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Research article

Protective effects of chlorogenic acid against ionizing radiation-induced testicular toxicity

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ABSTRACT

Background: Testicular tissues could damage by ionizing radiation (IR) during the treatment of pelvic cancers. The aim of this study was to investigate both the protective and therapeutic effects of chlorogenic acid (CGA) on IR-induced mouse testis tissue damage.

Methods: In this experimental study, 70 mice were divided into 3 groups, including group 1 (normal saline), group 2 (IR + normal saline), and group 3 (IR + 5, 10, 20, 40, and 80 mg/kg) CGA via I.P injection. Animals in groups 2 and 3 received a dose of 2.0 Gy total-body irradiation in a single fraction. At two determined time points (16 h and 35 days after exposure), the testis and caudal part of both epididymis were isolated and underwent subsequent analyses.

Results: The results showed that irradiation of mice caused massive damage to spermatogenesis, seminiferous tubules, basal lamina, Leydig cells, and sperm parameters. Further biochemical assessment of the data demonstrated that 40 mg/kg CGA almost restored MDA to a normal level. In addition, the level of SOD, TAC, and GSH were significantly increased in the 40 mg/kg CGA treated group. Molecular evidence confirmed the protective effects of CGA and also revealed that the ratio of *Bax/Bcl-2* in the presence of 40 mg/kg CGA was significantly decreased compared to IR and some treated groups.

Conclusion: The protective and therapeutic effects of CGA on testis were found to be positively correlated with the dose level.

1. Introduction

Ionizing irradiation (IR) therapy is one of the principal procedures employed in the treatment of many kinds of cancer (Jung et al., 2014). Pelvic irradiation for the treatment of pelvic cancers inevitably affects not only the tumor region but also other healthy organs such as the testis (radiosensitive organ) (Thibodeau et al., 2018). Radiation interaction with living tissues can cause detrimental effects on the nucleus, mitochondrial membranes, structural and cytoplasmic proteins, complex carbohydrates, RNA, and DNA (Rahman et al., 2012).

These side-effects of IR lead to cellular dysfunction and, in the male reproductive system, eventually cause low sperm counts (Clifton and Bremner, 1983), defective sperm function, and mutagenesis or apoptosis (Kanter et al., 2010) of radiosensitive cells (spermatogonia cells). According to the previous reports, in the male reproductive system, spermatogonia cells are completely killed with IR at the dose of 3 Gy, resulting in permanent infertility (van Beek et al., 1986) but spermatocytes and spermatids are generally less sensitive to irradiation (Ekici et al., 2016; Gao et al., 2017; Liu et al., 2006; Smina et al., 2015). Therefore, temporary or permanent infertility is a common problem of cancer treatment with radiation therapies and chemotherapy, especially for young male patients (Grewenig et al., 2015).

Clinical and experimental studies have also shown that IR exhibits oxidative stress-related injury in testis tissue and has deleterious effects on

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normal spermatogenic metabolism, proliferation, and differentiation (Adaramoye et al., 2012; Gao et al., 2017; Smina et al., 2015). In this regard, it has been identified that the destructive effects of IR are associated with an increase in oxidative stress molecules such as malondialdehyde (MDA) and a remarkable reduction in the level of glutathione (GSH) which leads to tissue dysfunction (Rani et al., 2016). On the other hand, compelling evidence reveals that the Sertoli cells (SCs) and Leydig cells (LCs) are highly resistant to radiation because the concentration of antioxidants increases in mentioned cells after irradiation (Albuquerque et al., 2013). It has been proposed that some agents with a potent antioxidant capacity may also be effective in preventing oxidative stress induced by IR (Adaramoye et al., 2012; Gao et al., 2017; Smina et al., 2015).

Chlorogenic acid (CGA) is one of the most widely available water-soluble polyphenols which have an aromatic ring structure along with one or more hydroxyl groups. It has been reported that there are a lot of sources, such as foods and herbs, in which CGA can be found including tomatoes, potatoes, carrots, apples, coffee beans, and so on (Jesús Santana-Gálvez, Luis Cisneros-Zevallos, & Daniel A Jacobo-Velázquez, 2017).

Furthermore, different scientific pieces of evidence clarify several important biological and therapeutic functions of CGA as a central nervous system stimulator, including its antioxidant activity, antibacterial, hepato-protective, cardio-protective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, antiviral, anti-microbial, and anti-hypertension characteristics (Tajik et al., 2017). Compared to other polyphenols, one of the main advantages of CGA is its antioxidant activity which could eliminate free radicals or inhibit oxidative damage (Guo et al., 2019; Nguyen et al., 2017, 2018). One study demonstrated that CGA protected the damaged DNA of blood lymphocytes induced by IR because of its antioxidant and radioprotective effects (Cinkilic et al., 2013).

It seems that the testis tissue damages induced by IR mostly occur via overproduction of oxidative stress markers and a decrease in the antioxidant level. Therefore, given the critical role of oxidative stress in testis, we sought to evaluate the effects of radiation resulting from IR on the testis at the histological, biochemical, and molecular levels. Moreover, this study aimed to investigate both the protective and therapeutic effects of CGA on IR-induced oxidative stress in mouse testis.

2. Materials & methods

2.1. Animals

Six to eight-week-old male mice within the weight range of 25–30 g were obtained from Central Animal House, Urmia University, West Azarbyjan (Iran). Animals (n = 70) were housed in polypropylene cages bedded with a standard animal pellet diet and were maintained under conventional and management conditions. The ethical code of this study is PU/IAEC/S/14/23 which is issued by Urmia University, West Azerbaijan, Iran. these experiments were conducted according to established animal welfare guidelines.

2.2. Experimental design

Animals, however, were allowed to acclimatize for one week before the experiment and randomly assigned into Group 1 (n = 10): control (normal saline), Group 2 (n = 10): IR + normal saline, and Group 3: IR + 5, 10, 20, 40, and 80 mg/kg CGA (Thermo Fisher Scientific) (I.P. injection) (n = 50); in these groups animals pretreatment (daily) with different concentrations of CGA 3 days before starting the study (day 1) and post-treatment (daily) continued for 35 days.

However, prior to irradiation (day 1), mice were anesthetized with a combination of ketamine (Bremer Pharma, GmbH, Germany) and xylazine (Serva. Feinbiochemica, New York) (100/10 mg/kg, I.P. injection). Then, mice were placed in a plastic box in a prone position and irradiated using a single posterior field covering the whole box plus a 5 cm margin to achieve maximum uniformity of dose distribution (Cinkilic et al., 2013). Animals received a dose of 2.0 Gy total-body irradiation in a single fraction at a source to skin distance of 100 cm at a half-thickness with a tissue-equivalent bolustorats' superior and inferior parts with a gantry 0 and 180° from the front and rear using the isometric method at an area size of 10 × 40 cm. The rate of irradiation was 2 Gy/min using a 320-kVp Electa X-ray machine.

The effects of the CGA supplement on IR-induced testicular injury were studied at two-time points: the first time point was 16 h after exposure because apoptosis of GC starts approximately 8 h after IR and increases to maximum effect in about 16 h (Ding et al., 2015), and the second time point was the last day of the study (day 35) because of differentiation of mouse testicular GCs from early spermatogonia to spermatozoa approximately 35 days (Ding et al., 2015).

However, blood (1 mL) was collected by cardiocentesis in all groups of the study, then mice were sacrificed with cervical dislocation. Also, their testes and caudal part of both epididymis were isolated and dissected mechanically free of surrounding epididymal fat. For histological studies, one testis (right) was fixed and then embedded in paraffin, followed by staining with hematoxylin-Eosin (H&E) (Sigma-Aldrich). For molecular and biochemical analysis, the other testis (left) was divided into two halves and was stored at −80 °C until processing.

2.3. Histomorphometric evaluations of testis

Morphological and histological examinations of all groups of study (n = 5 from each group) were evaluated using standard protocols of hematoxylin-eosin (H&E) staining and performed at a light microscope. The number of germinal cell layers and Johnsen's score were used to categorize spermatogenesis. The number of germinal epithelial layers was counted in ten seminiferous tubules as described by Miller et al. (Miller et al., 1990) and the mean number was calculated. Each tubular section was given a score from 10 to 1 according to the presence or absence of the main cell types arranged in the order of maturity as described by Johnsen (1970).

2.4. Sperm preparation

The caudal part of both epididymides (free of fat, vas deferens, and other tissues) was removed from each side and transferred to a Petri dish containing 1 mL of Hams'F10 medium supplemented with bovine serum albumin (Sigma, St. Louis, USA). Then, epididymis was minced and incubated at 37 °C in 5% CO_2 for 30 min to let the spermatozoa swim out into the medium.

2.5. Spermatozoa parameters

Sperm count was assessed according to the method described by Ranawat and Bansal (Ranawat and Bansal, 2009). For evaluating sperm count, a 1:50 diluted sperm sample with distilled water was mixed in a microtube, then 10 μl of sperm suspension was loaded on a cleaned Neubauer slide; the number of spermatozoa was counted and expressed as sperm counts per mm^3. Motility was assessed using techniques for assessment and quality control according to the World Health Organization (WHO) (World Health, 2010).

2.6. Eosin-Nigrosin staining

To evaluate the percentage of sperm viability, we obtained 20 μL of sperm samples from each mouse and placed them on a slide; then the same value of Eosin-Nigrosin (EN) solution was added and smears were prepared. Following drying the smears at room temperature and examining them using a light microscope with a magnification of 400×, we considered red sperms as dead ones and classified the white (unstained) ones as live (Björndahl et al., 2004).

2.7. Aniline blue staining

This staining was used to determine the percentage of sperms with chromatin condensation (replacement of histone with protamine during spermiogenesis). The air-dried smear of each sample was fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH = 7.2) for 30 min at room temperature, and then each smear was treated with 5% aqueous aniline blue (AB) stain for 5 min. At least 200 sperm cells were evaluated in each slide by light microscopy and the percentage of abnormal spermatozoa was reported. Normal sperms turned into pale blue-stained cells and abnormal immature sperms appeared as dark blue cells (Auger et al., 1990).

2.8. Morphological analysis of apoptosis

Sperm solution stained with Acridine orange (AO) and Ethidium bromide (EB) (Sigma-Aldrich, USA) was used to evaluate the percentage of sperms with DNA fragmentation. Briefly, 20 μL of sperm samples were obtained from each mouse and placed on a slide, then the same value of AO/EB (1 μl AO Stock (5 mg/mL) in 1 mL PBS with 1 μl EB Stock (3 mg/mL) for 1 min at 37 °C in dark) solution was added and smears were prepared. Under a fluorescent microscope, a minimum of 120 cells were counted, and three cellular states, that is, intact cells (green), early apoptotic (bright orange), and late apoptotic (red) were reported.

2.9. Real-time RT-PCR

After RNA extraction (by Qiagen (Germany) kit) and cDNA synthesis (by Thermo Fisher Scientific, Lithuania, USA) gene expression of apoptotic genes (*Bax* and *Bcl-2*) were determined by real-time RT-PCR on testis tissues using the Light Cycler 480 with SYBR Green detection and Amplicon Kit (Applied Biosystem). The primers used are reported in our previous study (Abedpour et al., 2018). The sequences for forward and reverse primers of *Bax* and *Bcl-2* are listed in Table 1. The expression level of each gene in an obtained sample was normalized using *β-actin* (as housekeeping genes). Relative expression levels were calculated based on Pfaffl or $2^{-\Delta\Delta CT}$ method. All experiments of RT-qPCR were replicated three times.

2.10. Assays for oxidative stress markers

Biochemical assays were carried out for evaluating oxidative stress markers (Malondialdehyde (MDA), glutathione (GSH), total antioxidant capacity (TAC), and superoxide dismutase (SOD) activity in all groups of the study by an ELISA Kit according to the provider's instruction (ZellBio GmbH, Biotechnology company in Lonsee, Germany). For this purpose, testicular tissue was homogenized in phosphate buffer (pH = 7.40) or 1.15% KCl buffer (1:9, w/v) using a manual glass homogenizer for approximately 5 min and centrifuged at 12,000 g at 4 °C for 20 min; then, the supernatant was separated and stored at –80 °C until analysis.

2.11. Statistical analysis

The obtained data were analyzed through Statistical Package for the Social Sciences (SPSS) program version 16 (SPSS Inc. Chicago, IL, USA) software. We used Kolmogorov–Smirnov test for data normality. One-way ANOVA followed by a post hoc Tukey test was performed to compare various groups with each other. The data normally distributed were expressed by mean ± standard deviation. In this study, differences with a *p*-value less than 0.05 were regarded to be statistically significant at a 95% confidence level.

Table 1. Sequences of apoptotic-related genes.

Symbol	Forward & Reverse
***Bax* (NM-007527.3)**	**F: CGGCGAAATGGAGATGAACTG**
	R: GCAAAGTAGAAGAGGGCAACC
***Bcl-2* (NM-177410)**	**F: GGTGTTCAGATGTCGGTTCA**
	R: CGTCGTGACTTCGCAGAG
***β-actin* (NM_031144.2)**	**F: CTATGTTGCCCTAGACTTCG**
	R: AGGTCTTTACGGATGTCAAC

3. Results

3.1. Histopathological findings

To assess the effects of long-term treatment with CGA in the testicular tissues of mice exposed to 2.0 Gy total-body, the last day of the study, 35 days after exposure, was chosen. The photomicrographs of testicular tissues are shown in Figure 1.

Histological analysis of the testes obtained from the control group demonstrated normal architecture. The lumen of seminiferous tubules with normal spermatogonia (which are in direct contact with epithelial basal lamina), spermatogenic cells (spermatogonia, primary spermatocytes, spermatids), and somatic testicular cells (Sertoli and Leydig cells) were seen in this group (Figure 1A). In addition, accurate spermatogenesis with abundant spermatids was observed. In contrast, in testis from the IR group (Figure 1B), multiple pathological changes such as shrunken tubules, disorganized/distorted seminiferous tubules with loosely arranged cells, detachment (desquamation) of spermatogenic cells, depletion in the germinal cell population, disrupted basement membrane, and empty tubules were detected as compared to control group. In addition, the lumen was full of cellular and spermatogenic debris. In comparison with the IR group, by increasing the concentration of CGA testicular injuries were reduced (Figure 1C, D, E, F, G).

However, Table 2 shows evaluations for the Johnsen score, the thickness of seminiferous epithelium (HE), and mean seminiferous tubule diameter (MSTD). According to Table 2, the Johnsen score evaluation confirmed that IR induced testicular injury. Moreover, morphometric findings in the IR group, as compared to the control, showed a significant decrease in HE and MSTD ($p < 0.05$). Based on our results, atrophy of seminiferous tubules, spermatogenic arrest, thickening of the basal lamina, Leydig cells hyperplasia, and reduction of Johnsen score were significantly improved by CGA treatment at post-IR (day 35) (Table 2). These positive changes were dependent on an increase in the concentration of CGA up to 40 mg/kg, but these effects were reduced at a concentration of 80 mg/kg.

3.2. Sperm viability and sperm chromatin

As represented in Figure 2 and Table 3, the sperm viability (EN^+ spermatozoa) showed a decreasing trend in the IR group when compared to the control and IR + CGA (at different concentrations of 10, 20, 40, and 80 mg/kg) groups. Overall, our results suggested that treatment with 40 mg/kg CGA significantly increased sperm viability on post-IR injury (day 35).

On the other hand, there was a significant increase in the rate of Aniline blue $(AB)^+$ spermatozoa in the IR and IR + 5 mg/kg CGA groups in comparison with others (Figure 3, Table 3). On the other hand, AB^+ spermatozoa were dependent on an increase in the concentration of CGA up to 40 mg/kg. However, these effects were increased at a concentration of 80 mg/kg, but no significant difference was observed when compared to the group of IR + 40 mg/kg CGA ($p > 0.05$). Overall, these results suggest that treatment with 40 mg/kg CGA significantly ameliorated sperm chromatin after IR injury on post-IR Day 35.

3.3. Sperm parameters

From a functional standpoint, as summarised in Table 3, IR significantly decreased the number and motility of sperm in the caudal epididymis on post-IR day 35. By contrast, treatment with some different concentrations, 20, 40, and 80 mg/kg CGA attenuated the testicular detriment after IR damage and significantly ($p < 0.05$) increased the sperm count and the rate of progressive motility. However, the dose of 40

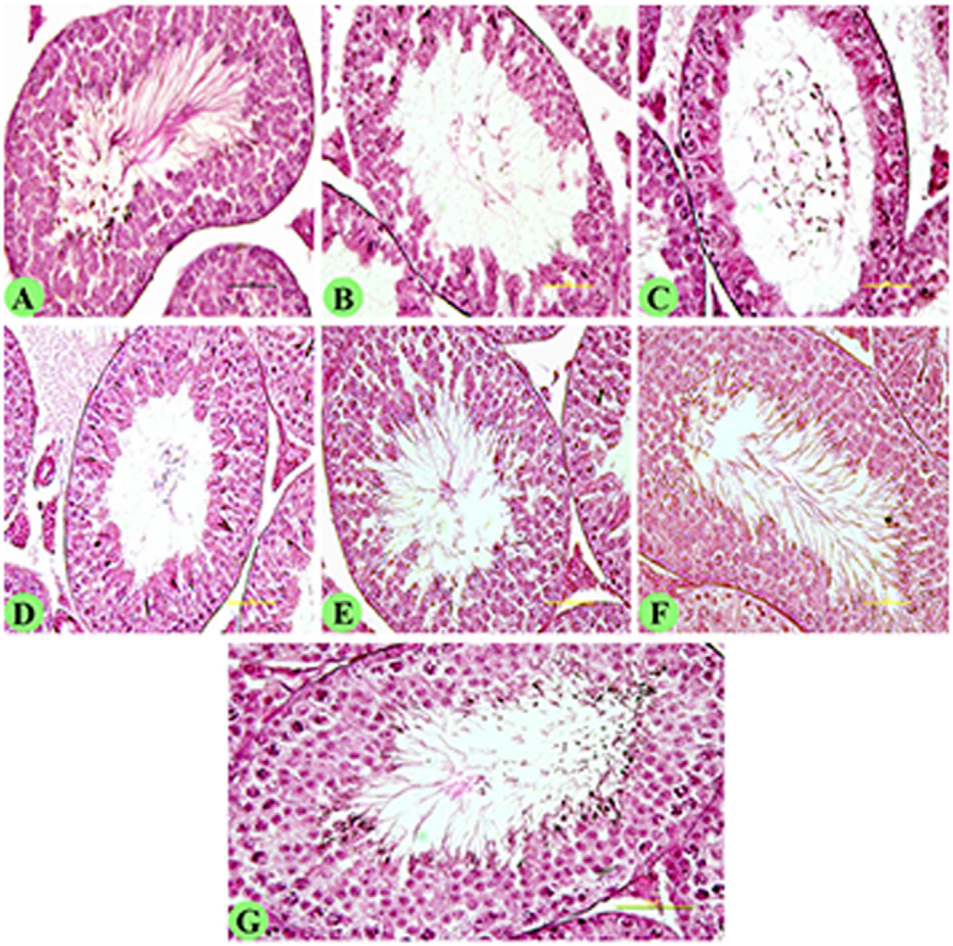

Figure 1. Photomicrographs of the effect of CGA on the histological architecture of testes in all groups of study after 35 days. Control group (A), IR group (B), and CGA with 5, 10, 20, 40, and 80 mg/kg (C, D, E, F, and G respectively). Animals in the control group show normal histoarchitecture, IR group animals shows shrunken tubule (S), spermatogenic debris in lumen (DB), destruction of seminiferous tubules (ST), empty tubule (E), depletion in the cell population (P), IR + CGA animals shows organized histoarchitecture of seminiferous tubules but revealed debris in the lumen as compared to the control and IR groups. (H&E, ×400).

mg/kg CGA showed the best result compared to the other CGA-treated groups.

3.4. Morphological analysis of apoptosis in sperm on the last day of the study

There was a significant dose-dependent increase (Figure 3) in intact sperm that was assessed by AO/EB fluorescent staining. Overall, our results suggested that the CGA 40 mg/kg treated mice showed a low amount of sperm with DNA damage.

3.5. RT-qPCR analysis

It has been reported that the most apoptotic rate occurs 16 h after exposure (Ding et al., 2015). Although the main purpose of the present study was to assess the effects of long-term treatment with CGA (35 days after exposure) on the expression of apoptotic-related genes, *Bax* and *Bcl-2*, in testicular tissues, the expression of mentioned genes was also analyzed at 16 h after exposure (Table 4).

Our results demonstrated that 16 h and 35 days after exposure, the relative expression of *Bax* was augmented in the IR-stimulated testis and almost restored to the normal control level in the presence of 40 mg/kg CGA ($p \leq 0.003$). There was no significant difference between IR and IR + 5 mg/kg CGA ($p > 0.05$) (Table 4).

In contrast, the expression of *Bcl-2* was significantly downregulated in the irradiated testis. However, this downregulation was effectively reversed by the 40 mg/kg CGA supplement ($p \leq 0.003$). There was no significant difference between IR and IR + 5 mg/kg CGA ($p > 0.05$).

On the other hand, at these two-time points, 16 h and 35 days after exposure, the ratio of *Bax/Bcl-2* showed that there was a significant difference between IR groups and some other groups including the control group and CGA-treated groups (10–80 mg/kg) ($p \leq 0.003$). However, there was no remarkable change between the control and some treated groups (40 and 80 mg/kg CGA) ($p > 0.05$). Interestingly, our results showed similar alterations 35 days after exposure, in comparison with the results of 16 h after exposure (Table 4).

3.6. Biochemical results

As mentioned before, the most apoptotic rate occurs 16 h after the exposure (Ding et al., 2015), hence, the levels of biochemical parameters definitely change at this time compared to the control and CGA-treated groups. However, although the purpose of the study was to assess the effects of long-term treatment (35 days after exposure) with CGA on some biochemical parameters (SOD, GSH, TAC, and MDA), these mentioned parameters were also investigated for the first period, 16 h after exposure. However, the data relating to the biochemical parameters are presented in Figure 4.

In the IR group, the levels of MDA at post-IR 16 h and day 35 were significantly higher than in the control group ($p < 0.05$), while the levels of SOD, GSH, and TAC remarkably decreased compared to the control group ($p < 0.05$).

For the MDA parameter at post-IR 16 h and day 35, pretreatment and post-treatment with CGA at some concentrations, including 10–80 mg/kg CGA, significantly decreased the MDA level compared to the IR group ($p < 0.05$), while the levels of SOD, GSH, and TAC only markedly increased in the groups treated with 20–80 mg/kg CGA compared to the IR group

Table 2. Assessment of radiation-induced testicular injuries in the presence of chlorogenic acid.

Groups	Height (Thickness) of Seminiferous Epithelium (HE) (μm)	Mean Seminiferous Tubule Diameter (MSTD) (μm)	Johnsen's Score
Control	82.47 ± 2.74	182.1 ± 5.65	8.54 ± 0.35
IR	58.32 ± 2.03[a]	153.4 ± 4.03[a]	4.78 ± 0.64[a]
IR + CGA (5 mg/kg)	65.32 ± 2.65[a]	162.7 ± 3.34[a]	6.52 ± 0. 58[a]
IR + CGA (10 mg/kg)	71.34 ± 2.73[ab]	172.4 ± 5.12[ab]	6.85 ± 0.23[ab]
IR + CGA (20 mg/kg)	78.45 ± 3.25[bcd]	178.6 ± 4.87[bcd]	7.34 ± 0.45[bcd]
IR + CGA (40 mg/kg)	81.65 ± 2.41[bcd]	181.8 ± 4.75[bcd]	8.35 ± 0.72[bcde]
IR + CGA (80 mg/kg)	80.76 ± 2.23[bcd]	180.8 ± 4.36[bcd]	8.03 ± 0.02[bcd]

As a matter of fact, the most apoptotic rate in testicular tissues occurs 16 h after exposure. Data obtained from RT-qPCR analysis (Table 4) showed that pre-treatment with chlorogenic acid (CGA) could decrease the expression of *Bax* and increase *Bcl-2* expression 16 h after exposure. Therefore, the last day of the study, day 35, was chosen for the assessment of testicular injuries to show the effect of long-term treatment with CGA. The Johnsen score, the thickness of the epithelium (HE), and the diameter of seminiferous tubules (MSTD) confirmed that IR induced testicular injury in different lineages of spermatogenic and somatic cells after 35 days. Values are shown as mean ± SD. SD: standard deviation. IR: irradiation. (a): significant difference with the control group; $p < 0.05$. (b, c, d, and e): these show a significant difference compared to the IR, IR + 5 mg/kg CGA, IR + 10 mg/kg CGA, and IR + 20 mg/kg CGA groups, respectively ($p < 0.05$).

($p < 0.05$). For all mentioned antioxidant parameters, there was no significant difference when the IR group compared to the groups treated with 5 and 10 mg/kg CGA, respectively ($p > 0.05$).

At two mentioned time points, 16 h and 35 days after exposure, the treated group with 40 mg/kg CGA showed the best results related to the other treated groups when all mentioned parameters were compared to the IR group ($p < 0.05$). Similarly, the results showed the same biochemical changes 35 days after exposure, in comparison with the results of the other time point, 16 h after exposure.

4. Discussion

This study aimed to investigate the protective and therapeutic effects of CGA on irradiation-induced testicular injury for assessing the importance of long-term treatment.

Our histological results showed that irradiation of mice caused massive damage to spermatogenesis, seminiferous tubules, basal lamina, and Leydig cells. Moreover, irradiation significantly decreased some sperm parameters such as count and motility, decreased sperm viability, increased sperm chromatin condensation, and reduced the Johnsen score as an index of healthy spermatogenesis, the mean seminiferous tubule diameter, and the thickness of the seminiferous epithelium (Figure 5).

Several scholars have reported that IR is highly toxic as regards testicular germ cells, testicular function, morphology, and spermatogenesis (Cordelli et al., 2012; Delessard et al., 2020; Okada and Fujisawa, 2019). Spermatogenesis is a complex and very delicate process. Cytotoxic agents, such as ionizing irradiation, by producing endogenous or exogenous factors deeply affect spermatogenic cells and may damage somatic testicular cells or germ cells at different developmental stages. A decrease in the thickness of the epithelium inhibits mitotic divisions of type B spermatogonia and the elongation G1 phase of the cell cycle finally brings about defects in sperm production. This sperm defect can lead to infertility in men and boys via induction of apoptosis in germ cells and spermatogonia cells (Hosseini et al., 2018).

In contrast, our morphological data demonstrated the drastic effects of pretreatment (before radiation) together with post-treatment (after radiation) of CGA on spermatogenesis in the testis exposed to radiation. Atrophy of seminiferous tubules, spermatogenic arrest, thickening of the basal lamina, Leydig cells hyperplasia, and reduction of Johnsen score was significantly improved by CGA treatment, especially for the dose of 40 mg/kg CGA, at post-IR day 35, which revealed the normal architecture of seminiferous tubules. Moreover, CGA treatment exerted beneficial effects on spermatogenesis and was significantly able to increase sperm viability and some sperm parameters, count, and motility, during the IR irradiation; besides it could preserve spermatozoa cells and testicular tissue structure. It seems that CGA exhibited two different effects on spermatogenesis: (1) to protect or strengthen the testicular cells against radiation injury by pretreatment, and (2) to support the recovery of the cells after radiation exposure by post-treatment.

Our findings, similar to several experimental studies, showed that IR exerts negative effects on the male reproductive system and causes abnormal changes in sperm function, chromosomal aberrations, and epigenetic changes that have been clarified by radiation workers (Cadet et al., 2012; Marjault and Allemand, 2016; Taneja et al., 2019).

To date, a large number of studies have been conducted to investigate the effective factors for the improvement of IR-induced spermatogenesis injury. However, although the real association between IR and antioxidants is still not completely revealed, the literature reveals that depending on the type of antioxidants, treatment duration, and even the diagnostics of the man's fertility, antioxidants are useful to ameliorate sperm count, motility, morphology, and DNA damages (Cadet et al., 2012; Marjault and Allemand, 2016; Taneja et al., 2019). This represents the idea that supplementation with metabolic and antioxidant compounds could be efficacious when included in strategies to improve fertility. For example, Moller et al. showed that antioxidants decreased

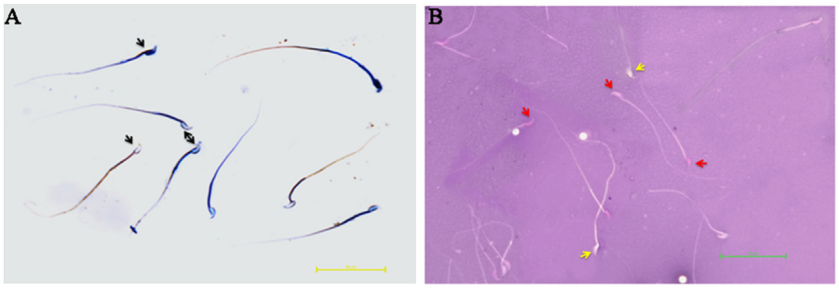

Figure 2. A) Using AB staining normal sperms (no absorbed color) become pale blue-stained cells (AB^-) and abnormal immature sperms (black arrow) appear dark blue cells (AB^+). B) the viability of sperm. Sperm with a redhead (red arrows) were deemed as dead sperm and those that showed white (yellow arrow) were classified as live. All analyzed data are presented in Table 3.

Table 3. Effect of chlorogenic acid on some sperm parameters and chromatin condensation in sperm.

Groups	Sperm Viability (%) (Mean ± SD)	Sperm with Chromatin Condensation (Mean ± SD)	Count ($\times 10^6$/mL) (Mean ± SD)	Progressive Motility (Mean ± SD)	Non-Progressive Motility (Mean ± SD)	Immotile Sperm (Mean ± SD)
Control	25.21 ± 0.03	12.15 ± 0.01	52.34 ± 0.35	55.74 ± 0.35	15.44 ± 0.47	28.41 ± 1.3
IR	14.38 ± 0.01^{a}	22.35 ± 0.04^{a}	24.67 ± 0.07^{a}	33.65 ± 0.67^{a}	29.21 ± 0.64^{a}	45.67 ± 1.04^{a}
IR + CGA (5 mg/kg)	15.47 ± 0.02^{a}	19.22 ± 0.03^{a}	32.74 ± 0.03^{a}	38.05 ± 0.04^{a}	22.38 ± 0.36^{a}	39.98 ± 0.68^{a}
IR + CGA (10 mg/kg)	17.22 ± 0.02^{ab}	18.75 ± 0.03^{ab}	38.21 ± 0.07^{ab}	41.76 ± 0.46^{ab}	21.79 ± 0.45^{ab}	36.12 ± 1.45^{ab}
IR + CGA (20 mg/kg)	17.24 ± 0.02^{abc}	16.84 ± 0.02^{ab}	40.52 ± 0.04^{abc}	45.28 ± 0.75^{abc}	19.46 ± 0.71^{ab}	35.82 ± 1.06^{abc}
IR + CGA (40 mg/kg)	23.52 ± 0.02^{bcde}	12.12 ± 0.01^{bcde}	48.22 ± 0.04^{bcde}	51.26 ± 0.08^{bcd}	15.32 ± 0.52^{bcde}	29.14 ± 0.76^{bcde}
IR + CGA (80 mg/kg)	20.26 ± 0.02^{bcde}	14.25 ± 0.01^{bcd}	45.32 ± 0.03^{bcd}	49.78 ± 0.23^{bcd}	17.85 ± 0.34^{bcd}	32.75 ± 0.54^{bcd}

The last day of the study, day 35, was chosen for the assessment of testicular injuries to show the effect of long-term treatment with CGA. Values are shown as mean ± SD. SD: standard deviation. IR: irradiation, CGA: chlorogenic acid. (a): significant difference with the control group; $p < 0.02$. (b): significant difference with IR group; $p < 0.04$. (c, d, and e): these show a significant difference compared to the IR + 5 mg/kg CGA, IR + 10 mg/kg CGA, and IR + 20 mg/kg CGA groups, respectively ($p < 0.05$). The viability and chromatin condensation of sperm was evaluated under a light microscope following EN staining (Figure 2A and B).

aberrant morphology in the sperms of Chernobyl survivors (Møller et al., 2005). Some antioxidant factors such as green tea, ascorbic acid, and alpha-tocopherol have been proved abilities to protect the testis against IR (Ding et al., 2015; Najafi et al., 2019). In other investigations, it has been also confirmed that antioxidants such as Coenzyme Q10 (10 mg/kg, orally, for 2 consecutive weeks) and/or melatonin (100 mg/kg, I.P, 30 min prior irradiation) could alleviate ionizing radiation-induced testicular damage in rats or mice (Khan et al., 2015; Said et al., 2019). One study has shown that pre-treatment with 100 mg/kg CGA (IP) 30 min before testicular detorsion led to the restoration of the Johnsen Score (Kazaz et al., 2022). Interestingly, it has been reported that higher doses of antioxidants could be linked to health risks in some cases because of the prooxidative effects at high concentrations (Salehi et al., 2018). For example; excessive vitamin C enhances the concentration of urinary oxalate subsequently could lead to an increase in the risk of calcium oxalate kidney stones (Salehi et al., 2018). In our study, the dose of 40 was better than the dose of 80 mg/kg CGA.

In this regard, in the present study, we focused, for the first time, on the beneficial and antioxidant effects of CGA on the amelioration of oxidative stress in IR-induced testicular damage.

Our biochemical assessment data demonstrated that CGA remarkably inhibited testicular oxidative stress after irradiation and almost restored MDA to its normal level. In addition, the level of SOD, TAC, and GSH significantly increased in CGA treated group (Figure 5).

The body of evidence indicates that non-enzymatic antioxidants including vitamins, glutathione and micronutrients, and nutrients have a

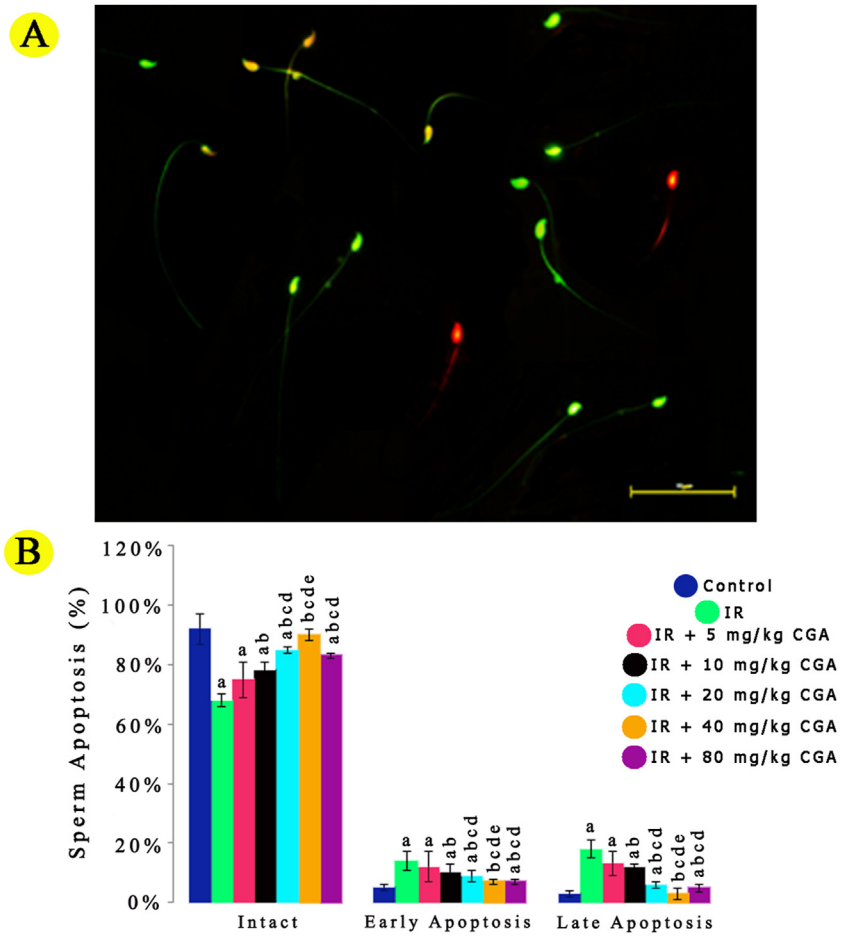

Figure 3. Acridine orange and ethidium bromide (AO/EB)-stained sperm: A: intact cells (green), B: early apoptotic (bright orange), and C: late apoptotic (red) under a fluorescent microscope. Data are expressed as the total number of intact and apoptotic cells (early + late) as a percentage of the total. a: significant difference with control group; $p < 0.05$. b: significant difference with 2nd group; $p < 0.05$. c: significant difference with 3nd group (IR + 5 mg/kg CGA); $p < 0.05$. d: significant difference with 3nd group (IR + 10 mg/kg CGA); $p < 0.05$. e: significant difference with 3nd group (IR + 20 mg/kg CGA); $p < 0.05$.

Table 4. mRNA expression of apoptotic-related genes analyzed by RT-qPCR.

	16 Hours After Exposure			35 Days After Exposure		
Groups/Genes	*Bax* (Mean ± SD)	*Bcl-2* (Mean ± SD)	*Bax/Bcl-2* (Mean ± SD)	*Bax* (Mean ± SD)	*Bcl-2* (Mean ± SD)	*Bax/Bcl-2* (Mean ± SD)
Control	3.22 ± 0.024	5.18 ± 0.021	0.62 ± 0.022	3.13 ± 0.024	5.13 ± 0.021	0.61 ± 0.022
IR	5.81 ± 0.027^{a}	2.08 ± 0.023^{a}	2.79 ± 0.024^{a}	5.58 ± 0.027^{a}	2.21 ± 0.023^{a}	2.52 ± 0.024^{a}
IR + CGA (5 mg/kg)	5.52 ± 0.021^{a}	2.58 ± 0.023^{a}	2.13 ± 0.023^{a}	5.37 ± 0.021^{a}	2.67 ± 0.023^{a}	2.01 ± 0.023^{a}
IR + CGA (10 mg/kg)	5.13 ± 0.022^{ab}	2.72 ± 0.021^{ab}	1.88 ± 0.023^{ab}	4.98 ± 0.022^{ab}	3.01 ± 0.021^{ab}	1.65 ± 0.023^{ab}
IR + CGA (20 mg/kg)	4.81 ± 0.026^{abc}	3.41 ± 0.025^{abcd}	1.41 ± 0.021^{abc}	4.52 ± 0.026^{abc}	3.30 ± 0.025^{abc}	1.36 ± 0.021^{abc}
IR + CGA (40 mg/kg)	3.34 ± 0.019^{bcde}	5.08 ± 0.018^{bcde}	0.65 ± 0.019^{bcde}	3.21 ± 0.019^{bcde}	5.1 ± 0.018^{bcde}	0.62 ± 0.019^{bcde}
IR + CGA (80 mg/kg)	3.64 ± 0.018^{bcde}	4.81 ± 0.020^{bcde}	0.75 ± 0.019^{bcde}	3.52 ± 0.018^{bcde}	4.88 ± 0.020^{bcde}	0.72 ± 0.019^{bcde}

It has been reported that the most apoptotic rate occurs 16 h after exposure. Therefore, the expression of apoptotic-related genes, *Bax* and *Bcl-2*, was analyzed in these two time periods, 16 h and day 35. Relative expression levels normalized against the internal control level, *β-actin*. The *Bax/Bcl-2* ratio considered an index shows that the cells survive or die (apoptosis). Data are shown as mean ± SD (standard deviation). (a, b, c, d, and e): these show a significant difference compared to the control, IR, IR + 5 mg/kg CGA, IR + 10 mg/kg CGA, and IR + 20 mg/kg CGA groups, respectively (0.003≤ع).

beneficial effect on fertility, in particular on sperm quality, and are, therefore, recommended as a potentially effective therapy for the treatment of male infertility. Research in this field confirmed that CGA has antioxidant effects (Ali et al., 2017; Dkhil et al., 2020; J. Santana-Gálvez, L. Cisneros-Zevallos, & D. A. Jacobo-Velázquez, 2017). It has been discerned that hydroxyl groups (OH) on phenolic acids act as positive moieties for their antioxidant properties, which is associated with the number of hydroxyl groups as follows: tri-hydroxy phenolic acids > di-hydroxy

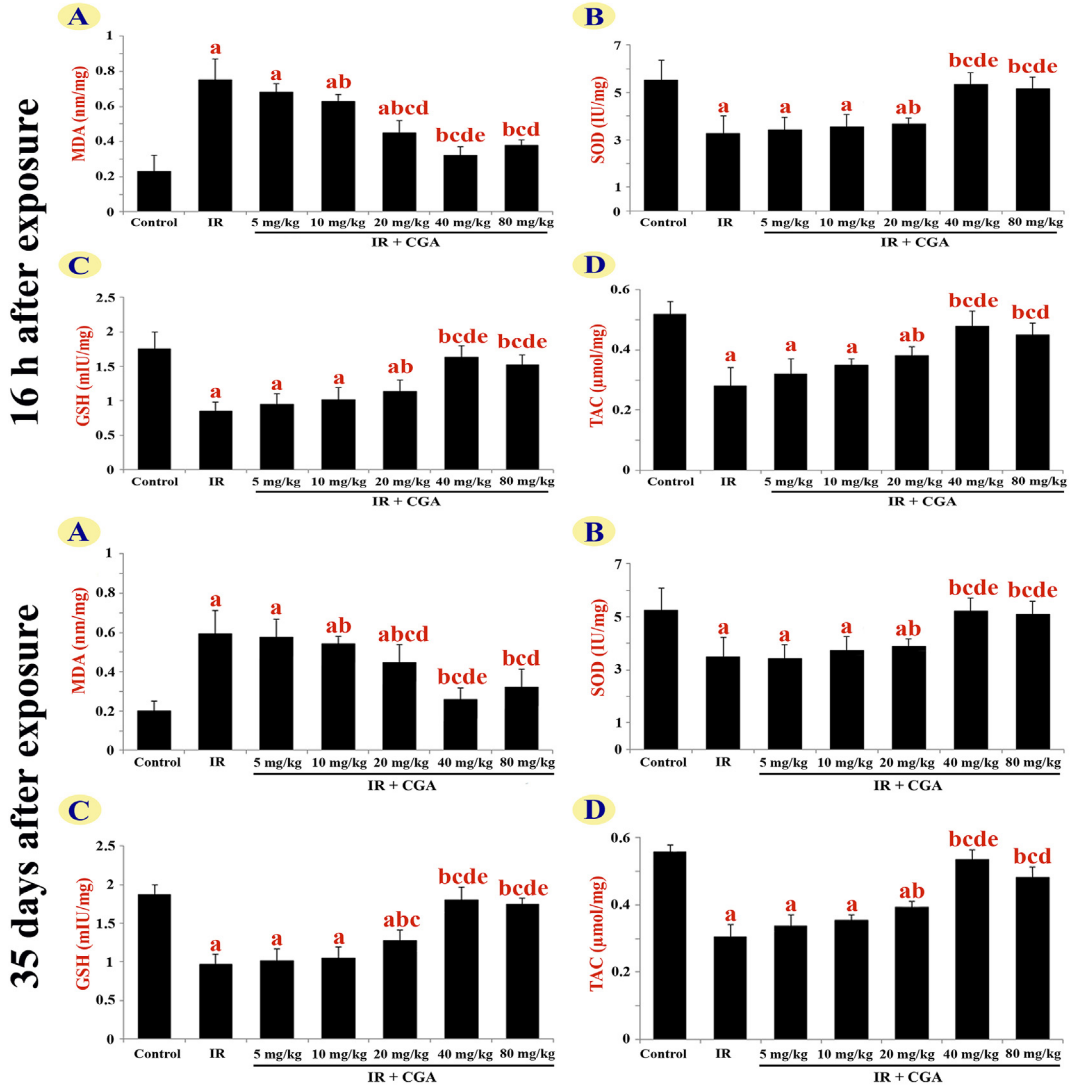

Figure 4. Effect of CGA on oxidative stress parameters. Values are Mean ± S.D. MDA: malondialdehyde. GSH: glutathione. TAC: total antioxidant capacity. SOD: superoxide dismutase. IR: ionizing radiation. CGA: chlorogenic acid. (a, b, c, d, and e): shows a significant difference with the control, IR, IR + 5 mg/kg CGA, IR + 10 mg/kg CGA, and IR + 20 mg/kg CGA ($p < 0.05$).

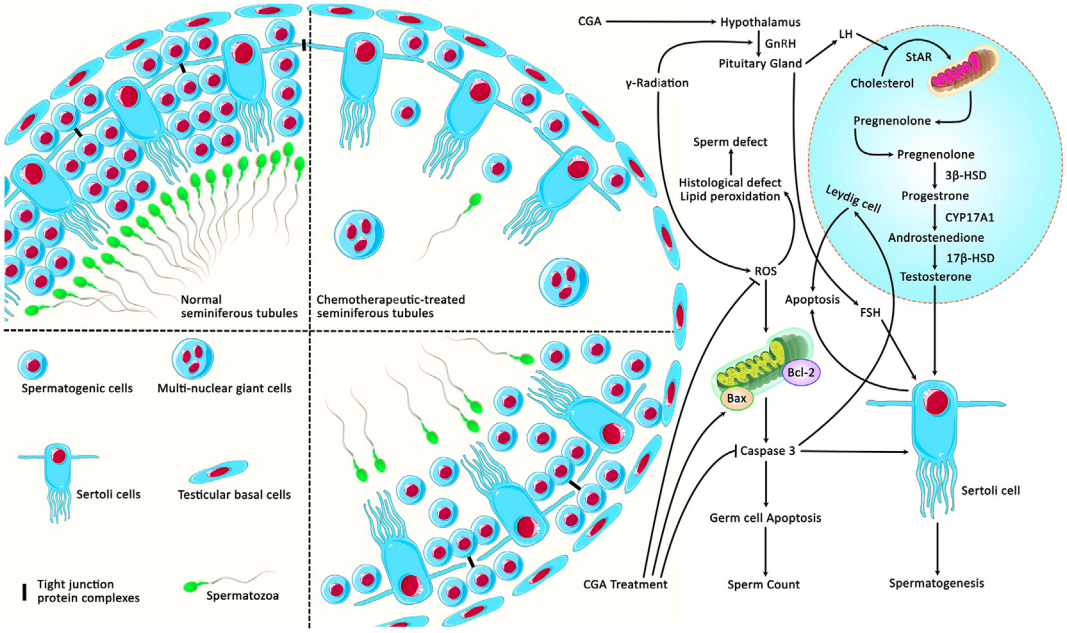

Figure 5. In this schematic graph, we try to explain the mechanism of irradiation (IR)-induced testicular injury and the protective effects of long-term treatment with chlorogenic acid (CGA). IR could lead to severe damage to testicular germ cells and disrupt the normal testicular structure. IR could also increase intracellular ROS production, subsequently resulting in an alternation in levels of cellular antioxidant parameters and DNA breaks, and finally, caspase-3 activation could lead to apoptosis. By increasing the rate of apoptosis, the number of spermatogenic and somatic cells probably decreased, hence, these damages in testicular tissues could lead to a decrease in normal sperm production. For understanding the impacts of IR injury on the hypothalamic-pituitary-gonadal (HPG) axis, we strongly suggest that the mRNA or protein expression of genes involved in steroidogenesis should be studied. However, on the one hand, long-term treatment with CGA could improve the structure of testicular histology, increase the ratio of *Bcl-2/Bax*, and restore sperm production and spermatogenesis.

(catechol) > mono-hydroxy (Naveed et al., 2018; Tajik et al., 2017). However, CGA has been reported to ameliorate oxidative stress-elicited tissue damage in different systems. For example, CGA successfully attenuates hydrogen peroxide-induced oxidative stress in human lens epithelial cells (Naveed et al., 2018; Tajik et al., 2017); CGA increases the expression of neuroprotective ribosomal proteins that exerted significant antioxidant and neuroprotective effects on the brain (Naveed et al., 2018; Tajik et al., 2017), and CGA pretreatment can inhibit ER stress-induced cell death and levels of indicators of ER stress caused by palmitic acid (Naveed et al., 2018; Tajik et al., 2017). Moreover, another study reported that co-administration with CGA (25 and 50 mg/kg, orally) for 14 consecutive days could abate tamoxifen-mediated reproductive toxicities and increases the antioxidant activities of GPx, GST, GSH in testicles of rats (Owumi et al., 2021). Their results have also shown the dose of 50 mg/kg CGA had a better effect related to the other dose.

Finally, our molecular evidence by RT-qPCR also confirmed these enhancing effects of CGA and demonstrated that the expression ratio of *Bax/Bcl-2* (key regulators of apoptosis) in the presence of CGA was significantly lower than in the other groups (Figure 5). Notably, our molecular findings supported both the histological and biochemical results. In a way that molecular data amplified the result of developmental ability and functional potential of CGA during IR of testis tissue. Our findings were following Dkhil et al. who reported the protective effect of CGA against the injuries induced by sodium arsenite ($NaAsO_2$) in the liver of mice. They demonstrated a significant decrease in the mRNA levels of the liver pro-apoptotic markers, but a significant increase was observed in the anti-apoptotic markers in the presence of CGA (Dkhil et al., 2020). E. Owumi et al. also reported that treatment with CGA (25 and 50 mg/kg, orally) for 14 consecutive days by downregulating apoptosis (*Caspase-3*) and reactive oxygen and nitrogen species (RONS) levels could improve reproductive function in rats which underwent reproductive toxicity with tamoxifen (Owumi et al., 2021).

At the end of the article, it should be noted that although it is stated that most changes related to apoptosis or biochemical factors occur 16 h after exposure, our study showed that there is no significant difference in the expression of apoptosis-related genes and biochemical factors for two mentioned time points, 16 h and 35 days after exposure.

Therefore, to save costs, the study of sperm parameters and the level of apoptosis in sperm as well as histological changes were performed only on the last day of the study, which was the goal of our study from the beginning.

5. Conclusion

The results of the current original investigation demonstrate that CGA may have several roles in the protection of testis against irradiation. Taken together, we believe that the CGA could exert radiation protective and therapeutic effects on testis by enhancing DNA damage repair activity, inhibiting apoptosis caused by DNA damage, and abating IR-induced oxidative stress. Therefore, the employment of CGA still appears to be an extremely attractive strategy in the future for fertility preservation in males exposed to IR. Although more research is still needed for a better understanding of the effect of CGA at the cellular and molecular levels, CGA protective function may likely involve the activation of several apoptosis-related key pathways.

Declarations

Author contribution statement

Neda Abedpour; Hamed Shoorei: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ahad Zeinali; Mojtaba Karimipour; Bagher Pourheidar; Gholam Hossein Farjah; Atefe Abak: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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