Radioprotective Potential of *Grewia Asiatica* Fruit Extract in Mice Testis

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Summary

The radioprotective effect of *Grewia asiatica* fruit (GAE) was studied. Method: For study Swiss albino mice were divided into five groups-1. Control (vehicle treated) 2. GAE treated (700 mg / Kg. b.wt/day for 15 days), 3. Irradiated (5 Gy), 4. GAE + Irradiated and 5. Irradiated + GAE treated. Testis were weighed and used for histopathological examination. Irradiation of animals resulted a significant depletion in testis weight, whereas GAE pre/post treated group showed significantly higher values compare to the irradiated group. Histopathological study showed that due to radiation exposure number spermatogina "A", spermatogonia "B", spermatocytes and spermatid count were declines significantly compare to the control group. Whereas these counts were higher in GAE pre/post treated irradiated group compare to the respective irradiated group till last autopsy interval i.e. 30 days post irradiation. Conclusion: GAE have the protective potential to ameliorate the damaging effect of radiation to the testis.

Key words: Radiation Protection, Mice testis, Testis histology. Spermatocytes count, Spermatid count

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Introduction

Nowadays, many children and young adults are successfully treated for several types of cancer. More than half of cancer patients are treated with radiation therapy. Despite its high therapeutic index, radiation therapy can cause disabling injuries to normal tissues, especially in long-term survivors.

According to the scientific standards of protection, the response of the gonads has been recognized as one of the most important criteria in regulating the action of ionizing radiation. Radiation acts as retrogressive agent in the production of gametes by its degenerative influence at the level of germs cells and/or steroids synthesizing mechanisms. This could also cause eventual sterility in the mammals induced by gamma radiation and the length of the sterile period depends on the stem cell survival and repopulation index (1, 2).

The effects of irradiation on gonadal tissue have been studied extensively in both man and rodents (3, 4). Histopathological examination of reproductive organs is an important tool in the interpretation of testicular toxicity when particular cell types (seminiferous epithelium, sertoli or Leydig cells) or uncertain spermatogenic stages are affected.

Thus, one of the great challenges of modern radiation therapy is to increase tolerance of normal tissue to ionizing radiation in order to improve the quality of life of cancer survivors and/or enhance local control using dose escalation.

Among different synthetic molecular radio protectors, WR-2721 and related compounds have been found to be most promising, but the side effects associated with them have restrained their use (5). In view of this, the search for newer and more effective agents is inevitable.

Naturally occurring antioxidants may provide an extended window of protection against low-dose, low-dose-rate irradiation, including therapeutic potential when administered after irradiation. A number of phytochemicals, including caffeine, genistein and melatonin, have multiple physiological effects, as well as antioxidant activity, which result in radioprotection in vivo (6).

In this context *Grewia asiatica* (Phalsa) cultivated on a commercial scale mainly in the northern and western states of India (7, 8), is known for its medicinal properties (9). *Grewia asiatica* contains anthocyanin type cyanidin 3- glucoside vitamin C, A, minerals, carotenes and dietary fibers etc (10). Earlier studies in laboratory showed that supplementation of *Grewia asiatica* fruit extract (GAE) can ameliorate radiation induced depletion in GSH and protein level and can inhibit the radiation induced lipid peroxidation in brain (11), cerebrum (12,13), liver (14,15), and blood (16), and dose reduction factor (DRF) of GAE is 1.53 (17). Sharma and Sisodia (18) also showed the radioprotective efficacy of GAE in mice testis in terms of nucleic acid and antioxidant level.

The present study has been undertaken to assessing the histological evidence of radioprotective efficacy of GAE in testis of Swiss albino mice.

Materials and methods

Animal care and handling

The animal care and handling was done according to the guidelines set by World Health Organization, Geneva, Switzerland and INSA (Indian National Science Academy, New Delhi, India). The Departmental Animal Ethical Committee (DAEC) approved this study. Swiss albino mice (6–8 weeks) old weighing 23±2 gm from an inbred colony was used for the present study. These animals were maintained under controlled conditions of temperature and light (Light: dark, 10 hrs: 14 hrs.). Four animals were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. They were provided standard mice feed (procured from Hindustan Levers Ltd., India) and water *ad libitum*.

Extract preparation (Drug)

Fresh fruits of *Grewia asiatica* collected locally in summer season were washed, shade dried and powdered after removal of seeds. Methanolic extract was then prepared by refluxing for 48 hours (4x12) at 40^{o}C. The extract thus obtained was vacuum evaporated so as to get in powdered form. The extract was redissolved in double-distilled water (DDW) just before the oral administration. For the various concentrations, a known amount of GAE was suspended in DDW and 50 µl of GAE suspension was given to each mouse by oral gavage as given by Ahaskar *et al* (17).

Source of irradiation

The cobalt teletherapy unit (ATC-C9) at Cancer Treatment Center, Radiotherapy Department, SMS Medical College and Hospital, Jaipur, Rajasthan, India was used for irradiation. Unanaesthestized animals were restrained in well-ventilated perspex box and whole body exposed to gamma radiation at a distance (SSD) of 77.5cm from the source to deliver the dose rate of 1.07 Gy/ min.

Dose selection

Dose selection of Grewia asiatica was carried out on the basis of drug tolerance study in our laboratory (17,19). Various doses of *Grewia asiatica* (100, 400, 700, 1000, 1300 mg/kg b.wt.) were tested against gamma irradiation (10Gy). Thus, 700-mg/kg b.wt. /day was used as optimum dose based on survivability of mice for further experimentation.

Experimental design

Mice selected from an inbred colony were divided into 4 groups (30 animals in each group).

1. Control (vehicle treated): Mice of this group received only DDW water for 15 days.

2. GAE treated: Mice of this group were administered orally once daily with GAE (700mg/kg of b.wt. /day) for 15 consecutive days.

3. Irradiated: Mice received DDW (volume equal to *Grewia asiatica* solution) for 15 days and were than whole body exposed to 5Gy of gamma-radiation.

4. GAE treated + Irradiated (GAE+IR): In this group after oral administration of GAE (700mg/kg of b.wt. /day) for 15 consecutive days as done in GAE treated group. Mice were whole body exposed to single dose of 5 Gy gamma-radiation one hour after administration of last dose of GAE,

5. Irradiated +GAE treated (IR+GAE): In this group, after whole body exposure to a single dose of 5 Gy gamma-radiation, oral administration of GAE (700mg/kg of b.wt. /day) was made once daily for 15 consecutive days one hour after radiation exposure.

Six mice from each group were necropsied by cervical dislocation at various intervals viz. 1, 3, 7, 15 and 30 days post irradiation. Testis weight was taken and tissue was used for quantitative histopathological study.

Histopathological studies

The samples were fixed in 10% neutral buffered formalin and were processed for making paraffin blocks and sections 5 μm thick were cut. The sections of tissues were passed through a graded series of alcohol and stained in Eosin and Harris Haematoxylin (20). Different testicular cells were counted by using a planimeter at high magnification (400x).

Statistical analysis

The results obtained in the present study were expressed as mean ± SEM in graphs. The statistical difference between various groups (a: Control v/s GAE treated, b: Control v/s Irradiated, c: Irradiated vs GAE treated + Irradiated, d: Irradiated vs Irradiated + GAE treated) were analyzed by the Student's *t*-test and significance levels were shown in graphs as p values of t-test: n= non-significant, $<0.1, *<0.05, **<0.01, ***<0.005, ****<0.001. One way ANOVA analyses between all groups at each interval were also carried out and result shown in the text.

Results

Statistically non-significant differences existed in weight of testis between group I (control) and group II (GAE treated) mice. In group III, irradiation induced continuous decline in testis weight upto day 15 (37.98%) thereafter increase was noted at day 30 *p.i* but weight was significantly ($P < 0.001$) lower compare to the weight of testis of control group at all the *p.i.* intervals. In group IV (GAE + IR group) and V (IR + GAE) early recovery in testis weight was noted as evident by increased weights after day 7 *p.i.* In both the experimental groups (IV & V) GAE supplementation resulted significantly higher values ($p<0.001$) of testis weight in comparison to group III (irradiated) at all the *post irradiation* intervals (Fig_1). When the testis weight of group I, III, IV and V was compared with each other at

each autopsy interval by one-way ANOVA, highly significant difference was observed on day 1 ($F_{3, 20}$=587.71, p<0), day 3 ($F_{3,20}$=747.7, p<0), day 7 ($F_{3,20}$=1016.39, p<0), day 15 ($F_{3,20}$=1320.17, p<0) and day 30 ($F_{3,20}$=1396.69, p<0) *p.i.* respectively.

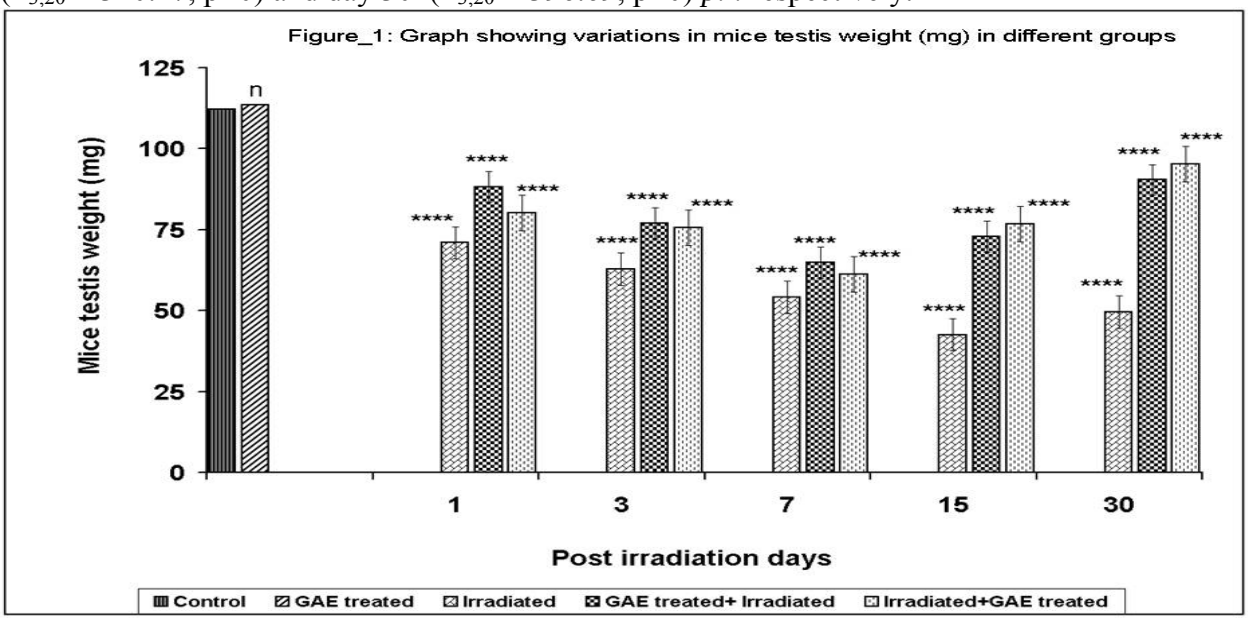

Figure_1: Graph showing variations in weight (mg) of mice testis in pre and post GAE (methanolic extract of *Grewia asiatica* fruit, 700mg/kg b.wt/day for 15 days) treated- irradiated group and irradiated group in comparison to control group and only GAE treated group

Supplementation of GAE for 15 days (group II) also induced significant (p<0.05) change in the number of spermatogonia 'A' when compared with group I (Control). In group III (irradiated) the spermatogonia 'A' population diminished after irradiation; their number decreased significantly and progressively till day 7 *p.i*. Thereafter, the percentage of spermatogonia 'A' improved till day 30 with a deficit of only 38.07% from control. Administration of GAE prior/post irradiation maintained a higher number of spermatogonia 'A' in comparison to irradiated group (Fig_2). One-way ANOVA comparison between the four groups *i.e.* control, IR, GAE + IR and IR + GAE group, at each autopsy interval showed highly significant differences in spermatogonia 'A' counts on day 1 ($F_{3,20}$=145.7, p<0.0001), day 3 ($F_{3,20}$=190.5, p<0.0001), day 7 ($F_{3,20}$=111.3, p<0.0001), day 15 ($F_{3,20}$=122.6, p<0.0001) and day 30 ($F_{3,20}$=27.06, p<0.0001) *p.i.* respectively.

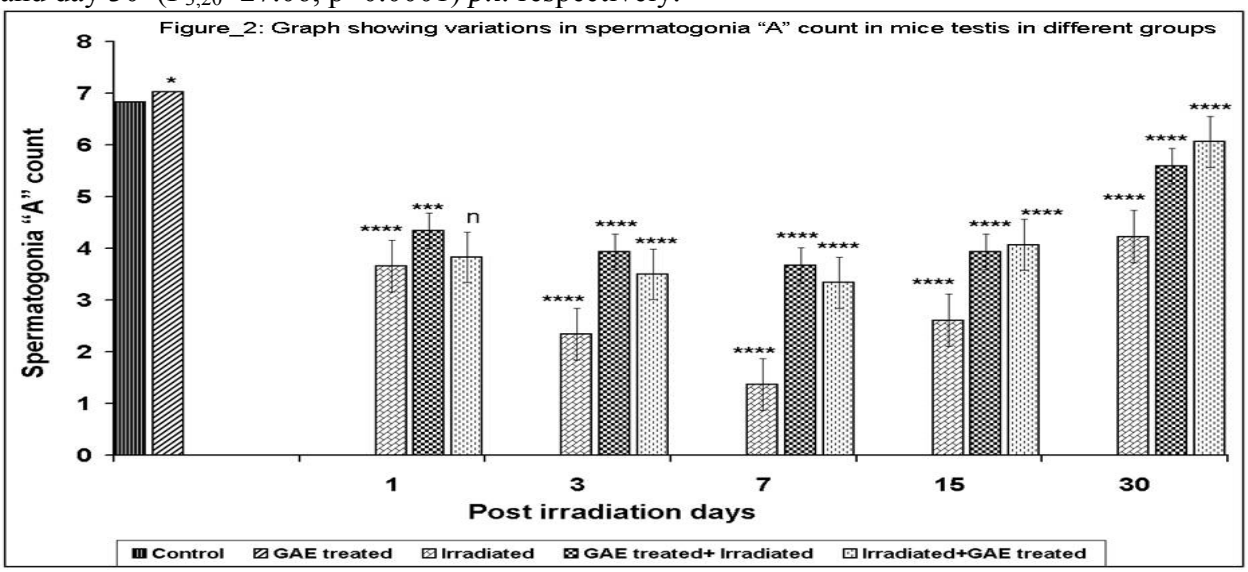

Figure_2: Graph showing variations in average spermatogonia "A" count in mice testis in pre and post GAE (methanolic extract of *Grewia asiatica* fruit, 700mg/kg b.wt/day for 15 days) treated- irradiated group and irradiated group in comparison to control group and only GAE treated group.

Statistically non significant differences existed between group I and II with respect to spermatogonia 'B' count. In group III irradiation resulted in significant deficit in number of spermatogonia 'B' continuously till day 7 *p.i.* Thereafter it increased continuously upto the last interval studied but still deficit of 54.91% existed from group I (control). In group IV and V (GAE+IR and IR+ GAE), similar pattern of decrease was noticed as in group III. The number of spermatogonia 'B' decreased till day 7 *p.i.*, thereafter the numbers increased till the last autopsy interval, but could not attain the control number. Considerably high ($p<0.001$) population of these cells was present as compared to corresponding group III (irradiated) at all the autopsy intervals (Fig_3). One-way ANOVA comparison between the four groups *i.e.* control, IR, GAE + IR and IR + GAE group, at each autopsy interval showed highly significant differences in spermatogonia 'B' count on day 1 ($F_{3,20}=2022$, $p<0.0001$), day 3 ($F_{3,20}=328.9$, $p<0.0001$), day 7 ($F_{3,20}=1399$, $p<0.0001$), day 15 ($F_{3,20}=274.1$, $p<0.0001$) and day 30 ($F_{3,20}=187.1$, $p<0.0001$) *p.i.* respectively.

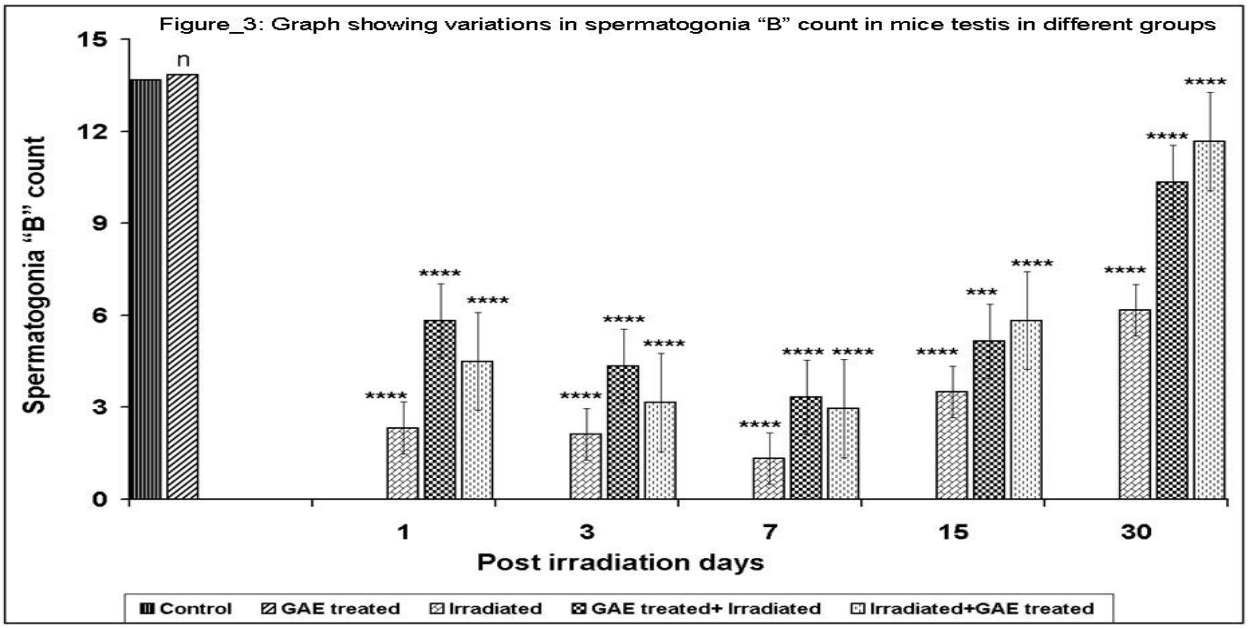

Figure_3: Graph showing variations in average spermatogonia "B" count in mice testis in pre and post GAE (methanolic extract of *Grewia asiatica* fruit, 700mg/kg b.wt/day for 15 days) treated- irradiated group and irradiated group in comparison to control group and only GAE treated group

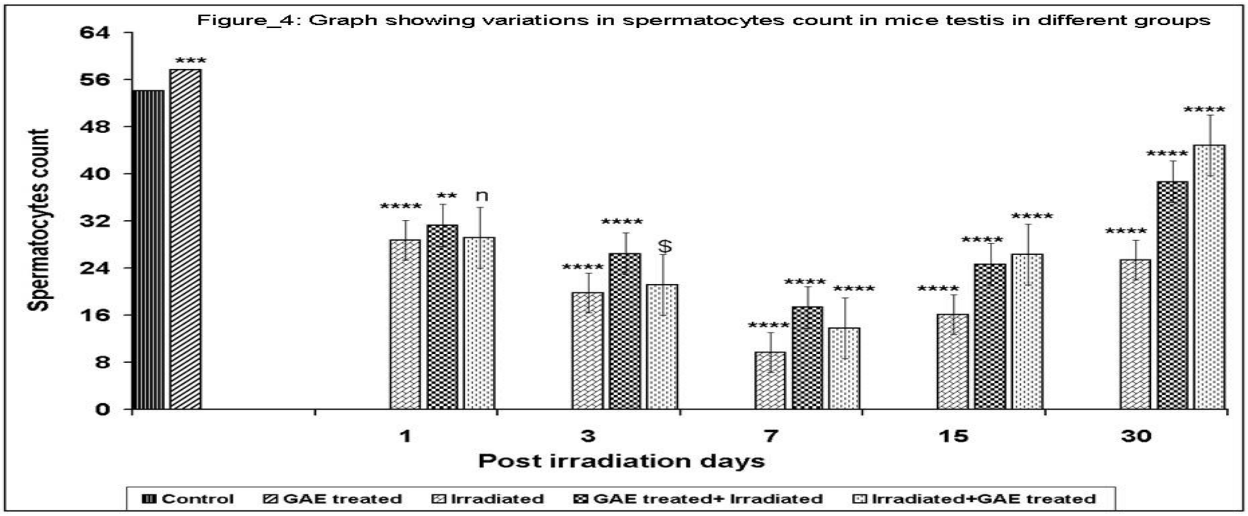

Figure_4: Graph showing variations in average spermatocytes count in mice testis in pre and post GAE (methanolic extract of *Grewia asiatica* fruit, 700mg/kg b.wt/day for 15 days) treated- irradiated group and irradiated group in comparison to control group and only GAE treated group.

Only GAE supplementation for 15 days (group II) showed significantly ($p<0.005$) increased number of spermatocytes in comparison to group I (control). Irradiation caused statistically significant ($p<0.001$) decrease in number of spermatocytes in group III at all the autopsy intervals. The initial decline in the number of spermatocytes noticed at day 1 continued upto 7 day *p.*i, thereafter, the number of spermatocytes started to increase in comparison to the group I (control) and by day 30, their number reached 47% of control. Supplementation of GAE (prior/post) (group IV & V) reduced the spermatocytes loss. In group IV and V (GAE+IR and IR+ GAE), the number of spermatocytes was considerably higher ($p<0.001$) in comparison to corresponding group III at each autopsy interval (Fig_4). One-way ANOVA comparison between the four groups *i.e.* control, IR, GAE + IR and IR + GAE group, at each autopsy interval showed highly significant differences in spermatocytes count on day 1 ($F_{3,20}=446.9$, $p<0.0001$), day 3 ($F_{3,20}=818.5$, $p<0.0001$), day 7 ($F_{3,20}=2195$, $p<0.0001$), day 15 ($F_{3,20}=1669$, $p<0.0001$) and day 30 ($F_{3,20}=395.1$, $p<0.0001$) *p.i.* respectively.

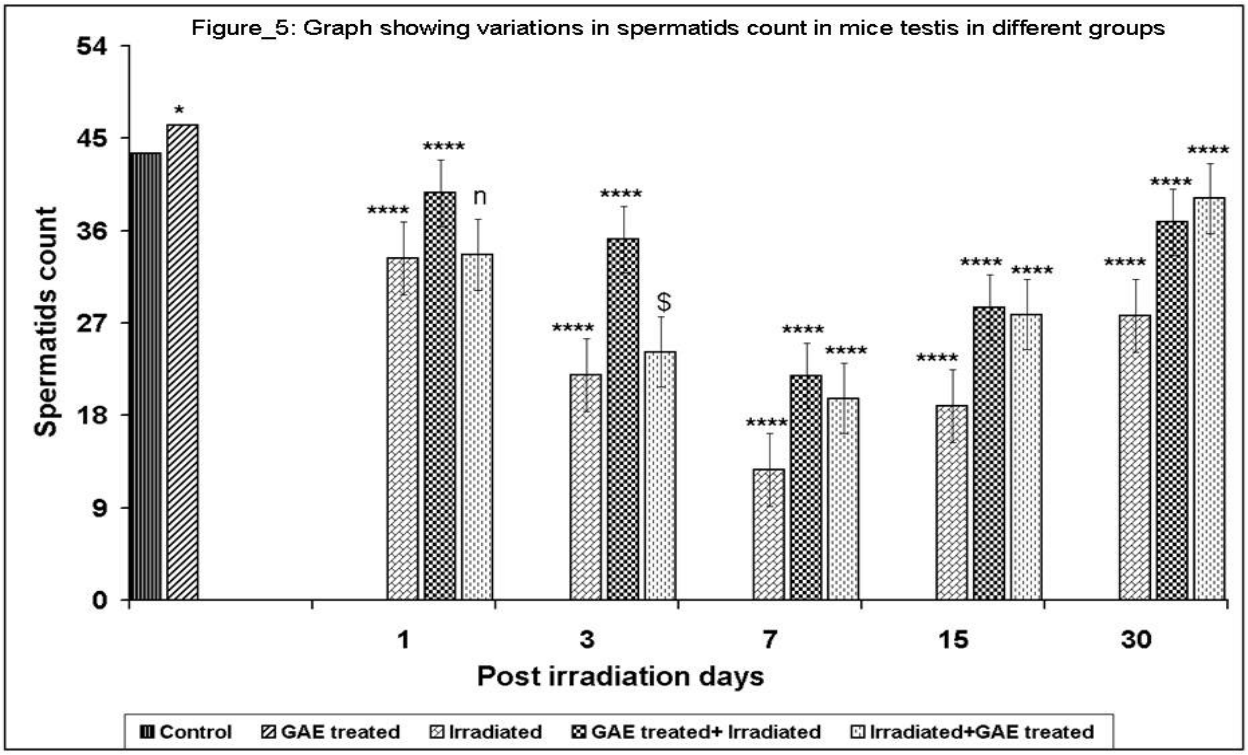

Figure_5: Graph showing variations in average spermatids count in mice testis in pre and post GAE (methanolic extract of *Grewia asiatica* fruit, 700mg/kg b.wt/day for 15 days) treated- irradiated group and irradiated group in comparison to control group and only GAE treated group.

Only GAE supplementation (group II) for 15 days showed significantly ($p<0.05$) decreased numbers of spermatids in comparison to group I. Irradiation (group III) caused statistically significant ($p<0.001$) decline in number of spermatids at all the autopsy intervals and at day 30, the deficit was only 36.29% from control. In group IV (GAE+IR) significantly ($p<0.001$) higher number of spermatids count in comparison to corresponding control at each autopsy interval was noted. Whereas in group V (IR+GAE) higher number of spermatids ($p<0.001$) specially at later interval as compared to corresponding control level were recorded (Fig_5). One-way ANOVA comparison between the four groups *i.e.* control, IR, GAE + IR and IR + GAE group, at each autopsy interval showed highly significant differences in spermatids count on day 1 ($F_{3,20}=60.27$, $p<0.0001$), day 3 ($F_{3,20}=120.2$, $p<0.0001$), day 7 ($F_{3,20}=465.7$, $p<0.0001$), day 15 ($F_{3,20}=274.3$, $p<0.0001$) and day 30 ($F_{3,20}=221.4$, $p<0.0001$) *p.i.* respectively.

Discussion

The present findings of this study revealed reduction in the average weight of testis and cell counts in irradiated animals. The depletion in spermatogenic elements might be possible cause of the reduction in the testes weight.

The main rationale to choose 30 day-time period of study after irradiation were: firstly, the length of time from initiation of stem cell division to formation of spermatozoa is around 33 days in mouse (2); secondly, sertoli cells are given the opportunity to remove dead germ cells and increase access to stem cell niches along the basement membrane after irradiation (21). The chosen period of time (30 day) provides sufficient time to monitor the potential recovery of spermatogenesis in surviving stem cells in the irradiated animals.

The stem spermatogonia are moderately radioresistant. The types A_l to B spermatogonia are the most radiosensitive (22). Recovery ensues because the surviving stem spermatogonia, which are more resistant, immediately begin regeneration of their own numbers and production of differentiated cells.

Due to high radiosensitivity of spermatogonia B, radiation doses as low as 15 cGY can depress spermatogenesis for 2 months, and 100 cGY can result in azoospermia for 1 year (23). Fajardo *et al*. (24) proposed that radiosensitivity depends on the proliferative capacity of cell. In adult testis, the spermatogonia B are actively proliferating which makes them most radiosensitive followed by primary and secondary spermatocyte.

Grover and Kumar (25) stated that type B spermatogonia are exquisitely sensitive to effects of radiation. The type A is more resistant because their longer cell cycle time allows considerable variation in radio-sensitivity among different phases of cell cycle. Similarly, Van Der Meer *et al* (26) reported that undifferentiated type A spermatogonia was resistant to irradiation and could repopulate the seminiferous epithelia and entire spermatogenic cell population after irradiation. Songthaveesin (27) reported a significant decrease in primary spermatocyte on exposure to radiation.

Adreieu *et al* (28) reported decrease in spermatogonia A, primary spermatocyte count and significant reduction in testis weight in relation to individual body weight on local testicular irradiation (6 Gy) in adult Wistar rats.

Sharma and Sisodia (18) also proved that GAE has the free radical scavenging activity by the DPPH* scavenging assessment (IC_{50} = 218.47 μg/ml) as well as the superoxide radicals generated from xanthine/xanthine oxidase assay were examined for the scavenging by the GAE extracts in comparison with cytochrome C (IC_{50} = 15.18 μg/ml). They also showed that GAE inhibits the protein carbonylation formation in BSA in *in vitro* study (IC_{50} = 26.66 μg/ml for GAE). They also showed that GAE pre/post treatment inhibits the radiation (5 Gy) induced elevation of lipid peroxidation and enhance the level of GSH and protein content in testis.

The exact mechanism by which GAE protects against radiation-induced damage is not fully understood. But as Sharma and Sisodia (18) demonstrated that the GAE has a strong radical scavenging activity in $DPPH^*$ and O_2^- assays in a concentration dependent manner and *in vitro* radioprotective activity in protein carbonyl estimation assay suggests that the radioprotective potential of GAE may be due to free radical scavenging power by the antioxidant present in it like anthocyanin Vit. C and carotenes.

This suggests that *Grewia asiatica* fruit extract (Phalsa) could be a potential candidate for screening as a radioprotector for clinical applications.

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