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Ameliorating Effects of Silymarin in Combination with Zinc Against Radiation

Efectos de Mejora de la Silimarina en Combinación con Zinc Contra la Radiación

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SUMMARY: This study was aimed to evaluate the radioprotective effects of Silymarin (SIL) and zinc alone, and in combination with each other against irradiation (IR) testicular toxicity in mice. Sixty-four male adult NMRI mice were allocated into eight groups, including the control group; SIL treated group; zinc treated group; SIL+Zinc treated group; IR group which irradiated with 2 Gy gamma rays; IR+SIL group; IR+Zinc group; and IR+SIL+Zinc group. Treatments commenced for eight consecutive days and then mice were exposed to IR. One day after irradiation, four animals of each group were sampled and both testes were harvested for determination of the biochemical (MDA, GSH, SOD, and CAT) and molecular (Caspase-3, P21, TNF-α, IL-1β, and NF-κβ) parameters. Fourteen days after the irradiation, four other animals in each group were harvested to assessments of histological, stereological, and sperm parameters as well as the testosterone levels. We found that the sperm parameters, testosterone levels, the numerical density of spermatogonia, primary spermatocytes, spermatids, sertoli, leydig cells, and seminiferous tubules, and biochemical factors (except MDA) were significantly higher in the IR+SIL, IR+Zinc, and IR+SIL+Zinc groups, especially in IR+SIL+Zinc ones, compared to IR group. This is while expression of apoptotic and inflammatory genes, and MDA levels considerably decreased in the IR+SIL, IR+Zinc, and IR+SIL+Zinc groups compared to IR group and it was more pronounced in the IR+SIL+Zinc ones. Generally, SIL and zinc attenuated cellular and molecular disorders induced by gamma-radiation in the testis and these protective effects were more pronounced in the combined treatment group.

KEY WORDS: Radiation; Silymarin; Zinc; Oxidative Stress; Radioprotective; Testicular Toxicity.

INTRODUCTION

The main function of the testes is producing and storing sperm (spermatogenesis) which is essential for reproduction, and also the synthesis of hormones playing an important role in the development of male sex characteristics (Djureinovic *et al*., 2014). Spermatogenesis is a highly synchronized, regular, long and complex process that takes place in the germinal epithelium of the testes (Raoofi *et al*., 2023). 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 (Fatehi *et al*., 2018). 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 (Ehghaghi *et al*., 2022). Payne *et al*. (1992) 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. 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) (Huang *et al*., 2022). X- and γ-ray can affect the cells directly/indirectly and produce free radicals, oxidative stress and reactive oxygen species (ROS) (Soliman *et al*., 2020). Radioprotector agents can protect normal cells against the destructive effect of radiation in radiotherapy (Musa *et al*., 2019).

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Radioprotectors decrease bystander effects of ionizing radiation, consequently, reducing the side effects of radiotherapy (Sheikholeslami *et al*., 2021). There is a long history of the application of radioprotectors, namely chemical drugs as well as antioxidants. 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 (Raj *et al*., 2022). Silymarin (SIL), 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. Combined, these compounds represented about 65–80 % of the total extract composition (Singh *et al*., 2023). In recent years, SIL is widely prescribed as a complementary and alternative treatment in preventing and reversing various diseases/disorders (Soleimani *et al*., 2019; Gillessen & Schmidt, 2020; Fallah *et al*., 2021). In addition, SIL has been shown to be more potent in antioxidant capacity as compared to vitamin E (Poulos *et al*., 2022). Ramadan *et al*. (2002) documented that the SIL act as a radioprotective agent against radiation induced hepatotoxicity in rat. In an *in vitro* study, Adhikari & Arora (2015) showed that specific dose (25 mg/ml) of SIL can ameliorate the deleterious effects of γ–radiation on human embryonic kidney cells.

Moreover, zinc as antioxidant and anti-inflammatory has an important role in many biological and physiological processes such as cell membrane integrity and scavenger of free radicals (Bashandy *et al*., 2016; Fallah *et al*., 2018). Previous studies show radioprotective effects of zinc on many systems especially reproductive, gastrointestinal, blood cells, visual, and endocrine (Ramadan *et al*., 2013; Akbulut *et al*., 2018). Protective role of zinc in reproductive system is significant via increasing germ cell survival and sperm count, promotion of sperm capacitation, secretion of higher level of testosterone, and progression of spermatogenesis (Ramadan *et al*., 2013).

Therefore, with regard to the antioxidant, anti-inflammatory, and anti-apoptotic properties of SIL and zinc, the aim of the current study was to evaluate the protective effects of SIL, zinc, and SIL plus zinc against gamma-ray-induced testicular toxicity in the mice.

MATERIAL AND METHOD

Ethics Approval. All experimental protocols were approved by Ethics Committee of Sabzevar University of Medical Sciences, Sabzevar, Iran (Ethic no: IR.MEDSAB.REC. 1400.094). The animals were handled in accordance to the protocol of animal management and welfare of the University of Helsinki, Finland. Animals were kept in a laboratory standard hutch with no limitation access to a rodent's food and drinking water. All methods were carried out in accordance with relevant guidelines and regulations. The study was carried out in compliance with the ARRIVE guidelines. All materials used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA), except where assigned otherwise.

Animal and experimental design. A total of 64 male adult NMRI mice (25–30g) were recruited. The animals were randomly planned into 8 equal groups, including (I) the untreated (Control) group; (II) SIL treated group which received only SIL daily for 8 days; (III) Zinc treated group which received only zinc daily for 8 days; (IV) SIL+Zinc treated group which received SIL and zinc at the same volume and times as the SIL and zinc groups; (V) IR group which irradiated with 2 Gy gamma rays to whole body without any drug treatment; (VI) IR+SIL group which received SIL for eight consecutive days and then mice were irradiated with 2 Gy gamma rays; (VII) IR+Zinc group which received zinc for eight consecutive days and then mice were irradiated with 2 Gy gamma rays; and (VIII) IR+SIL+Zinc group which received a combination of SIL and zinc for eight consecutive days and then mice were irradiated with 2 Gy gamma rays. The selected doses and administration method for the SIL (200 mg/kg) and zinc (50 mg/kg) were based on previous studies and our pilot experiments (Qin *et al*., 2019; Abo El-Atta & Ahmed, 2020; Rahimi-Madiseh *et al*., 2020). Moreover, both drugs were administered by intraperitoneal (ip) injection dissolved in volume 50 µl diluted in saline.

One day after irradiation, four animals of each group were anesthetized by ip injection of ketamine & xylazine (10 mg/kg: 80 mg/kg) and both testis were harvested for determination of the biochemical and molecular parameters (Amiri *et al*., 2022), and then the mice were euthanized with a sodium thiopental overdose (200 mg/kg, ip). While histological, sperm parameters, as well as the testosterone levels assessments had been performed on 14th day as described in Figure 1. For this purpose, first the animals were anesthetized and then blood was collected by cardiac puncture to evaluate testosterone levels. Immediately after that, the scrotum opened and the tails of the epididymis were isolated for semen analysis. Finally, testis tissues were harvested and one of them was isolated for structural and stereological evaluations (Amiri *et al*., 2022). The study has been carried out through the unbiased application of stereological tools.

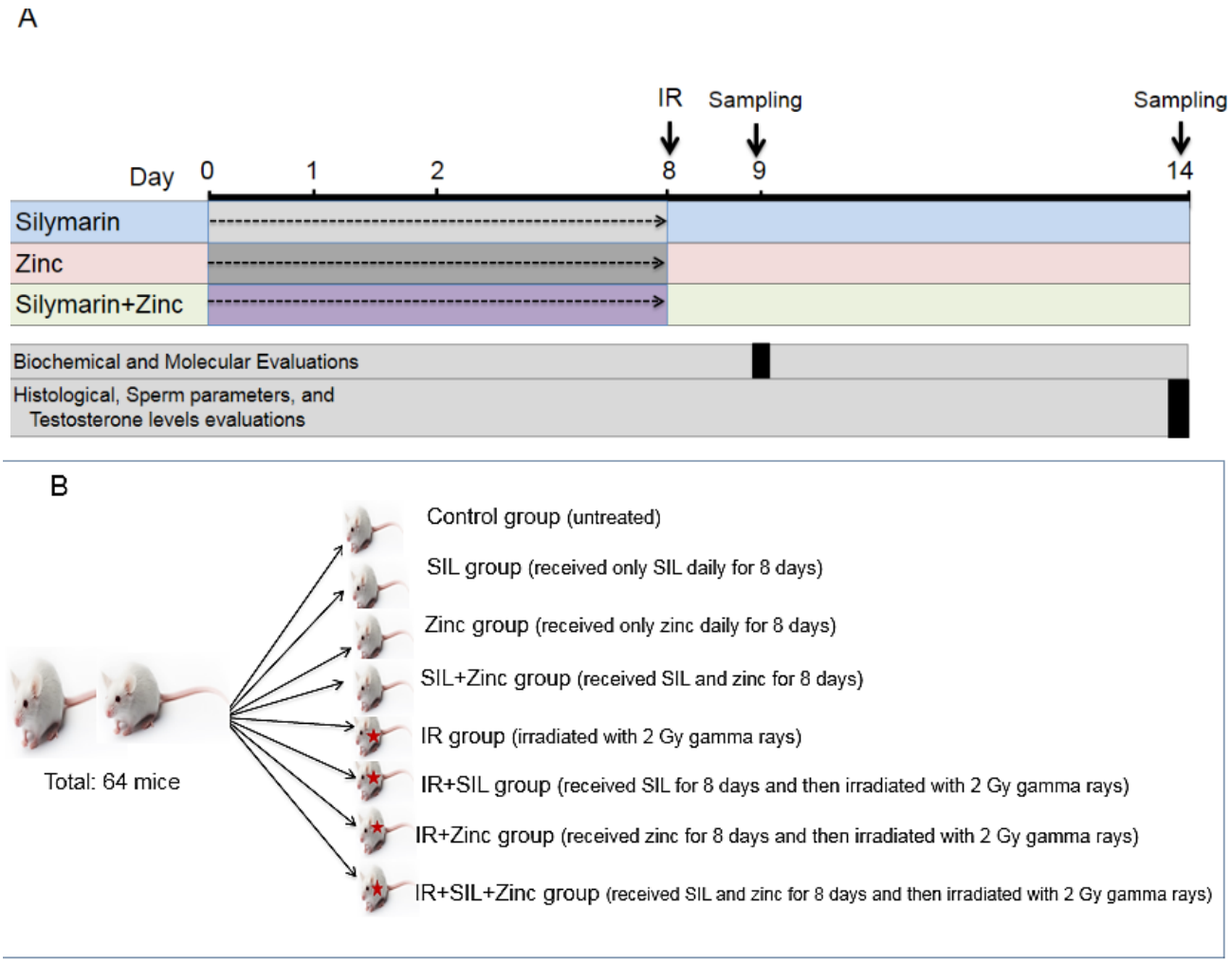

Fig. 1. Scheme of the experimental design. **A.** To irradiation, a Cobalt-60 machine (Teratron 780, Canada) in a radiotherapy unit was used. In IR received groups, SIL, zinc, and SIL+zinc were received for eight consecutive days. Sampling was done in 2 time points. Twenty four hours after IR, 4 mice from each group were sampled for qRT-PCR and biochemical evaluations. The remaining 4 mice in each group were sampled for histological, sperm parameters, and testosterone levels evaluations on day 14. **B.** Sixty four male adult NMRI mice were randomly planned into 8 equal groups, including (I) Control group; (II) SIL group; (III) Zinc group; (IV) SIL+Zinc group; (V) IR group; (VI) IR+SIL group; (VII) IR+Zinc group; and (VIII) IR+SIL+Zinc group (The red star indicates IR in the studied groups).

Irradiation method. A Cobalt-60 machine (Teratron 780, Canada) in a radiotherapy unit was used as a source of irradiation. The mice were restrained in well-ventilated boxes and exposed the whole body radiation. One dose of 2 Gy was applied as a single fraction at a dose rate of 50 cGy/min. Moreover, the source-to-skin distance was chosen 80 cm (Bagheri *et al*., 2020).

Testosterone evaluation. The collected blood was placed in a centrifuge with 2500 x g for 15 min and blood serum was isolated. To determination of testosterone levels, ELISA Kits (Shanghai Yi Li Biological Technology, Shanghai, China) was used. For this purpose, the samples were added to 96-well microplates and absorbance was read at 640 nm using a microplate reader (Biocompare; Agilent Technologies) (Nasiry *et al*., 2021). All samples were performed in three replicates.

Semen Analysis. After isolation the semen samples from the epididymis tails, immediately transferred to 1 mL of Ham's F-10 medium and incubated for 20 min at 37° C. To evaluate the sperm count, counting chamber was used. In order to evaluate the sperm motility, 10 mL of the samples were separated and assessed with inverted microscope. The sperm viability was exanimated by eosin-nigrosine staining. Moreover, sperm morphology was assessed using Diff Quik staining kit (Tavares *et al*., 2013).

Histological and stereological assessments. At the end of the study in each group, after sacrificing the animals, the right testicle was removed and fixed in 4 percent paraformaldehyde. Then, after tissue processing and molding in paraffin, ten sections with equal distances from each other were selected and stained with hematoxylin and eosin (H&E). Histological evaluation was Johnson test to assess the tissue structure. Stereological evaluations were the total volumes of testis tissue, length density of seminiferous tubules, and numerical densities of spermatogonia, primary spermatocytes, spermatids, sustentacular cell (Sertoli cell), and interstitial cell (Leydig cell). To measure the total volumes, a thickness of 5 μm was used, and a thickness of 20 μm was used to determine the numerical density of cells and length density of seminiferous tubules (Howard & Reed, 2004).

Testicular tissue structure. After selecting and staining tissue sections, 5 areas of each section with a magnification of 400 were photographed. The areas were in the upper and lower right and left corners as well as the center of the tissue. Then, using Johnsen scoring system, the extent of tissue structure damage was assessed. In this scoring system, the minimum and maximum scores are 1 and 10, respectively, which are related to the absence of any epithelium in the seminiferous tubules and the completeness of spermatogenesis, and the presence of countless spermatozoa, respectively (Nasiry *et al*., 2021).

Testicular total volumes. To measure the volume of the testicular tissue, stereological evaluation was performed based on the Cavalieri method (Howard & Reed, 2004). For this purpose, a grid of points was superimposed on the micrographs which were taken from the 10 selected sections of each sample. Next, the total volumes were estimated via counting the points that overlay on each tissue and using the following formula:

$$V_{total} = \sum P \times \frac{a}{p} \times t$$

Σ: total counted points; a (area)/p (point); t: thickness.

The numerical density of cells and seminiferous tubules. To the assessment of the numerical density (Nv) of spermatogonia, primary spermatocytes, spermatids, Sertoli, and Leydig cells, the optical dissector method was used using the following formula (Howard & Reed, 2004):

$$N_v = \frac{\sum Q}{\sum P \times h \times \frac{a}{f}} \times \frac{t}{BA}$$

ΣQ: cell number (nucleus); h: height of the dissector; Σp: the total number of each cells that counted inside the probe; a (area)/ f (field); BA: block advance of the microtome; t: section thickness.

Moreover, the below formula was used to assess length density of seminiferous tubules:

ΣQ: the total number of seminiferous tubules; Sp: the total number of each cells that counted inside the probe; a (area)/

$$L_v = \frac{2\sum Q}{\sum P \times \frac{a}{f}}$$

f (field)

Biochemical evaluation. After washing the harvested samples with sterile PBS to remove excess tissue residues, they were immediately frozen at -80°C for further analysis. To assess the biochemical status at the site of injury, concentrations of catalase (CAT), glutathione (GSH), and superoxide dismutase (SOD), as antioxidant biomarkers, and malondialdehyde (MDA), as oxidant factor, have been investigated. Briefly, to measure the levels of CAT, hydrogen peroxide was used together with phosphate buffer and the absorbance was recorded at a wavelength of 245 nm (Aebi, 1974). To measure GSH and SOD levels, trichloroacetic and pyrogallol were used, respectively, and absorptions were recorded at 420 nm and 412 nm wavelengths, respectively, using spectrophotometry (Ellman, 1959; Sun *et al*., 1988). Finally, the concentration of MDA was measured using thiobarbituric acid and the absorbance was recorded at a wavelength of 520 nm (Mihara & Uchiyama, 1978).

Gene expression evaluation. In the present study, we measured the expression level of three genes effective in apoptosis (Caspase-3 and P21), and three inflammatory genes (TNF-α, IL-1β, and NF-κβ) using quantitative real-time PCR (qRT-PCR) method. For this purpose, the total RNA was gathered using RNeasy Mini Kit (Qiagen). Next, cDNA synthesis was done using iScript™ cDNA Synthesis Kit (Bio-Rad) (Nasiry *et al*., 2022). In qRT-PCR, three biological replicates were performed using a real-time PCR instrument (Bio-Rad) and harvested data were analyzed using the fold change method. The sequences of primers used are shown in Table I. In addition, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Table I. Primer sequences used for gene expression analysis using quantitative RT-PCR.

Gene	Sequence (5 ' > 3 ')
Caspase-3	**F:** AAGATACCGGTGGAGGCTGA **R:** AAGGGACTGGATGAACCACG
P21	**F:** CCTGGTGATGTCCGACCTG **R:** CCATGAGCGCATCGCAATC
TNF-α	**F:** TTGACCTCAGCGCTGAGTTG **R:** CCTGTAGCCCACGTCGTAGC
IL-1 β	**F:** TGCCACCTTTTGACAGTGATG **R:** TGATGTGCTGCTGCGAGATT
NF-κβ	**F:** GCCAGACACAGATGATCGCC **R:** GTTTCGGGTAGGCACAGCAA
GAPDH	**F:** TGGCCTTCCGTGTTCCTAC **R:** GAGTTGCTGTTGAAGTCGCA

Statistical analysis. Utilizing SPSS software (version 21), the data was analyzed. A one-way analysis of variance test (ANOVA) with Tukey's post hoc test was used to evaluate the relationships between groups. The outcomes were shown as mean ± standard deviation (SD). The threshold for statistical significance was a p-value of 0.05.

RESULTS

As it is clear in all the graphs, the control and non-irradiated groups were better in comparison with other groups. Therefore, a comparison of the results has been done between the irradiated groups.

Sperm characteristics. The results of sperm characteristics are shown in Figure 2A-D. The results showed that the IR+SIL, IR+Zinc, and IR+SIL+Zinc groups had significantly higher sperm count (P<0.01, P<0.05, and P<0.0001, respectively), viability (P<0.01, P<0.05, and P<0.0001, respectively), motility (P<0.05, P<0.05, and P<0.001, respectively), and morphology (P<0.05, P<0.05, and P<0.001, respectively) percentage compared to the IR group. Moreover, a comparison of the sperm characteristics between treated groups showed that the IR+SIL+Zinc group compared with the IR+SIL and IR+Zinc groups had significantly higher amounts of cell count (P<0.05 and P<0.01, respectively), viability (P<0.01 and P<0.001, respectively), and morphology (both, P<0.05).

Testosterone levels. The results of testosterone levels evaluation are shown in Figure 3. We found that there were significantly more testosterone levels in the IR+SIL, IR+Zinc, and IR+SIL+Zinc groups in comparison to IR group (P<0.05, P<0.05, and P<0.001, respectively). Furthermore, testosterone level in the IR+SIL+Zinc group was higher than the IR+SIL and IR+Zinc groups, but they were not significant (P>0.05).

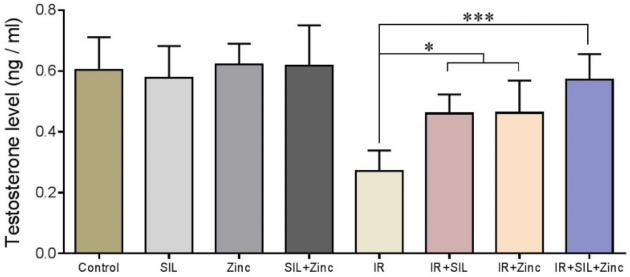

Fig. 3. Effects of Silymarin-zinc combination on testosterone levels in the mice induced by irradiation. Data are presented as Mean ± SD. *p<0.05; ***p<0.001.

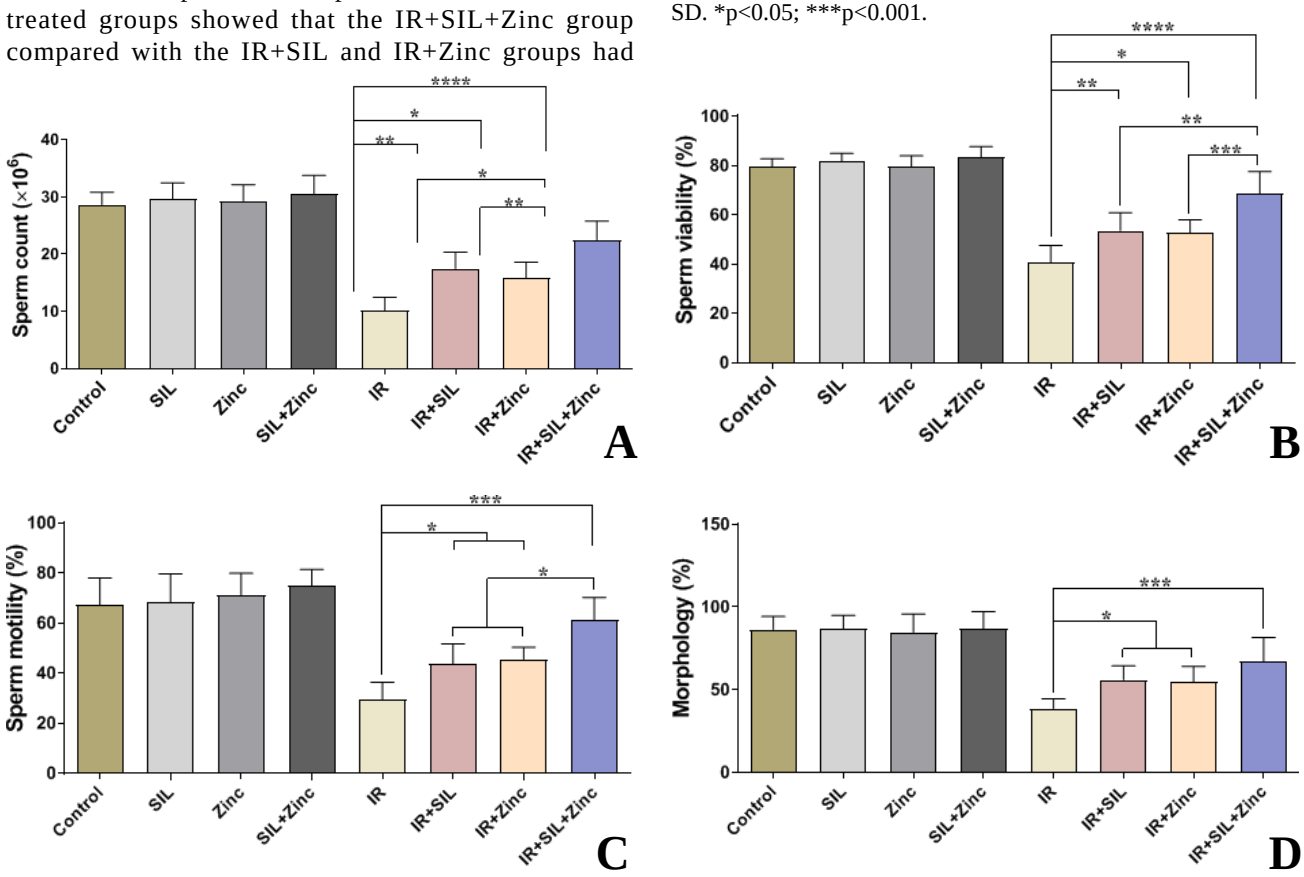

Fig. 2. Effects of Silymarin-zinc combination on sperm characteristics in the mice induced by irradiation. Comparing (A) sperm count, (B) viability, (C) motility, and (D) morphology in the study groups. Data are presented as Mean ± SD. *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

Histological evaluations and Stereological parameters.

Structure of testes. To investigate the histological structure and the amount of stereological parameters, H&E staining was performed (Fig. 4A). The results of tissue structure evaluation using Johnsen scoring system showed that the IR+SIL, IR+Zinc, and IR+SIL+Zinc groups were considerably higher scores in comparison to IR group ($P<0.01$, $P<0.001$, and $P<0.0001$, respectively). Moreover, the tissue structure status was considerably better in the IR+SIL+Zinc group in comparison to the IR+SIL and IR+Zinc groups (both, $P<0.05$) (Fig. 5B).

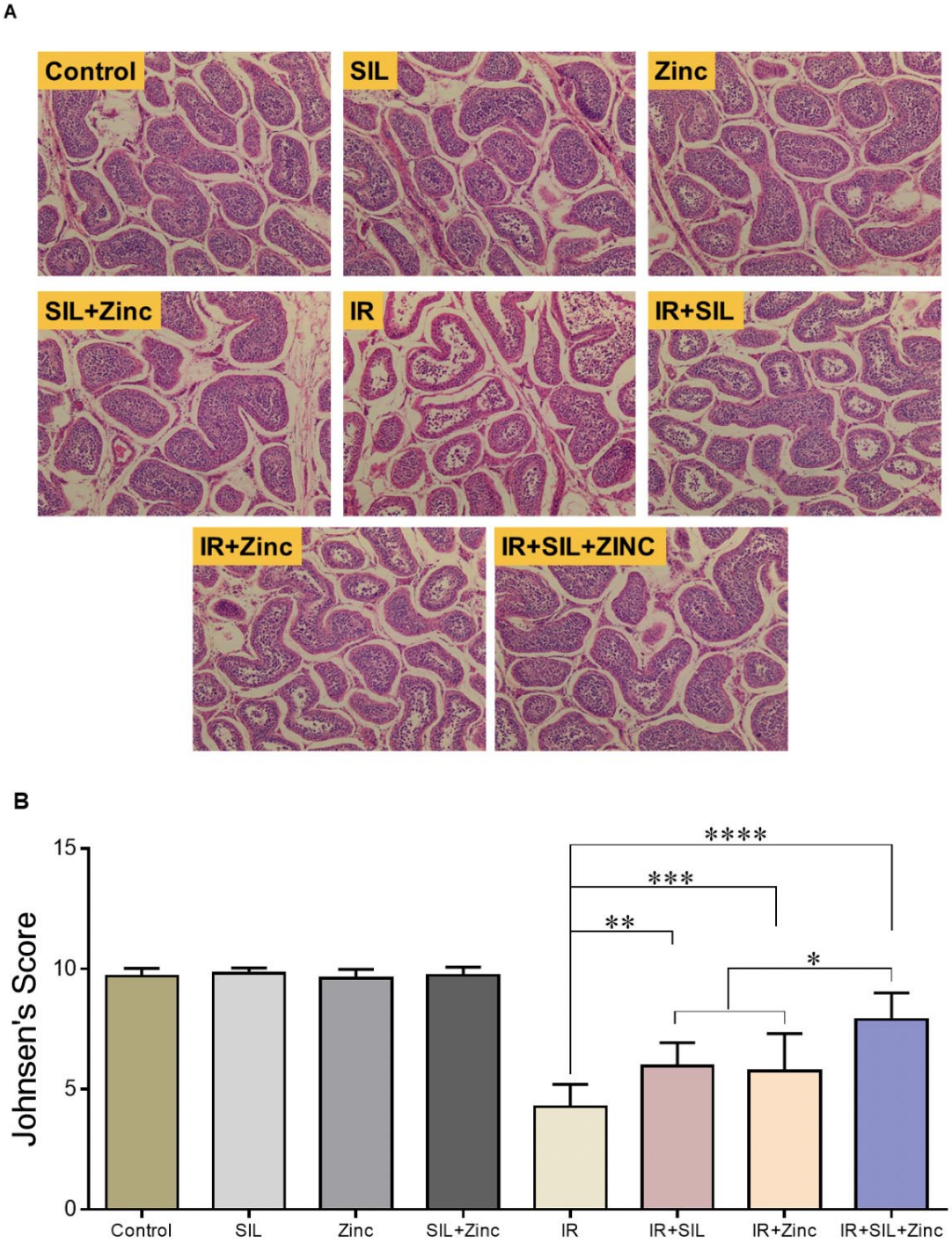

Fig. 4. Effects of Silymarin-zinc combination on histological structure in the mice induced by irradiation. (A) Histological photomicrographs stained with H&E. (B) Comparing histological structure based on Johnson scores in experimental groups. Data are presented as Mean ± SD. *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

Volume of testis. The results indicated that there was a significantly higher volume of testis in the IR+SIL+Zinc group in comparison to the IR group (P<0.05). Furthermore, testis volume in the IR+SIL and IR+Zinc groups were higher than the IR group, but they were not significant (P>0.05) (Fig. 5A).

The numerical cells densities and length density of seminiferous tubules. The quantitative results for stereological evaluations are shown in Figure 5B-C. Considering spermatogonia, we observed that these cells were significantlyhigher in the IR+SIL+Zinc group compared to the IR group (P<0.001).

Comparison of primary spermatocyte density between groups showed there was significantly more cells in the IR+SIL, IR+Zinc, and IR+SIL+Zinc groups compared to the IR group (P<0.05, P<0.05, and P<0.01, respectively).

Evaluation of spermatid revealed that the numerical density of these cells increased considerably in the IR+SIL and IR+SIL+Zinc groups compared to the IR group (P<0.05 and P<0.001, respectively). In addition, in the IR+SIL+Zinc group, there were significantly more cells in comparison to the IR+SIL and IR+Zinc groups (both, P<0.05).

Considering the Sertoli cells, we found that the IR+SIL+Zinc group had significantly higher cells in comparison to the IR group (P<0.05).

Finally, in comparison of numerical density of Leydig cells, we observed that the IR+SIL+Zinc group had considerably more cells in comparison to the IR group (P<0.01).

Comparison of the length density of seminiferous tubules indicated that the IR+SIL+Zinc group had significantly more tubules in comparison to the IR group (P<0.01).

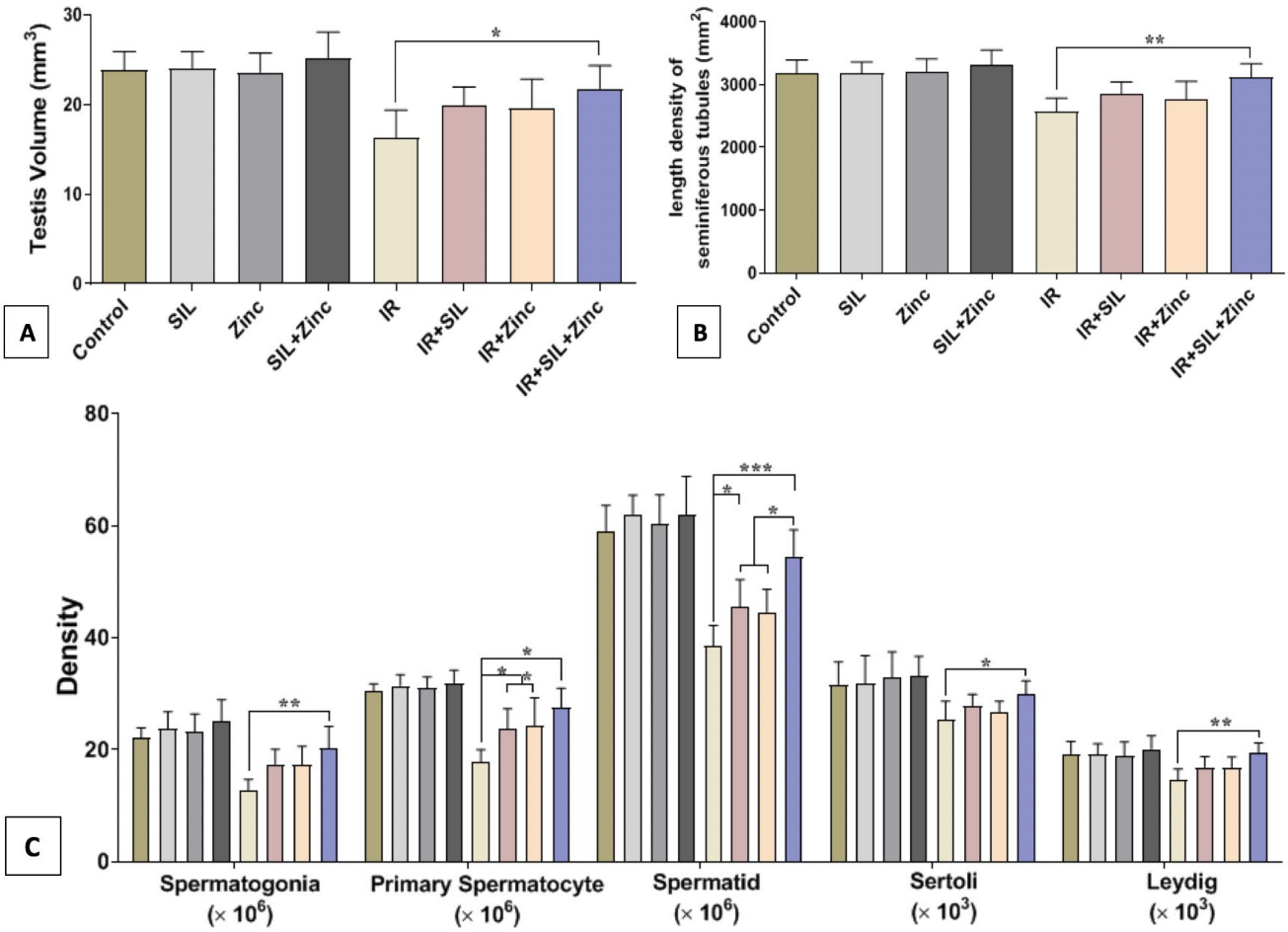

Fig. 5. Effects of Silymarin-zinc combination on stereological parameters in mice induced by irradiation. (A) Volumes of testis calculated by Cavalieri method. (B) Length density of seminiferous tubules and (C) numerical density of spermatogonia, primary spermatocyte, spermatid, Sertoli, and Leydig cells. Data are presented as Mean ± SD. *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

Biochemical parameters. The results of the evaluation of biochemical biomarkers are shown in Figure 6A. Assessment of the MDA levels indicated that the IR+SIL, IR+Zinc, and IR+SIL+Zinc groups had considerably lower MDA levels than the IR group (P<0.01, P<0.01, and P<0.001, respectively).

Determination of antioxidative biomarkers levels showed that the IR+SIL, IR+Zinc, and IR+SIL+Zinc groups compared with the IR group had significantly higher GSH (P<0.01, P<0.05, and P<0.001, respectively), SOD (P<0.05, P<0.05, and P<0.01, respectively), and CAT (P<0.05, P<0.05, and P<0.001, respectively) levels.

Gene expression levels. In evaluating the expression levels of apoptotic genes among the study groups, we found that the IR+SIL, IR+Zinc, and IR+SIL+Zinc groups had considerably lower levels of caspase-3 (P<0.01, P<0.01, and P<0.0001, respectively) and P21 (P<0.001, P<0.01, and P<0.0001, respectively) genes in compared to IR group. Furthermore, the expression level of caspase-3 gene in the IR+SIL+Zinc group was significantly lower than the IR+SIL and IR+Zinc groups (both, P<0.05) (Fig. 6B).

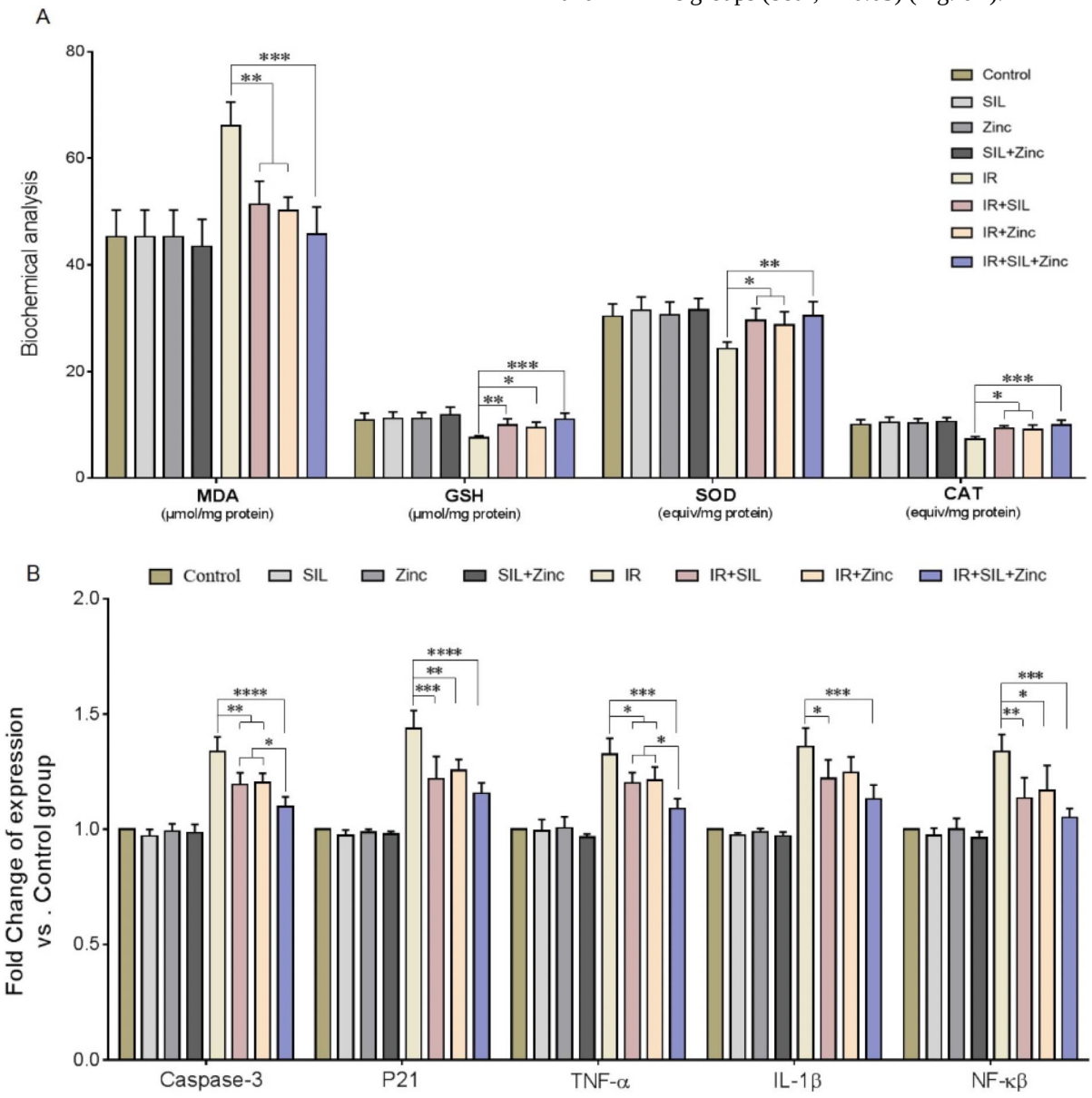

Fig. 6. Effects of Silymarin-zinc combination on testicular oxidative biomarkers and gene expression in the mice induced by irradiation. (A) Concentrations of oxidant (MDA) and antioxidant (GSH, SOD, and CAT) factors on the testis tissues were determined by the biochemistry method. (B) The amount of transcripts for three genes effective in apoptosis (Caspase-3 and P21), and three inflammatory genes (TNF-α, IL-1β, and NF-κβ) were analyzed using qRT-PCR. Data are presented as Mean ± SD. *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

Regarding the inflammatory genes, we found that in the IR+SIL, IR+Zinc, and IR+SIL+Zinc groups, compared to the IR group, the expression levels of TNF-α ($P<0.05$, $P<0.05$, and $P<0.001$, respectively) and NF-κβ ($P<0.01$, $P<0.05$, and $P<0.001$, respectively) genes were significantly reduced. However, regarding the IL-1β gene, only the IR+SIL and IR+SIL+Zinc groups were significantly decreased compared to the IR group ($P<0.05$ and $P<0.001$, respectively). Moreover, the expression level of TNF-α gene in the IR+SIL+Zinc group was significantly lower than the IR+SIL and IR+Zinc groups (both, $P<0.05$) (Fig. 6B).
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DISCUSSION

Our results showed that gamma radiation caused destruction of testicular tissue, reduction of spermatogenic cell line, reduction of testosterone concentration, occurrence of oxidative stress and expression of inflammatory and apoptotic genes in testicular tissue. However, we found that the administration of Silymarin and zinc alone reduced the damage and protected the testicular tissue, and these protective effects were significantly better in the combined treatment group.

Due to the growing trend of cancer, especially in young people, and the lack of available suitable medication for protecting patients, as well as the importance of fertility for these people, herbal drugs with low side effects and high efficacy promise an effective strategy.

Studies have shown that free radicals trigger phosphorylation and activation of certain messenger proteins such as histone and creatine kinases. These changes result in a significant increase in the production rate of ROS and enhance the caspase-3 pathway in the sperm cell, resulting in apoptosis during spermatogenesis or sperm maturation and influencing fertility (Kesari & Behari, 2012). Because ionizing radiation plays a significant role in the developmentof inflammation and increase in the expression of the cyclooxygenase-2 (COX-2) enzyme, it seems that increasing this enzyme in vascular tissues with high division potential increases the production and activation of certain factors such as prostaglandin-2 (PGE-2), NF-kB, lipoxygenase, IL-6, and TNF-α. These factors play an important role in the destruction of cell membranes and cell death so that interleukins and prostaglandins cause neutrophil accumulation, ROS production, induction of oxidative stress, and also the production of arachidonic acid from cell membrane phospholipids (Alahmar, 2019; Dutta *et al*., 2019). The human body has a defense system to fight free radicals known as the antioxidant system. The imbalance between the free radical amount produced and the antioxidant capacity results in oxidative stress. The most important free radicals in human semen include superoxide anion radical, hydrogen peroxide and hydroxyl radical. These free radicals are typically produced during oxygen metabolism (Desai *et al*., 2009; Kesari & Behari, 2012). Besides that, the structure of sperm DNA can also be damaged during the oxidative stress process.

Bases and phosphodiester bonds in this structure are susceptible to free radical-induced peroxidation damage that can lead to certain abnormalities such as bases exchange and dislocation, producing sites containing free bases, bases deletion, and chromosomal rearrangements. The apoptotic process can help eliminate abnormal germ cells and prevent their excessive production (Gavella & Lipovac, 2000; Tomás-Zapico & Coto-Montes, 2005; Zini & Libman, 2014). Free radicals are able to trigger the apoptotic process cascade. Damage in DNA can accelerate the apoptosis process, which ultimately leads to a reduction in sperm count, a decrease in membrane thickness, degeneration of interstitial tissue, and possibly cell inability to survive (Tremellen, 2008; Zini & Libman, 2014) which was also observed in the present study. IR reduces the production of protein and glucose in spermatogonia and primary spermatocytes, leading to disruption of cell divisions in germ cells and eventually pyknosis and karyolysis. Radiation also increases the death of germ cells in the testicular tissue by activating the caspase pathways, which are the main mediators of apoptosis, thus leading to decreased thickness of the germinal epithelium and testicular tissue atrophy (Adhikari *et al*., 2013). SIL stabilizes membrane structures and inhibits the release of inflammatory mediators such as cyclooxygenase and lipoxygenase. SIL prevents activation of NF-κβ and cell proliferation-associated kinases, such as TNF-α, and reduces cell cytotoxicity to prevent apoptosis. Due to its antioxidant a property, SIL is also able to scavenge free radicals and ROS (Dehmlow *et al*.,1996; Adhikari *et al*., 2013).

In a study by Ramadan *et al*. (2002) they investigated the radioprotective effect of SIL on rat liver after 3 Gy of gamma radiation and reported that radiation caused a significant increase in serum alkaline phosphatase (AP) level, one day after radiation. They also reported that in rats receiving one single dose of SIL at 70 mg/kg one hour before radiation, the activities of AP and other liver enzymes, such as glutathione peroxidase (GSH), returned to normal levels. In another study by Katiyar *et al*. (2011) the protective effect of SIL on apoptosis of human epidermal keratinocytes induced by UV-B irradiation was assessed, and they observed that administration of 10 and 20 mg/ml of SIL 3 h before radiation reduced 40 % and 73 % of apoptotic cells due to radiation, respectively. Razavi-Azarkhiavi *et al*. (2014) investigated the protective effect of SIL on bleomycin-

induced pulmonary toxicity in BALB/c mice, and found that administration of 50 and 100 mg/kg of SIL after bleomycin administration reduced lipid peroxidation, and also the levels of glutathione (GSH) and catalase (CAT) in the lung. Their results also showed a significant reduction in the TNF-a and NF-kB levels after the administration of SIL. The results of Ramadan *et al*. (2002), Katiyar *et al*. (2011) and Razavi-Azarkhiavi *et al*. (2014) are consistent with the findings of the present study and confirm the protective role of SIL against radiation-induced malignant effects.

Chromatin and flagellate in mammalian sperm contain high amounts of protamine rich in thiols and sulfhydryl groups; semen also contains many antioxidants such as turin, vitamin C, CAT and SOD that contribute to maintaining sperm and protecting it against oxidative stress. Also, they maintain the motility of sperm when they are deprived of their supporting environment, i.e., seminal plasma in the testis. The decrease in the count of progressive sperm seen in this study, may be due to (radiation-induced) free radical binding to thiol or sulfhydryl groups of sperm protein or inhibition of mitochondrial enzymes responsible for sperm motility. The results of this study indicated that SIL could act as a strong radio protector through regulation of cell membrane permeability, by inhibiting the 5-lipoxygenase pathway and ROS, as well as reducing the DNA damage by suppressing the NF-κβ (Ramadan *et al*., 2002; Adhikari *et al*., 2013; Surai, 2015). Overall, of the properties of SIL, which have already been proven by various studies, antioxidant and anti-apoptotic properties can be mentioned. Increased antioxidant properties of SIL lead to trapping more free radicals and increasing cell glutathione content and SOD activity (Ramadan *et al*., 2002; Take *et al*., 2009; Surai, 2015).

The protective effect of zinc, as a modulator factor, against lipid and protein oxidation in membrane structure can also be related to its ability to scavenge oxidation-triggering agents that are produced during the oxidation of proteins and lipids (Amara *et al*., 2008).

Improvement of sperm parameters and testicular cell structure in zinc receptor groups can be due to protective properties and increased antioxidant levels of cells. Hamam *et al*. (report that zinc protects normal cells via mechanisms, increased sperm DNA integrity, decrease in the ROS content, and also improves reproductive function during prepubertal period-cisplatin administration.

Due to the inherent toxicity of synthetic and chemical substances at active concentrations that protect against radioactive substances and ionizing radiation, researchers have focused on natural products and are seeking out the main solution in the active ingredients of medicinal plants. An ideal radioprotector should be cheap and have negligible toxicity at a wide range of concentrations, be absorbed quickly, have an appropriate dose reduction coefficient (acceptable half-life), and act through different mechanisms, so the pharmacodynamics characteristics of SIL and zinc can fulfill these criteria. Moreover, the current study showed SIL was an inhibitor and potent radioprotector that can promise new therapies. Success in the development of radioprotectors depends on certain factors such as the proper understanding of molecular biology of radiation-induced damage, tissue and organ response to radiation, and differentiation between tumor cells and normal cells. As future research, it is suggested that further research should be planned to elucidate the role of SIL and zinc combination antioxidant molecules on gene expression and its downstream pathways. Since zinc has the ability to activate SOD and enhance free radical scavenging by antioxidant defense of cells, it therefore plays a regulator role in the organization of DNA, stability of cell membranes, sperm development as well as cell division (Mruk *et al*., 2002).

CONCLUSION

Our results demonstrated that the IR causes sperm parameters reduction and testicular dysfunction through inflammation, oxidative stress, and apoptosis induction. This was while Silymarin and zinc, especially in combined administration can improve sperm parameters and infertility issues related to IR.

JAVAN, R.; ROSTAMZADEH, A.; MOJADADI, M.S.; HOSSEINPUR, K.; NASIRY, D. & RAOOFI, A. Efectos de mejora de la silimarina en combinación con zinc contra la radiación. *Int. J. Morphol., 43(3)*:1070-1081, 2025.

RESUMEN: Este estudio tuvo como objetivo evaluar los efectos radioprotectores de la silimarina (SIL) y el zinc, por separado y en combinación, contra la toxicidad testicular por irradiación (IR) en ratones. Se dividieron sesenta y cuatro ratones machos adultos con NMRI en ocho grupos: grupo control; grupo tratado con SIL; grupo tratado con zinc; grupo tratado con SIL + zinc; grupo IR irradiado con rayos gamma de 2 Gy; grupo IR + SIL; grupo IR + zinc; y grupo IR + SIL + zinc. Los tratamientos comenzaron durante ocho días consecutivos y posteriormente los ratones fueron expuestos a IR. Un día después de la irradiación, se tomaron muestras de cuatro animales de cada grupo y se retiraron ambos testículos para la determinación de los parámetros bioquímicos (MDA, GSH, SOD y CAT) y moleculares (Caspasa-3, P21, TNF-α, IL-1β y NF-κβ). Catorce días después de la irradiación, se sacaron otros cuatro animales de cada grupo para evaluaciones de parámetros histológicos, estereológicos y espermáticos, así como los niveles de testosterona. Encontramos que los parámetros espermáticos, los niveles de testosterona, la densidad numérica de espermatogonias, espermatocitos primarios, espermátidas, células sustentaculares, células intersticiales, túbulos

seminíferos, y los parámetros bioquímicos (excepto MDA) fueron significativamente mayores en los grupos IR+SIL, IR+Zinc e IR+SIL+Zinc, especialmente en los IR+SIL+Zinc, en comparación con el grupo IR. Esto se debe a que la expresión de genes apoptóticos e inflamatorios, así como los niveles de MDA, disminuyeron considerablemente en los grupos IR+SIL, IR+Zinc e IR+SIL+Zinc en comparación con el grupo IR, siendo más pronunciada en los grupos IR+SIL+Zinc. En general, SIL y zinc atenuaron los trastornos celulares y moleculares inducidos por la radiación gamma en los testículos, y estos efectos protectores fueron más pronunciados en el grupo de tratamiento combinado.

PALABRAS CLAVE: Radiación; Silimarina; Zinc; Estrés Oxidativo; Radioprotector; Toxicidad Testicular.

REFERENCES

Abo El-Atta, H. & Ahmed, D. Testicular dysfunction in malathion-induced toxicity in male rats: protective role of NAC and silymarin. *Mansoura J. Forensic Med. Clin. Toxicol., 28(2)*:33-45, 2020.

Adhikari, M. & Arora, R. Nano-silymarin provides protection against g-radiation-induced oxidative stress in cultured human embryonic kidney cells. *Mutat. Res. Genet. Toxicol. Environ. Mutagen., 792*:1-11, 2015.

Adhikari, M.; Dhaker, A.; Adhikari, J.; Ivanov, V.; Singh, V.; Chawla, R.; Kumar, R.; Sharma, R.; Karamalakova, Y. & Gadjeva, V. In vitro studies on radioprotective efficacy of silymarin against g-irradiation. *Int. J. Radiat. Biol., 89(3)*:200-11, 2013.

Aebi, H. *Catalase*. In: Bergmeyer, H. U. (Ed.). Methods of Enzymatic Analysis. New York, Academic Press, 1974. pp.673-84.

Akbulut, A.; Sadic, M.; Yumusak, N.; Aydinbelge, F. N.; Koca, G. & Korkmaz, M. The effects of zinc in the gastrointestinal system as a radioprotective agent. *Int. J. Radiat. Res., 16(3)*:333-9, 2018.

Alahmar, A. T. Role of oxidative stress in male infertility: an updated review. *J. Hum. Reprod. Sci., 12(1)*:4-18, 2019.

Amara, S.; Abdelmelek, H.; Garrel, C.; Guiraud, P.; Douki, T.; Ravanat, J. L.; Favier, A.; Sakly, M. & Ben Rhouma, K. Preventive effect of zinc against cadmium-induced oxidative stress in the rat testis. *J. Reprod. Dev., 54(2)*:129-34, 2008.

Amiri, F. T.; Raeispour, M.; Farzipour, S. & Hosseinimehr, S. J. Radioprotective effect of febuxostat against testicular damage induced by ionizing radiation in mice. *Curr. Radiopharm., 15(2)*:134-40, 2022.

Bagheri, H.; Salajegheh, A.; Javadi, A.; Amini, P.; Shekarchi, B.; Shabeeb, D. & Najafi, M. Radioprotective effects of zinc and selenium on mice spermatogenesis. *J. Biomed. Phys. Eng., 10(6)*:707-12, 2020.

Bashandy, S. A. E. M.; Omara, E. A. A.; Ebaid, H.; Amin, M. M. & Soliman, M. S. Role of zinc as an antioxidant and anti-inflammatory to relieve cadmium oxidative stress induced testicular damage in rats. *Asian Pac. J. Trop. Biomed., 6(12)*:1056-64, 2016.

Dehmlow, C.; Erhard, J. & de Groot, H. Inhibition of Kupffer cell functions as an explanation for the hepatoprotective properties of silibinin. *Hepatology, 23(4)*:749-54, 1996.

Desai, N. R.; Kesari, K. K. & Agarwal, A. Pathophysiology of cell phone radiation: oxidative stress and carcinogenesis with focus on male reproductive system. *Reprod. Biol. Endocrinol., 7*:114, 2009.

Djureinovic, D.; Fagerberg, L.; Hallström, B.; Danielsson, A.; Lindskog, C.; Uhlén, M. & Pontén, F. The human testis-specific proteome defined by transcriptomics and antibody-based profiling. *Mol. Hum. Reprod., 20(6)*:476-88, 2014.

Dutta, S.; Majzoub, A. & Agarwal, A. Oxidative stress and sperm function: a systematic review on evaluation and management. *Arab. J. Urol., 17(2):87*-97, 2019.

Ehghaghi, A.; Zokaei, E.; Modarressi, M. H.; Tavoosidana, G.; Ghafouri-Fard, S.; Khanali, F.; Motevaseli, E. & Noroozi, Z. Antioxidant and anti-apoptotic effects of selenium nanoparticles and Lactobacillus casei on mice testis after X-ray. *Andrologia, 54(11)*:e14591, 2022.

Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys., 82(1)*:70-7, 1959.

Fallah, A.; Mohammad-Hasani, A. & Colagar, A. H. Zinc is an essential element for male fertility: a review of Zn roles in men's health, germination, sperm quality, and fertilization. *J. Reprod. Infertil., 19(2)*:69-81, 2018.

Fallah, M.; Davoodvandi, A.; Nikmanzar, S.; Aghili, S.; Mirazimi, S. M. A.; Aschner, M.; Rashidian, A.; Hamblin, M. R.; Chamanara, M.; Naghsh, N.; *et al*., Silymarin (milk thistle extract) as a therapeutic agent in gastrointestinal cancer. *Biomed. Pharmacother., 142*:112024, 2021.

Fatehi, D.; Mohammadi, M.; Shekarchi, B.; Shabani, A.; Seify, M. & Rostamzadeh, A. Radioprotective effects of Silymarin on the sperm parameters of NMRI mice irradiated with g-rays. *J. Photochem. Photobiol. B Biol., 178*:489-95, 2018.

Gavella, M. & Lipovac, V. Antioxidative effect of melatonin on human spermatozoa. *Arch. Androl., 44(1)*:23-7, 2000.

Gillessen, A. & Schmidt, H. H. J. Silymarin as supportive treatment in liver diseases: A narrative review. *Adv. Ther., 37(4)*:1279-301, 2020.

Hamam, E. T.; Awadalla, A.; Shokeir, A. A. & Aboul-Naga, A. M. Zinc oxide nanoparticles attenuate prepubertal exposure to cisplatin-induced testicular toxicity and spermatogenesis impairment in rats. *Toxicology, 468*:153102, 2022.

Howard, V. & Reed, M. *Unbiased Stereology. Three-Dimensional Measurement in Microscopy*. 2nd ed. London, Garland Science, 2004.

Huang, Z.; Peng, R.; Yu, H.; Chen, Z.; Wang, S.; Wang, Z.; Dong, S.; Li, W.; Jiang, Q.; Li, F.; *et al*., Dimethyl sulfoxide attenuates radiation-induced testicular injury through facilitating DNA double-strand break repair. *Oxid. Med. Cell. Longev., 2022*:9137812, 2022.

Katiyar, S. K.; Mantena, S. K. & Meeran, S. M. Silymarin protects epidermal keratinocytes from ultraviolet radiation-induced apoptosis and DNA damage by nucleotide excision repair mechanism. *PLoS One, 6(6)*:e21410, 2011.

Kesari, K. K. & Behari, J. Evidence for mobile phone radiation exposure effects on reproductive pattern of male rats: role of ROS. *Electromagn. Biol. Med., 31(3)*:213-22, 2012.

Mihara, M. & Uchiyama, M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal. Biochem., 86(1)*:271-8, 1978.

Mruk, D. D.; Silvestrini, B.; Mo, M. Y. & Cheng, C. Y. Antioxidant superoxide dismutase - a review: its function, regulation in the testis, and role in male fertility. *Contraception, 65(4)*:305-11, 2002.

Musa, A. E.; Omyan, G.; Esmaely, F. & Shabeeb, D. Radioprotective effect of hesperidin: a systematic review. *Medicina (Kaunas), 55(7)*:370, 2019.

Nasiry, D.; Khalatbary, A. R.; Ahmadvand, H. & Talebpour Amiri, F. B. Effects of Juglans regia L. leaf extract supplementation on testicular functions in diabetic rats. *Biotech. Histochem., 96(1)*:41-7, 2021.

Nasiry, D.; Khalatbary, A. R.; Ghaemi, A.; Ebrahimzadeh, M. A. & Hosseinzadeh, M. H. Topical administration of Juglans regia L. leaf extract accelerates diabetic wound healing. *BMC Complement. Med. Ther., 22(1)*:255, 2022.

Payne, C. M.; Bjore Jr., C. G. & Schultz, D. A. Change in the frequency of apoptosis after low- and high-dose X-irradiation of human lymphocytes. *J. Leukoc. Biol., 52(4)*:433-40, 1992.

Poulos, J. E.; Kalogerinis, P. T.; Milanov, V.; Kalogerinis, C. T. & Poulos, E. J. The effects of vitamin E, silymarin and carnitine on the metabolic abnormalities associated with nonalcoholic liver disease. *J. Diet. Suppl., 19(3)*:287-302, 2022.

Qin, Z.; Zhu, K.; Xue, J.; Cao, P.; Xu, L.; Xu, Z.; Liang, K.; Zhu, J. & Jia, R. Zinc-induced protective effect for testicular ischemia-reperfusion injury by promoting antioxidation via microRNA-101-3p/Nrf2 pathway. *Aging (Albany NY), 11(21)*:9295-309, 2019.

Rahimi-Madiseh, M.; Mohammadi, M.; Hassanvand, A.; Ahmadi, R.; Shahmohammadi, M. & Rostamzadeh, A. Assessment of the toxicity effects of nicotine on sperm and IVF and the potential protective role of silymarin—an experimental study in mice. *Middle East Fertil. Soc. J., 25*:14, 2020.

Raj, S.; Manchanda, R.; Bhandari, M. & Alam, M. S. Review on natural bioactive products as radioprotective therapeutics: present and past perspective. *Curr. Pharm. Biotechnol., 23(14)*:1721-38, 2022.

Ramadan, F. L.; Abdel-Monem, D. D. & Ismail, N. H. Protective role of L-carnitine and zinc against g-radiation induced cardiac and testicular disorders in albino rats. *J. Nucl. Technol. Appl. Sci., 1(2)*:217-33, 2013.

Ramadan, L. A.; Roushdy, H. M.; Senna, G. M. A.; Amin, N. E. & El-Deshw, O. A. Radioprotective effect of silymarin against radiation induced hepatotoxicity. *Pharmacol. Res., 45(6)*:447-54, 2002.

Raoofi, A.; Omraninava, M.; Javan, R.; Maghsodi, D.; Rustamzadeh, A.; Nasiry, D. & Ghaemi, A. Protective effects of epigallocatechin gallate in the mice induced by chronic scrotal hyperthermia. *Tissue Cell, 84*:102165, 2023.

Razavi-Azarkhiavi, K.; Ali-Omrani, M.; Solgi, R.; Bagheri, P.; Haji-Noormohammadi, M.; Amani, N. & Sepand, M. R. Silymarin alleviates bleomycin-induced pulmonary toxicity and lipid peroxidation in mice. *Pharm. Biol., 52(10)*:1267-71, 2014.

Sheikholeslami, S.; Khodaverdian, S.; Dorri-Giv, M.; Hosseini, S. M.; Souri, S.; Abedi-Firouzjah, R.; Zamani, H.; Dastranj, L. & Farhood, B. The radioprotective effects of alpha-lipoic acid on radiotherapy-induced toxicities: a systematic review. *Int. Immunopharmacol., 96*:107741, 2021.

Singh, M.; Kadhim, M. M.; Turki Jalil, A.; Oudah, S. K.; Aminov, Z.; Alsaikhan, F.; Jawhar, Z. H.; Ramírez-Coronel, A. A. & Farhood, B. A systematic review of the protective effects of silymarin/silibinin against doxorubicin-induced cardiotoxicity. *Cancer Cell Int., 23(1)*:88, 2023.

Soleimani, V.; Delghandi, P. S.; Moallem, S. A. & Karimi, G. Safety and toxicity of Silymarin, the major constituent of milk thistle extract: an updated review. *Phytother. Res., 33(6)*:1627-38, 2019.

Soliman, M. M.; Elshazly, S. A. & Aldhahrani, A. Gamma-irradiation-induced testicular oxidative stress and apoptosis: Mitigation by l-carnitine. *J. Biochem. Mol. Toxicol., 34(11)*:e22565, 2020.

Sun, Y.; Oberley, L. W. & Li, Y. A simple method for clinical assay of superoxide dismutase. *Clin. Chem., 34(3)*:497-500, 1988.

Surai, P. F. Silymarin as a natural antioxidant: an overview of the current evidence and perspectives. *Antioxidants (Basel), 4(1)*:204-47, 2015.

Take, G.; Erdogan, D.; Helvacioglu, F.; Göktas, G.; Ozbey, G.; Uluoglu, C.; Yücel, B.; Guney, Y.; Hicsonmez, A. & Ozkan, S. Effect of melatonin and time of administration on irradiation-induced damage to rat testes. *Braz. J. Med. Biol. Res., 42(7)*:621-8, 2009.

Tavares, R.; Silva, A.; Lourenço, B.; Almeida-Santos, T.; Sousa, A. & Ramalho-Santos, J. Evaluation of human sperm chromatin status after selection using a modified Diff-Quik stain indicates embryo quality and pregnancy outcomes following *in vitro* fertilization. *Andrology, 1(6)*:830-7, 2013.

Tomás-Zapico, C. & Coto-Montes, A. A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes. J. *Pineal Res., 39(2)*:99-104, 2005.

Tremellen, K. Oxidative stress and male infertility--a clinical perspective. *Hum. Reprod. Update, 14(3)*:243-58, 2008.

Zini, A. & Libman, J. *Oxidative Stress and Male Infertility*. In: Laher, I. (Ed.). Systems Biology of Free Radicals and Antioxidants. Berlin, Springer, 2014. pp.2815-33.

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