jacobson, J. Corselli, P.J. Chan, Addition of eosin to the aniline blue assay to enhance detection of immature sperm histones, Fertil. Steril. 90 (2008) 1999–2002.

[34] C. Shaha, R. Tripathi, D.P. Mishra, Male germ cell apoptosis: regulation and biology, Philos. Trans. R. Soc. Lond. B Biol. Sci. 365 (2010) 1501–1515.

[35] S. Khan, J.S. Adhikari, M.A. Rizvi, N.K. Chaudhury, Radioprotective potential of melatonin against ^{60}Co γ-ray-induced testicular injury in male C57BL/6 mice, J. Biomed. Sci. 22 (2015) 1–15.

[36] A. Bonisoli-Alquati, A.P. Møller, G. Rudolfsen, N. Saino, M. Caprioli, S. Ostermiller, T.A. Mousseau, The effects of radiation on sperm swimming behavior depend on plasma oxidative status in the barn swallow (*Hirundo rustica*), Comp. Biochem. Physiol. A Mol. Integr. Physiol. 159 (2011) 105–112.

[37] G. Delaney, S. Jacob, C. Featherstone, M. Barton, The role of radiotherapy in cancer treatment, Cancer 104 (2005) 1129–1137.

[38] S.K. Katiyar, S. Meleth, S.D. Sharma, Silymarin, a flavonoid from milk thistle (*Silybum marianum* L.), inhibits UV-induced oxidative stress through targeting infiltrating CD11b + cells in mouse skin, Photochem. Photobiol. 84 (2008) 266–271.

[39] M. Marzban, M. Anjamshoa, P. Jafari, H. Masoumi, R. Ahadi, D. Fatehi, Effects of gamma rays on rat testis tissue according to the morphological parameters and immunohistochemistry: radioprotective role of silymarin, Electron. Physician 9 (2017) 4524.

[40] H.H. Mansour, H.F. Hafez, N.M. Fahmy, Silymarin modulates cisplatin-induced oxidative stress and hepatotoxicity in rats, J. Biochem. Mol. Biol. 39 (2006) 656.

[41] H.R. Momeni, N. Eskandari, Effect of vitamin E on sperm parameters and DNA integrity in sodium arsenite-treated rats, Iran J. Reprod. Med. 10 (2012) 249–256.

[42] V. Lobo, A. Patil, A. Phatak, N. Chandra, Free radicals, antioxidants and functional foods: impact on human health, Pharmacol. Rev. 4 (2010) 118.

[43] V. Hajhashemi, G. Vaseghi, M. Pourfarzam, A. Abdollahi, Are antioxidants helpful for disease prevention? Res. Pharma. Sci. 5 (2010) 1.

[44] L.A. Pham-Huy, H. He, C. Pham-Huy, Free radicals, antioxidants in disease and health, IJBS 4 (2008) 89.

[45] M. Ahmadi, A. Rostamzadeh, F. Fathi, M. Mohammadi, M.J. Rezaie, The effect of Melatonin on histological changes of ovary in induced polycystic ovary syndrome model in mice, Middle East Fertil. Soc. J. 22 (2017) 255–259.

[46] M. Mohammadi, M.J. Rezaie, A. Rostamzadeh, A. Allahveisi, H.R. Mohammadi, F. Mohammadi, Signaling pathways of melatonin in prevention of liver disorders via suppressing of oxidative stress in cellular level, Biomed. Pharmacol. J. 9 (2016) 555–561.