Radioprotective role of *Punica granatum* fruit rind extract: A biochemical study on mouse testis

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► ***Original article***

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ABSTRACT

***Background*:** To evaluate the radioprotective potential of *Punica granatum* fruit rind extract (PGRE) in mouse testes. ***Materials and Methods*:** Adult male mice were divided into four groups. Group I was kept without any treatment. Group II was irradiated with 8Gy Co^{60} gamma irradiation and Group III was given PGRE one hour before irradiation. Group IV was fed with PGRE at the rate of 10 mg/kg body weight. Mice were sacrificed at various post irradiation intervals and testes were removed, weighed and analysed biochemically for reduced glutathione content, Lipid peroxidation, Total protein, DNA and RNA content. Results were subjected to students't' test. ***Results*:** Testes weight of both the II group and III group decreased till 3rd post irradiation day. Protein and RNA contents increased up till the 3rd post irradiation day and decreased till 28th day in group II. Group IV maintains a higher level of protein and RNA content in comparison to II group. GSH and DNA content decreased in II group in comparison to normal and in III group they are found to be significantly higher at all the post irradiation intervals. ***Conclusion*:** *Punica granatum* fruit rind extract pretreatment renders protection against biochemical changes in mouse testes.

Keywords: *Radioprotection, punica granatum, gamma radiation, Swiss albino mouse, Testis.*

INTRODUCTION

Accidental radiation exposure can occur without any prior warning during radiography, nuclear medicine therapy, radiotherapy, radiological imaging, radionuclide production, biomedical research, military, transportation, nuclear reactors and space flight. Ionizing radiation causes damage to living tissues through a series of molecular events. The absorbed energy of ionizing radiation can break chemical bonds and cause ionization of different atoms and molecules, including water and different biologically essential macromolecules as DNA, membrane lipids and proteins. Deleterious effects of radiation on biological systems develop in a temporal sequence across various levels of organization, starting from the induction of primary lesions in the biomolecules and structures, eliciting repair processes, leading to the cell death or transformation responsible for morbidity, genetic disorder and cancer. To maintain the redox balance in order to protect themselves from these effects the living cells have evolved an endogenous antioxidant defense mechanism which include non enzymatic entities like glutathione, ascorbic acid, uric acid, etc. and also enzymes like catalase, superoxide dismutase (SOD), glutathione peroxidase, etc. [1]. Various pathological changes in living systems are produced by radiations which can be reduced by certain synthetic

chemicals such as Cysteine, Cysteamine, 2-MPG, WR-2721, Lipoic acid and Deoxyspergualin. Many of the amino acids, vitamins, glucosides, nucleic acid derivatives etc. were tried for their radioprotective potential but could not be translated into targeted use either due to less efficacy or associated toxicity with their effective doses [2, 3]. Therefore, plants and natural products offer an alternative solution due to their low toxicity at their optimum protective dose levels. Several polyherbal formulations, single plant extract and purified phytochemicals, like polyphenols and flavonoids have been studied for their radioprotective property. Radioprotective plants contain antioxidants, immunostimulants, cell proliferation stimulators and anti- inflammatory agents.

Punica granatum, commonly known as pomegranate, is a member of Punicaceae family. It is extensively cultivated in Iran, India, Afganistan and Mediterranean countries and to some extant in USA, China, Japan and Russia. Its root, leaves, fruit, fruit rind and seeds are used for medicinal purposes. It is digestive, carminative, enhances semen formation, activates memory, destroys disturbances caused by wind, bile, phlegm, improves formation of hemoglobin and is a very good blood purifier. Pomegranate fruit rind powder is a source of β- Carotene, Potassium, Phosphorous and Calcium. It provides natural antiviral activity [4] antifungal activity [5], antioxidant activity [6] and antibacterial benefits [7]. It contains tannins, anthocyanins, flavonoids, pectins, ellagitannins (Punicalin, Punicalagin, granatin, gallagyldilactone, casurinin), pedunculagin, tellimagrandin, corilagin [8], galic acid, ellagic acids, ursolic acid [9] and catechin [10].

Testes produce sperm, the male reproductive cell and androgen, the male hormones. Testes are one of the most radiosensitive organ [11,12]. The loss of male germ cell after exposure to ionizing radiation has been attributed to apoptosis [13]. Testes contain several types of germ cells, including stem cells, spermatogonia, spermatocytes and spermatids. These different types of germ cells are different in their susceptibility to radiation [14-15]. Irradiation of the testes may result in temporary or permanent sterility.

Present study is an attempt to assess the possible protective role of *Punica granatum* fruit rind extract against the radiation response in the testes of *Swiss albino mouse.*

MATERIALS AND METHODS

Animals

Adult male *Swiss albino mouse* (*mus musculus norvegicus*) 6-8 weeks old, weighing 25±2g each from an inbred colony, were selected. They were maintained under controlled conditions of temperature 37±5°C and 12hrs natural day light and dark night cycle. They were provided standard mouse feed and water *ad-libitum.* Animal care and handling were performed according to guidelines of World Health Organization (Geneva, Switzerland) and the Indian National Science Academy (New Delhi, India). The experimental protocols were approved by the Departmental Animal Ethics Committee.

Source of irradiation

Cobalt teletherapy unit (ATC-C9) at the cancer treatment center, Radiotherapy department, SMS medical college and Hospital, Jaipur, was used for irradiation. Animals were restrained in well- ventilated Perspex boxes and were whole bodily exposed to 8Gy gamma radiation at a dose rate of 1.45Gy/min.

Preparation of Plant extract

Rind of PG fruits was cut into pieces and shade dried. The extract was prepared by soxhlet apparatus in Acetone.

Selection of optimum dose

Various doses of PG RE (5,10,15,20,30,40 and 50mg/kg body weight) were tested against gamma irradiation (8Gy). Experimental animals were observed for 30 days for any sign of radiation sickness, mortality, behavioral toxicity or morbidity. The dose which facilited maximum survival on LD50/30 basis was selected as optimum dose. The optimum dose (10mg/kg body weight) obtained was used for the experiments.

Experimental design

The animals were divided into four groups keeping equal numbers in each group.

Group I- Animals were kept as such without any treatment (Normal).

Group II- Animals were irradiated with 8Gy Co^{60} gamma radiation only (Control).

Group III– Animals were given PGRE (10mg/ kg body weight) one hour before irradiation (Experimental).

Group IV- Animals were fed with PGRE only at the same dose rate (extract only).

The animals from each group were sacrificed by cervical dislocation at 3hrs, 1,3,7,14 and 28 days after treatment and six animals were sacrificed each time.

Biochemistry

Both the testes were removed, weighed and analysed biochemically for estimation of its Glutathione [16], lipid peroxidation [17], total protein [18], DNA [19] and RNA content [20] quantitatively.

Statistical analysis

The results obtained in the present study were expressed as mean ± Standard error. Statistical differences between various groups were analysed by applying Students *'t'-test* and significance was observed at the P<0.05, P< 0.01 and P<0.001 levels.

RESULTS

Testes weight

Testes weight in the control group increased after 3 hrs of irradiation and than decreased till 3rd post irradiation day in comparison to normal. Than it started to increase uptill the last interval (28thday). Testes weight in experimental group followed the same pattern but it remains higher than their corresponding control at all the intervals (table 1, figure1).

Total protein content

Protein content of the control group increased significantly till 3rd day in comparison

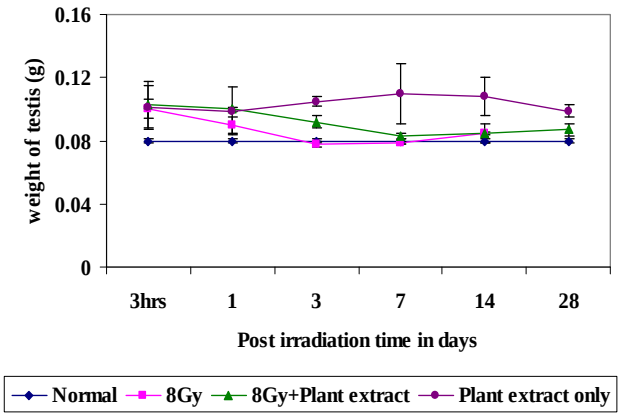

Figure1. Variations in the weight of testis (g) of Co^{60} gamma ray irradiated Swiss albino mouse with and without *punica granatum* pretreatment.

Table 1 . Variations in the weight of testis (in g) of Co^{60} gamma ray irradiated.

Treatment	Post irradiation time					
	3 hrs	1 day	3 day	7 day	14 day	28 day
8 Gy	0.100±0.006 P<0.001*	0.090±0.005 P<0.05*	0.078±0.002 NS	0.079±0.001 NS	0.085±0.001 P<0.001*	ANS
8 Gy + Plant extract	0.103±0.015 NS	0.100±0.001 P<0.05**	0.092±0.004 P<0.001**	0.083±0.002 P<0.05**	0.085±0.006 NS	0.087±0.004
Plant extract only	0.101±0.014	0.099±0.015	0.105±0.003	0.110±0.019	0.108±0.012	0.099±0.004

***Swiss albino mouse* with and without *Punica granatum* pretreatment**

The weight of testis of healthy normal *Swiss albino mouse* without any treatment is = 0.080±0.001

P value = Control Vs Normal* Control Vs Experimental** NS= Not Significant

Experimental = The plant extract was given 1hr before irradiation at the dose rate of 10mg/kg body weight.

Control = Irradiated only ANS – Animals not survived

to normal which started to decrease uptill the last interval. At all the post irradiation intervals protein content was significantly higher in experimental group as compared to the control group (table 2, figure 2).

Lipid peroxidation content

Testes showed gradual and continuous augmentation in the level of LPO content after gamma irradiation till 7th day post irradiation in both the control and experimental groups.

Table 2 . Variations in Total Protein content (mg/g of tissue) of testis of Co^{60} gamma**.**

Treatment	Post irradiation time					
	3 hrs	1 day	3 day	7 day	14 day	28 day
8 Gy	124.87±1.68 NS	133.00±1.125 P<0.01*	140.48±3.37 P<0.001*	134.95±2.81 P<0.05*	130.75±1.70 NS	ANS
8 Gy + Plant extract	129.43±1.125 P<0.01**	141.78±2.45 P<0.001**	151.54±2.25 P<0.01**	147.96±2.81 P<0.001**	139.83±1.49 P<0.001**	128.45±1.125
Plant extract only	127.47±1.13	133.98±2.25	144.38±1.69	141.13±1.12	135.28±1.12	129.10±2.25

The Total protein content of testis of healthy normal *Swiss albino mouse* without any treatment is = 127.79±1.69
P value = Control Vs Normal* Control Vs Experimental** NS= Not Significant
Experimental = The plant extract was given 1hr before irradiation at the dose rate of
10mg/kg body weight. Control = Irradiated only ANS – Animals not survived

Table 3 . Variations in Lipid peroxidation content (nmol MDA/mg of protein) of testis of Co^{60} gamma ray irradiated *Swiss albino mouse* with and without *Punica*.

Treatment	Post irradiation time					
	3 hrs	1 day	3 day	7 day	14 day	28 day
8 Gy	2.338±0.156 P<0.001*	3.075±0.221	3.465±0.355 P<0.001*	4.392±0.270	4.037±0.179	ANS
8 Gy + Pant Extract	1.784±0.068 P<0.001**	2.265±0.123 P<0.001**	2.312±0.043 P<0.001**	3.850±0.123 P<0.05**	3.552±0.156 P<0.05**	2.785±0.195
Plant extract only	1.406±0.022	1.437±0.035	1.501±0.073	1.554±0.086	1.505±0.050	1.424±0.024

The Lipid peroxidation content of testis of healthy normal *Swiss albino mouse* without any treatment is = 1.391±0.123
P value = Control Vs Normal* Control Vs Experimental** NS= Not Significant
Experimental = The plant extract was given 1hr before irradiation at the dose rate of
10mg/kg body weight. Control = Irradiated only ANS – Animals not survived

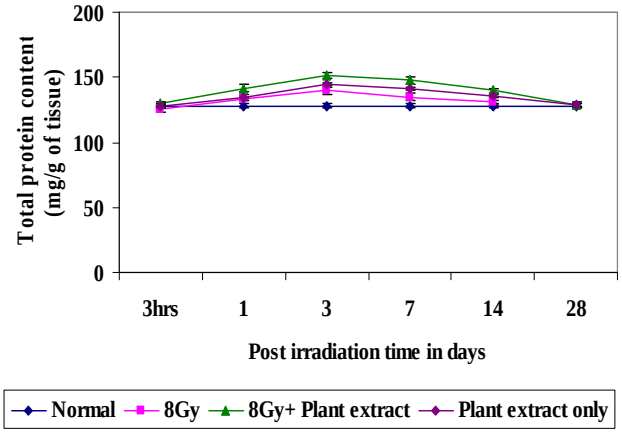

Figure 2. Variations in total protein content (mg/g of tissue) of testis of Co^{60} gamma ray Swiss albino mouse with and without *punica granatum* pretreatment.

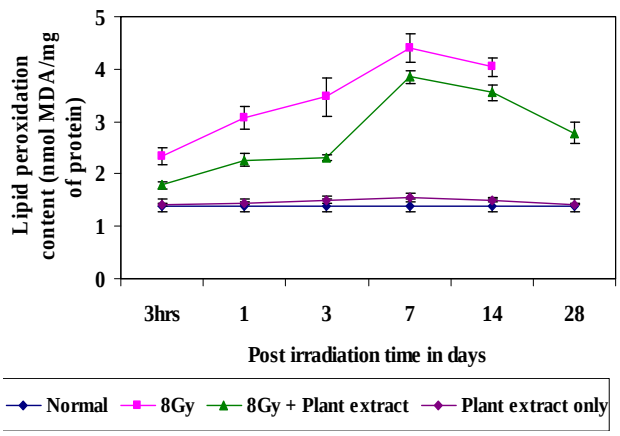

Figure 3. Variations in lipid peroxidation content (nmolMDA/ mg of protein) of testis of Co^{60} gamma ray Swiss albino mouse with and without *punica granatum* pretreatment.

Thereafter, depletion in LPO content was recorded till the last interval. In the experimental group it was lower as compared to the control group (table 3, figure 3).

Reduced glutathione content (GSH)

GSH content decreased continuously up to 7th day post irradiation. Administration of PG extract prior to irradiation maintains a higher level of GSH content than the corresponding control at all the intervals (table 4, figure 4).

DNA content

DNA content decreased significantly in the control group than normal and maximum decreased was observed after 24 hours of irradiation. In PG extract pretreated group it remained higher in comparison to control till 28thday (table 5, figure 5).

RNA content

RNA content increased significantly in irradiated group till 3rd day. In experimental group it remained significantly higher in comparison to corresponding control group. Maximum level of RNA was observed on 3rd day post irradiation and it decreased till the last interval (table 6, figure 6).

Table 4. Variations in Reduced glutathione content (GSH) (µmole/g) of testis of Co^{60} gamma ray irradiated *Swiss albino mouse* with and without *Punica granatum* pretreatment.

Treatment	Post irradiation time					
	3 hrs	1 day	3 day	7 day	14 day	28 day
8 Gy	0.427±0.011 NS	0.417±0.012 NS	0.381±0.005 P<0.001*	0.375±0.010 P<0.001*	0.385±0.011 P<0.01*	ANS
8 Gy + Plant extract	0.457±0.010 P<0.05**	0.443±0.005 P<0.05**	0.434±0.020 P<0.01**	0.424±0.016 P<0.01**	0.427±0.011 P<0.05**	0.434±0.010
Plant extract only	0.420±0.005	0.427±0.015	0.430±0.011	0.453±0.009	0.414±0.025	0.430±0.015

The reduced glutathione content of testis of healthy normal *Swiss albino mouse* without any treatment is = 0.420±0.011
P value = Control Vs Normal* Control Vs Experimental** NS= Not Significant
Experimental = The plant extract was given 1hr before irradiation at the dose rate of 10mg/kg body weight.
Control = Irradiated only
ANS – Animals not survived

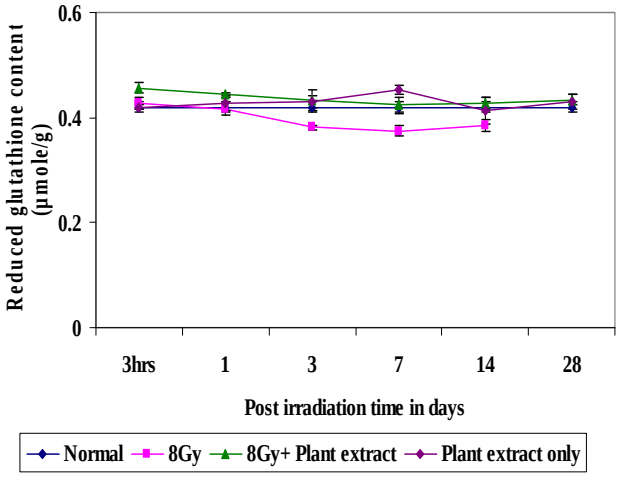

Figure 4. Variations in reduced glutathione content (µmole/g) of testis of Co^{60} gamma ray Swiss albino mouse with and without *punica granatum* pretreatment.

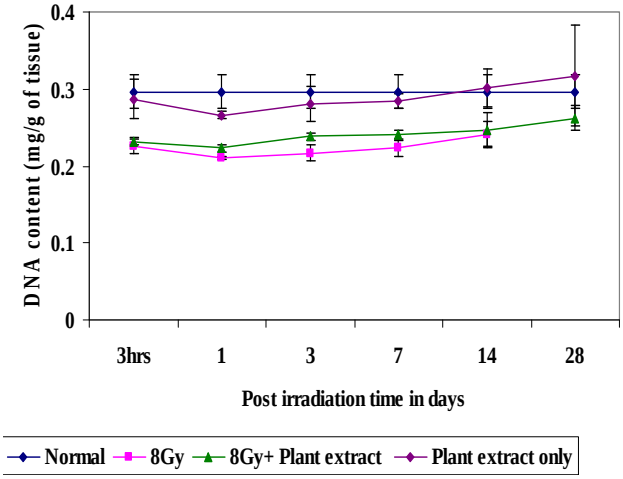

Figure 5. Variations in DNA content (mg/g of tissue) of testis of Co^{60} gamma ray Swiss albino mouse with and without *punica granatum* pretreatment.

Table 5. Variations in DNA content (mg/g of tissue) of testis of Co^{60} gamma ray irradiated *Swiss albino mouse* with and without *Punica granatum* pretreatment.

Treatment	Post irradiation time					
	3 hrs	1 day	3 day	7 day	14 day	28 day
8 Gy	0.226±0.009 P<0.001*	0.211±0.002 P<0.01*	0.217±0.010 P<0.001*	0.223±0.010 P<0.001*	0.241±0.016 P<0.05*	ANS
8 Gy + Plant extract	0.232±0.005 NS	0.223±0.005 P<0.01**	0.238±0.005 P<0.05**	0.241±0.005 P<0.001**	0.247±0.023 NS	0.262±0.016
Plant extract only	0.287±0.025	0.266±0.005	0.281±0.023	0.284±0.010	0.302±0.025	0.317±0.065

The DNA content of testis of healthy normal *Swiss albino mouse* without any treatment is = 0.296±0.022
P value = Control Vs Normal* Control Vs Experimental** NS= Not Significant
Experimental = The plant extract was given 1hr before irradiation at the dose rate of 10mg/kg body weight.
Control = Irradiated only ANS – Animals not survived

Table 6. Variations in RNA content (mg/g of tissue) of testis of Co^{60} gamma ray irradiated *Swiss albino mouse* with and without *Punica granatum* pretreatment.

Treatment	Post irradiation time					
	3hrs	1day	3day	7day	14day	28day
8Gy	1.101±0.018 NS	1.132±0.009 P<0.001*	1.190±0.028 P<0.001*	1.068±0.030 NS	0.994±0.013 P<0.001*	ANS
8Gy+Plant extract	1.213±0.011 P<0.001**	1.231±0.028 P<0.001**	1.271±0.020 P<0.001**	1.180±0.038 P<0.01**	1.080±0.020 P<0.001**	1.033±0.031
Plant extract only	1.004±0.009	1.018±0.028	1.039±0.020	1.033±0.016	0.948±0.010	0.929±0.012

The RNA content of testis of healthy normal *Swiss albino mouse* without any treatment is = 1.068±0.017
P value = Control Vs Normal* Control Vs Experimental** NS= Not Significant
Experimental = The plant extract was given 1hr before irradiation at the dose rate of 10mg/kg body weight.
Control = Irradiated only ANS – Animals not survived

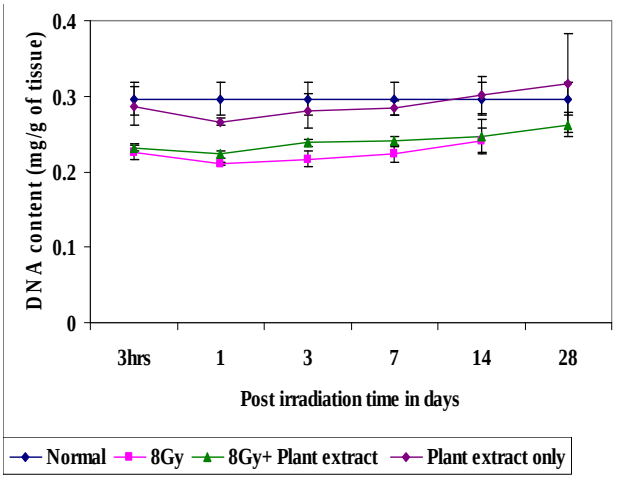

Figure 5. Variations in DNA content (mg/g of tissue) of testis of Co^{60} gamma ray Swiss albino mouse with and without *punica granatum* pretreatment.

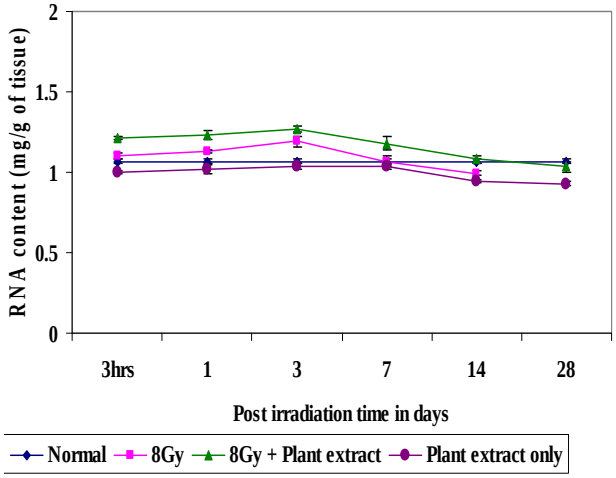

Figure 6. Variations in RNA content (mg/g of tissue/g) of testis of Co^{60} gamma ray Swiss albino mouse with and without *punica granatum* pretreatment.

DISCUSSION

Testicular tissue represents one of the most radiosensitive components in animals. Its importance as a subject of study on the cell and tissue levels comes from its activity in cell proliferation and differentiation, as well as its functional importance in reproduction. Gamma radiation exerts its harmful effects through generation of free radicals such as hydroxyl, superoxide and peroxyl radicals which react with macromolecules such as DNA, RNA, proteins and membrane lipids. The free radicals known as reactive oxygen species (ROS) could damage radiosensitive cells such as spermatogonia by disturbing normal metabolism, proliferation and differentiation, which may lead to mutagenesis, apoptosis and necrosis.

Elevated levels of Reactive oxygen species (ROS) may influence some transcription factors, enzyme activities, cell proliferation and various important signal transduction pathways, leading to male reproductive dysfunctions. The effect of gamma radiation on the function of gonads revealed complete sterility after using suitable doses of radiation. Radiation damages Leydig cells which are responsible for the synthesis and secretion of androgen that regulates the development and function of the male reproductive system and promotes spermatogenesis and sperm vitality [21]. A decrease in the level of serum testosterone is also reported. Radiation causes reduction of tubule diameter and decrease or loss of germ cells in various developing stages, especially spermatogenic elements [22]. Differentiating spermatogonia (A1, A2, Intermediate and B) are the most sensitive cells in the mouse testis whereas the stem and postgonial cells are relatively radioresistant.

Radiation downregulate dual character, steroidogenic and spermatogenic activity through the generation of oxidative stress, s uppression of antioxidant mechanism and by activating numerous molecular pathways involved in germ cells life and death decision making that ultimately altered normal testicular architecture. According to Makinta *et al.* [23] irradiation causes an overproduction of estrogens, which suppresses the hypothalamic-pituitary axis and inhibits LH and FSH secretions. Both LH and FSH deficiencies have negative effects on the testicular index and local reproductive hormones. Elevated estrogen level influence the epididymal internal milieu negatively, resulting in rigid, flagella bending sperm tail, impaired progressive movement of the spermatozoa and hence infertility. Pre irradiation injection of single or fractionated doses of oestrogen (0.09mg/100g body weight) could result in improving the effect on the structural changes of testes in the gamma irradiated rat [24].

Pretreatment with various plant extracts is known to reduce radiation induced damage in the testis. Their possible mechanisms of action may be metal chelation, DNA -protein cross linking and induction of chromatin compaction, reduced lipid peroxidation, DNA repair and recovery, anti-inflammatory response, reduced generation of reactive oxygen/nitrate species, hypoxia induction, increased cell proliferation, increased glutathione level, free radical scavenging, stabilization of cytoplasmic and mitochondrial membrane potential and in some cases induction of cell cycle arrest [25].

In the irradiated animals, a gradual loss in testes weight was observed. This may be related to actual loss in the number of germinal cells [26, 27]. The destruction at a specific stage of cell division caused disruption in the maturation process of the organ and therefore the number of immature cells increased, leading to a loss in testes weight. The loss of cells also affects t estosterone levels which ultimately disturb the maintenance of gonads [28].Decrease in testes weight after radiation exposure may be due to the actual loss of the germinal epithelial cells and not in the interstitial tissue or sertoli cells. In the *Punica granatum* pretreated irradiated animals weight of testes was increased as compared to their respective control. In the present study maximum decrease in the amount of DNA in irradiated control group was observed 24 hours after irradiation. Pretreatment with *Punica granatum* fruit rind extract increases DNA level in the experimental group.

Ionizing radiations induce damage to DNA by

direct ionization and also through generation of hydroxyl radicals that attack DNA resulting in single strand breaks (SSB), double strand breaks (DSB) and oxidative damage to sugar and base residues, which can be converted into DNA strand breaks later [29-30]. Due to induction of DNA double strand breaks, the ionizing radiations are extremely effective in producing chromosomal aberrations leading to genomic instability [31].

In irradiated group RNA and protein content increased till 3rd day and than decreased till the last interval. Pretreatment of *Punica granatum* fruit rind extract maintained a higher level of protein content in comparison to irradiated group till 28th day. Increase in protein content may also be due to elimination of most of the degenerated cells from the tissue or due to increased demand of proteins in repair process for recovery [32]. The decrease of protein noted may be due to its lyses, by irradiation or may be at the synthesis level, or by the inhibition of release of synthesized polypeptides from polysomes [33].

Radiation exposure induced a significant depletion in GSH level at early intervals, which may be due to its enhanced utilization as an attempt to detoxify the acute radiation induced free radical damage as glutathione is a major endocellular nonenzymatic antioxidant and executes its radioprotective function through free radical scavenging mechanism [34]. Pretreatment of Pomegranate rind extract reduced the depletion of GSH levels and provide protection to the testes. Punicalagin originating from the peels of pomegranate is one of the major polyphenol contributing to the total antioxidant capacity whilst anthocyanins play only a minor role in this activity [35]. Ellagic acid and Punicalagin which play an important role in the antioxidant activity of pomegranate peel [36].

Biomembrane of testes tissue is rich in polyunsaturated fatty acid content and radiation induced damage is mediated by peroxidation of membrane lipids. In the present study lipid peroxidation content of irradiated group was found significantly higher than normal. It was found maximum on 7th day post irradiation. In *Punica granatum* pretreated and then irradiated group it remained significantly lower than the group which was irradiated without PG at all the intervals. LPO produces a progressive loss of cellular integrity, fluidity of sperm membrane and its motility, impairment in membrane transport function and disruption of cellular ion homeostasis in testes [37]. Pomegranate fruit rind extract exhibit antioxidant effect which could be due to the available constituents. All the compounds including the isomers of punicalagin, tannin derivatives and anthocyanins (delphinidin, cyanidin and pelargonidin, 3-glucoside and 3,5-diglucosides) have free radical scavenging and antilipid peroxidation activity [38]. Punicalagin [2,3-(S)-hexa hydroxydiphenyl-4,6-(S,S)-gallogyl-D-glucose] inhibit lipid peroxidation due to its ability to provide electrons to eliminate the free radicals resulting from lipid peroxidation [39, 40].

The antioxidant constituents of pomegranate are compounds with phenolic hydroxyl groups and double bonds including tannins, flavonoids and unsaturated fatty acids. Reducing power of pomegranate peel extract is associated with the presence of reductones [41]. According to Gordon [42] antioxidative action of reductones is based on the breaking of the free radical chain by the donation of a hydrogen atom. Reductones react with certain precursors of peroxides, thus preventing peroxide formation [43]. Antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals or chelate metal cations. The protective effects of antioxidants in biological systems are due to their capacity to scavenge free radicals. The radicals scavenging ability of punicalagin is because of multiple – phenolic hydroxyl groups, which increase the antioxidative activity by additional resonance stability and O- quinone or P-quinone formation [44, 45]. The pomegranate extract containing ellagic acid and gallic acid act as a potent free radical scavenger, reduced the levels of hydrogen peroxide and enzyme inactivation, restoring enzyme activity. Pomegranate peel is rich in phenolic compounds and different activities of the pomegranate extract can be ascribed to their different phenolic composition.

Murthy *et al.* [6] reported that the pomegranate peel methanolic extract is capable of enhancing the activity of hepatic enzymes

which are involved in combating ROS. It provides protection against CCl_4 toxicity on the liver.

According to Li *et al.* [46] peel of pomegranate have higher antioxidant activity than its pulp and seed. Ricci *et al.* [47] reported the antioxidant capacities of some extracts from *Punica granatum* arils, juice and rind and correlated them to their polyphenol content. Okonogi *et al.* [48] reported that the extract of pomegranate peel have highest antioxidant activity with an IC50 of 0.003 mg/ml in a DPPH assay and highest TEAC value of 4.59mM/mg. *Punica granatum* peel extract decreased lipid peroxidation in hepatic, cardiac and renal tissues [49]. Toklu *et al.* [50] reported that chronic pomegranate peel extract supplementation alleviated oxidative injury of the liver and improved the hepatic structure and function in rats exposed to bile duct ligation. Madrigal Carballo *et al.* [51] suggested that pomegranate polyphenolic molecules undergo redox reactions because phenolic hydroxyl groups readily donate hydrogen to reducing agents.

CONCLUSION

Results from the present study suggest that oral pretreatment of *Punica granatum* fruit rind extract protects mouse testes against radiation induced damage. It works at a very low dose rate without causing side effects. It might have protected by scavenging free radicals and increasing antioxidant status. It can be considered as a radioprotector after further investigation. It also has possibility to be used to restore fertility in irradiated patients.

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