A concept of radiation hormesis. Stimulation of antioxidant machinery in rats by low dose ionizing radiation

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Abstract
Objective: The concept of radiation hormesis has been the matter of discussion with regard to beneficial effects to biological systems from low doses of ionizing radiations. However, its molecular basis is not well understood till now and the present study is a step forward to elucidate how low levels of ionizing radiation prove beneficial for functioning of biological systems. **Materials and Methods:** Female Wistar rats weighing 100-120g were divided into four different groups. Each group consisted of eight animals. The animals in Group I served as normal controls for Group II animals which were subjected to whole body X-rays exposure of 20rads and were sacrificed 6 hours following exposure. Group III animals served as normal controls for group IV animals which were given whole body X-rays radiation of 20rads and were sacrificed 24 hours following exposure. **Results:** The levels of reduced glutathione (GSH), total glutathione (TG) were increased in liver, kidney, brain and blood after 6hrs as well as 24hrs following X-rays exposure. On the contrary, no significant change in the oxidized glutathione (GSSG) content was observed following X-rays irradiation in any of the organs. Further, the low dose of X-rays resulted in a significant decrease in the lipid peroxidation (LPO) in liver, kidney and brain, whereas it caused an increase in LPO levels in blood. The enzyme activities of catalase (CAT) as well as glutathione-S-transferase (GST) were also increased in different organs after X-rays exposure. Furthermore, low dose irradiation with X-rays caused a significant increase in the counts of total leukocytes, lymphocytes and eosinophils, whereas it decreased the counts of neutrophils as well as monocytes. Hence, our results clearly indicate that low dose X-rays radiation exposure stimulates endogenous antioxidant defense machinery and also causes an increase in whole blood lymphocytes and eosinophils responsible for providing key defenses. **Conclusion:** Low doses of X-rays exposure may afford radiation hormesis by providing protection to organs from oxidative injury and support immune reaction.

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Introduction

It has earlier been shown that exposure to low levels of ionizing radiations provide key benefits to biological systems [1-4]. Reports have indicated that low doses of ionizing radiations would stimulate key molecular indices that have bearing on various types of cells of physiological systems [5, 6]. In the light of such reports, it has been accepted that the phenomenon of "Radiation Hormesis" exists, which means that low dose ionizing radiation exposure stimulates-urges beneficial biological effects (The word hormesis comes from the Greek verb "hormo" meaning "to exercise vital powerful energy-impulse". The word hormone also comes from "hormo").

Experimental studies have shown that low doses of X-rays irradiation can activate the immune system by regulating the activities of lymphocytes, macrophages and cytokines [7, 8]. It has also been reported that exposure to low dose ionizing radiations in mice has the potential to promote cellular repair mechanism by activating several cellular proteins, growth factors and DNA repair machinery [9-11]. Scientists have also proposed that low dose of ionizing radiations in rats afford protection mainly via regulating processes such as apoptosis, free radical generation and inflammation [12, 13].

In recent years, exposure to ionizing radiations has also attracted the attention of scientists towards cancer research and thus has been considered as an adjunct to chemotherapy as well as to analgesic therapy in experimental models of cancer [14, 15]. In vitro and in vivo studies have also demonstrated the anti-neoplastic and anti-cancer role of low dose ionizing radiations [8, 16, 17]. Beneficial effects of low dose X-rays irradiation have also been seen during aging, neurodegeneration and cold induced brain injury [18-20].

Thus, cumulative evidence has shown that irradiation therapy can modulate several

molecular mechanisms, which are responsible for providing protection against various diseases and toxic conditions. However, the exact molecular mechanisms involved in affording protection are yet to be explored. So, it was worthwhile to investigate the effects of low dose of X-rays radiation on various antioxidants as well as blood cells in rats.

Materials and Methods

Chemicals

Various chemicals used in the study were procured from Merck Ltd. and Sigma Pvt. Ltd.

Experimental design

Healthy female Wistar rats weighing 100-120g were obtained from the central animal house of Panjab University, Chandigarh, India. The animals were housed in polypropylene cages in a well ventilated animal room until the end of the experimental period. The animals had free access to drinking water and standard animal feed (obtained from Ashirwad Industries, Kharar, Punjab, India) throughout the treatment period. All procedures were done in accordance with the ethical guidelines for care and use of laboratory animals which were approved by Institutional Animal Ethics Committee (IAEC), Panjab University Chandigarh, India. To carry out the studies, the animals were segregated into the following four groups of 8 animals each.

The animals in Group I served as normal controls and were not given any radiation treatment. The animals in Group II were given whole body X-rays exposure of 20rads and were sacrificed 6 hours after X-rays exposure. Group III animals served as normal controls for group IV animals. The animals in Group IV were given whole body X-rays irradiation of 20 rads but were scarified 24 hours after X-rays exposure. All the animals were provided with standard laboratory food as well as water ad libitum throughout the experiment. The radiation dose was determined by using ferrous sulphate-benzoic acid-xylenol orange (FBX) dosimeteric method of Gupta et al., 1983 and X-rays exposure was given by using a calibrated X-rays rate meter (Allengers Medical Systems Ltd.). At the end of the treatment schedule, the animals were sacrificed by decapitation under light ether anesthesia.

Blood samples

To study the effects of low level radiations on blood cells, blood samples from normal and by X-rays irradiated rats were obtained by ocular vein puncture using sterilized capillaries in heparinized glass tubes.

Biochemical estimations

At the end of radiation exposure schedule, all the animals were sacrificed and different tissues which included liver, brain and kidneys were removed immediately. Later, the isolated tissues were placed in ice-cold isotonic saline. Tissue homogenates (10% w/v) were prepared in ice-cold 10mM PBS (phosphate-buffered saline, 0.15M NaCl), pH 7.4 and post-mitochondrial supernatants (PMS) were used for each of the biochemical estimations, described below:

Reduced glutathione

Estimation of GSH was performed by following the method of Hissin and Hilf (1976) [21]. Briefly, the samples were mixed with TCA and centrifuged for 2000g for 5 minutes. Further, the supernatants were mixed with phosphate buffer as well as Ellman reagent and absorbance was read at 412nm.

Total glutathione

This assay was done according to the method of Zahler and Cleland (1968) [22]. In this method, the samples were mixed with dithioerythritol (DTE), sodium arsenite, sodium acetate, Ellman reagent (DTNB) and phosphate buffer. The above solution was kept for incubation and absorbance was read at 412nm.

Glutathione-S-transferase

This enzyme activity was estimated by using the method of Habig et al. (1974) [23]. In this protocol, the samples were mixed with potassium phosphate buffer, reduced glutathione and CDNB. The absorbance was measured for 3 minutes at 340nm.

Oxidized glutathione

The levels of oxidized glutathione were obtained by subtracting reduced glutathione from total glutathione.

Lipid peroxidation

The measurement of lipid peroxidation was done by following the method of Wills (1966) [24]. Tissue homogenates (10% w/v) were mixed with ice-cold Trichloroacetic acid (TCA) and the reaction mixtures were centrifuged at 800g for 10 minutes. Further, the supernatants were mixed with Thiobarbituric acid (TBA) and the color was developed by boiling the total solution at 100°C for 10 minutes, followed by cooling at room temperature. The amount of malondialdehyde formed was measured by the reaction with TBA and optical density was read at 532nm.

Catalase

The method of Luck (1971) [25] was used for the estimation of catalase. In this method, the samples were mixed with phosphate buffer and H_2O_2. Further, the absorbance was measured for 3 minutes at 240nm.

Protein

Protein contents were estimated by using the standard method of Lowry et al. (1951) [26].

Total leucocyte counts (TLC) and differential leucocyte counts (DLC)

Total leucocyte counts and DLC analyses of the blood samples were done by using the method of Dacie and Lewis (1975) [27]. For TLC, blood samples were diluted with freshly prepared in Turk's fluid (2% acetic acid in distilled water with a pinch of crystal violet) in the ratio of 1:20 (v/v). Further, a drop of diluted blood was poured immediately on Neubaur's chamber and the cells were counted in $1mm^2$ at

four corners and one in the center of the Neubauer's chamber.

For DLC, blood smears were formed on the glass slides, which were air dried and fixed in methanol for 10 minutes. Further, the slides were stained with freshly prepared Giemsa stain for 30 minutes and then were counted using light microscope.

Statistical analyses

The statistical significance of the data was determined by using one-way analysis of variance (ANOVA) followed by a multiple post-hoc test (Student Newman Keuls). The results were represented as mean±SD of 6 observations. The comparisons were made as follows:

$^{x}P\leq0.05$, $^{y}P\leq0.01$, $^{z}P\leq0.001$ by Newman-Keuls test when the values are compared with Group I.

$^{a}P\leq0.05$, $^{b}P\leq0.01$, $^{c}P\leq0.001$ by Newman-Keuls test when the values are compared with Group III.

Results

The levels of reduced glutathione (GSH) and total glutathione (TG) were found to be significantly increased in liver and brain after 6hrs of X-rays exposure but no significant changes were observed in GSH levels of the kidney and blood when compared with normal controls (Tables 1, 3, 5 and 7). However, 24hrs after X-rays exposure, a significant increase in GSH levels was observed in the kidney, brain and blood, whereas no change was seen in the liver.

Further, no significant changes were observed in the levels of oxidized glutathione (GSSG) in the liver, kidney, brain and blood when they were compared to normal controls at 6hrs as well as 24hrs after X-rays (Tables 1, 3, 5 and 7). In addition, no significant changes were seen in the activity of glutathione-S-transferase (GST) in the liver after X-rays exposure at both the above time intervals (Table 2). On the contrary, GST activity was found to be significantly raised in the kidney as well as brain after 6hrs X-rays exposure and in blood samples after 24hrs X-rays exposure.

A significant decrease in lipid peroxidation was seen in the kidney and brain after X-rays exposure at both the above time durations when compared with normal controls but in liver the decrease was witnessed after 24 hours of exposure. On the contrary, the LPO levels were found to be increased at 6hrs and 24hrs after X-rays exposure in blood (Tables 2, 4, 6 and 8).

Catalase activity was significantly increased in the liver, kidney, brain and blood 6hrs after X-rays and a pronounced increase was seen in the kidney and blood at 24hrs after X-rays exposure (Tables 2, 4, 6 and 8). However, no statistically significant change in catalase activity was observed in the liver and brain 24hrs after X-rays exposure.

Further, X-rays irradiation caused a significant increase in the levels of TLC, lymphocytes and eosinophils and a significant decrease in the number of neutrophils both after 6 and 24 hours of exposure. An appreciable decrease was witnessed in the levels of monocytes only after 6 hours (Table 9).

Table 1. *Effects of low doses of X-rays on the levels of GSH, TG and GSSG in the liver.*

Groups	GSH	TG	GSSG
Group I	4.30±1.02	7.60±1.89	3.30±0.87
Group II	9.72±1.20^{z}	12.11±0.52^{y}	2.39±0.68
Group III	4.35±0.96	7.90±1.06	3.55±0.10
Group IV	4.45±0.69	9.45±1.45	5.00±0.76

GSH: (μmol of GSH/mg tissue); TG: (μmol GSH/mg tissue); GSSG: (μmol GSH/mg tissue). All the values are expressed as Means±SD. $^{x}P\leq0.05$, $^{y}P\leq0.01$, $^{z}P\leq0.001$ by Newman-Keuls test when the values are compared with Group I.

Table 2. *Effects of low doses of X-rays on the levels of CAT, LPO and GST in the liver.*

Groups	CAT	LPO	GST
Group I	135.00±0.21	0.33±0.02	2.37±0.20
Group II	501.44±0.10^{z}	0.27±0.01^{y}	2.75±0.50
Group III	169.79±0.46	0.47±0.07	2.00±0.90
Group IV	207.41±0.47	0.18±0.08^{b}	1.90±0.40

CAT: (nmol H_2O_2 decomposed/min/mg protein); LPO: (nmol MDA formed/min./mg protein); GST: (μmol conjugate formed/min/mg protein). All the values are expressed as Means±SD. $^{x}P\leq0.05$, $^{y}P\leq0.01$, $^{z}P\leq0.001$ by Newman-Keuls test when the values are compared with Group I. $^{a}P\leq0.05$ $^{b}P\leq0.01$, $^{c}P\leq0.001$ by Newman-Keuls test when the values are compared with Group III.

Table 3. *Effects of low doses of X-rays on the levels of GSH, TG and GSSG in the kidney.*

Groups	GSH	TG	GSSG
Group I	6.58±0.18	8.01±1.16	1.43±0.98
Group II	7.68±0.64	10.80±0.59^{y}	3.12±0.05
Group III	7.02±0.61	8.77±1.20	1.75±0.59
Group IV	11.74±0.08^{b}	13.33±0.85^{c}	1.59±0.77

GSH: (μmol of GSH/mg tissue); TG: (μmol GSH/mg tissue); GSSG: (μmol GSH/mg tissue). All the values are expressed as Means±SD. $^{x}P\leq0.05$, $^{y}P\leq0.01$, $^{z}P\leq0.001$ by Newman-Keuls test when the values are compared with Group I. $^{a}P\leq0.05$, $^{b}P\leq0.01$, $cP\leq0.001$ by Newman-Keuls test when the values are compared with Group III.

Table 4. *Effects of low doses of X-rays on the levels of CAT, LPO and GST in the kidney.*

Groups	CAT	LPO	GST
Group I	371.10±0.04	0.18±0.04	2.50±0.17
Group II	422.20±0.22^{x}	0.08±0.01^{z}	4.60±0.70^{z}
Group III	343.13±0.03	0.16±0.02	2.60±0.31
Group IV	442.36±0.19^{c}	0.05±0.01^{b}	2.90±0.80

CAT: (nmol H_2O_2 decomposed/min/mg protein); LPO: (nmol MDA formed/min./mg protein); GST: (μmol conjugate formed/min/mg protein). All the values are expressed as Means±SD. $^{x}P\leq0.05$, $^{y}P\leq0.01$, $^{z}P\leq0.001$ by Newman-Keuls test when the values are compared with Group I. $^{a}P\leq0.05$, $^{b}P\leq0.01$, $^{c}P\leq0.001$ by Newman-Keuls test when the values are compared with Group III.

Table 5. *Effects of low doses of X-rays on the levels of GSH, TG and GSSG in the brain.*

Groups	GSH	TG	GSSG
Group I	1.20±0.12	3.23±0.72	2.03±0.60
Group II	4.70±0.10^{y}	6.00±0.35^{z}	1.30±0.25
Group III	1.66±0.16	3.35±0.76	1.69±0.60
Group IV	2.71±0.10^{b}	4.53±0.75^{y}	1.82±0.65

GSH: (μmol of GSH/mg tissue); TG: (μmol GSH/mg tissue); GSSG: (μmol GSH/mg tissue). All the values are expressed as Means±SD. $^{x}P\leq0.05$, $^{y}P\leq0.01$, $^{z}P\leq0.001$ by Newman-Keuls test when the values are compared with Group I. $^{a}P\leq0.05$, $^{b}P\leq0.01$, $^{c}P\leq0.001$ by Newman-Keuls test when the values are compared with Group III.

Table 6. *Effects of low doses of X-rays on the levels of CAT, LPO and GST in the brain.*

Groups	CAT	LPO	GST
Group I	333.01±0.78	0.19±0.01	0.38±0.05
Group II	515.05±0.61^{z}	0.13±0.01^{x}	0.59±0.02^{x}
Group III	340.03±0.35	0.21±0.05	0.42±0.01
Group IV	352.04±0.08	0.15±0.03^{a}	0.49±0.04

CAT: (nmol H_2O_2 decomposed/min/mg protein); LPO: (nmol MDA formed/min./mg protein); GST: (μmol conjugate formed/min/mg protein). All the values are expressed as Means±SD. $^{x}P\leq0.05$, $^{y}P\leq0.01$, $^{z}P\leq0.001$ by Newman-Keuls test when the values are compared with Group I. $^{a}P\leq0.05$ $^{b}P\leq0.01$, $^{c}P\leq0.001$ by Newman-Keuls test when the values are compared with Group III.

Table 7. *Effects of low doses of X-rays on the levels of GSH, TG and GSSG in the blood.*

Groups	GSH	TG	GSSG
Group I	4.10±0.01	8.80±0.08	4.70±0.07
Group II	5.20±0.07	9.60±0.10^{z}	4.40±0.03
Group III	4.90±0.03	7.90±0.09	3.00±0.06
Group IV	16.90±0.10^{b}	20.20±0.20^{y}	3.30±0.15

GSH: (μmol of GSH/mg tissue); TG: (μmol GSH/mg tissue); GSSG: (μmol GSH/mg tissue). All the values are expressed as Means±SD. $^{x}P\leq0.05$, $^{y}P\leq0.01$, $^{z}P\leq0.001$ by Newman-Keuls test when the values are compared with Group I. $^{a}P\leq0.05$ $^{b}P\leq0.01$, $^{c}P\leq0.001$ by Newman-Keuls test when the values are compared with Group III.

Table 8. *Effects of low doses of X-rays on the levels of CAT, LPO and GST in the blood.*

Groups	CAT	LPO	GST
Group I	24.74±1.70	0.15±0.01	0.64±0.10
Group II	33.57±1.00^{z}	0.30±0.02^{y}	0.71±0.07
Group III	26.80±0.05	0.16±0.05	0.62±0.11
Group IV	59.84±1.50^{c}	0.41±0.04^{c}	0.83±0.08^{a}

CAT: (nmol H_2O_2 decomposed/min/mg protein); LPO: (nmol MDA formed/min./mg protein); GST: (μmol conjugate formed/min/mg protein). All the values are expressed as Means±SD. $^{x}P\leq0.05$, $^{y}P\leq0.01$, $^{z}P\leq0.001$ by Newman-Keuls test when the values are compared with Group I. $^{a}P\leq0.05$, $^{b}P\leq0.01$, $^{c}P\leq0.001$ by Newman-Keuls test when the values are compared with Group III.

Table 9. *Effects of low doses of X-rays on TLC, neutrophils, lymphocytes, eosinophils and monocytes of female Wistar rats.*

Groups	TLC	Neutrophils	Lymphocytes	Eosinophils	Monocytes
Control	7730 ±126	1174 ±214	5526 ±144	232 ±36.5	773 ±20.3
6 hours X-rays exposure	8600 ±183^{x}	679 ±124^{z}	6922 ±84.33^{x}	688 ±22.9^{z}	386 ±34.8^{y}
24hours X-rays exposure	9455 ±148^{y}	747 ±133^{y}	7616 ±111^{y}	458 ±25.5^{x}	765 ±42

Counts expressed in per mm^3. All the values are expressed as Mean±SD. $^{x}P\leq0.05$, $^{y}P\leq0.01$, $^{z}P\leq0.001$ by Newman-Keuls test when the values are compared with control

Discussion

During the past few years, remarkable progress has been made towards understanding the mechanism of action of low dose ionizing radiations in biological systems. Epidemiological and experimental data from studies have shown the beneficial effects of low doses of ionizing radiations [1, 12, 28]. The present study conducted investigations to understand the effects of low doses of X-rays exposure on antioxidant defense system in liver, kidney, brain and blood of rats.

Glutathione is a vital cellular antioxidant and has an important role in maintaining the cellular redox potential by regulating the generation of free radicals. In the present study, we observed that low doses of X-rays exposure to healthy rats were able to increase the levels of GSH and TG in various organs, which signifies the activation of glutathione system. This activation is understandably due to the difference in the radio-sensitivity in different types of cells. The sensitivity of cells to ionizing radiation depends on the rate of differentiation, accompanying factors of the tissue as well on the efficiency of the intrinsic antioxidant defense system. During our study, the observed increased levels of GSH and TG after X-rays exposure are apparently due to the induction of GSH biosynthesis genes, which result in the elevation of endogenous GSH levels and that would have enhanced the body's natural anti-oxidant defenses as well as related cellular functions. Earlier reports have also shown the similar effects on glutathione levels after exposure to low dose of ionizing radiations in animal models [29, 30]. On the contrary, we did not find any significant change in the GSSG activity after X-rays exposure, which is arguably due to regulation of mechanisms at the GSH and TG levels.

Low dose X-rays exposure causes activation of cellular defense system that is amply supported by an increase in the activity of GST. It is a natural antioxidant present in the body and acts as a detoxifying agent. The raised activity of GST after irradiation could be an adaptive response against reactive

oxygen species production. This enzyme is mainly involved in processes such as repair of oxidized biomolecules, regeneration of protein S-thiolates and biosynthesis of cellular metabolites. Low dose radiation causes production of reactive oxygen species which are regulated by modulation of cells' own antioxidant levels. In order to balance the cellular redox potential, X-rays exposure has resulted in raising the activity of natural antioxidants. Earlier reports also suggest an increased behavior of GST after low dose exposure of ionizing radiation [19, 31, 32].

Catalase is another crucial antioxidant that coverts H_2O_2 into H_2O and oxygen and thus provides resistance to cellular proteins/DNA from oxidative stress. We observed that low level X-rays irradiation was able to increase the activity of catalase in different organs. This elevated activity is due to body's defense mechanism, which resultantly gets activated to withstand the onslaught of increased oxidative stress induced by free radicals as a result of X-rays exposure. These results do suggest that low doses of X-rays generated active oxygen species such as peroxides, superoxides and peroxyl radicals in different organs, which resultantly were contained by the action of antioxidant enzymes.

Further, lipid peroxidation is the process mediated through the free radicals metabolites, which eventually disturb the oxidant/antioxidant ratio of cells and thereby cause impairment in the cellular integrity. In the current study, the X-rays exposure led to an appreciable decrease in the LPO levels, and this suppression could arguably be due to the usage of these free radicals within the cells. Several processes in the cells use these free radicals as carriers/transcription factors to stimulate the body defense system. Furthermore, our findings with respect to glutathione system have also clearly demonstrated that low dose X-rays exposure affords cellular protection by enhancing the cellular antioxidant machinery. On the contrary, an increase in LPO levels in blood is understandably due to its high vulnerability to free radicals. Similar effects on LPO levels were observed by Yoshimoto et al. (2012) during low dose X-rays exposure on cold-induced brain injury in mice [19].

In the present study, we have observed in rats a significant increase in the lymphocytes and easinophiles counts after low dose X-rays exposure, which indicates that the immune system plays an important role to combat against the radiation induced adverse effects in blood. Immune response may be found whenever the body is under stress [11]. Several scientists have also revealed that low dose irradiation can activate immune system [8, 33]. On the contrary, the monocytes and neutrophils counts were decreased that can be attributed to the cytotoxic activity of X-rays exposure. The decrease in monocytes and neutrophils counts also indicates the high radio-sensitivity of hematopoietic tissue. Studies have indicated that the response to radiation exposure depends upon various factors such as the sensitivity of the organ and the type of radiation [34, 35]. So, we kept our experiments under steady conditions. Our findings suggest that oxidant-antioxidant balance has an essential role in regulating cellular against free radicals onslaught as a consequence of radiation exposure.

In conclusion, exposure to low dose of ionizing radiations stimulates the body's defense system by enhancing the production of white blood cells as well as by upregulating the activities of various antioxidants and can be used as a novel therapeutic intervention during stress conditions.

The authors declare that they have no conflicts of interest.

Authors' Contributions

SS, NS, VCD and DKD have designed and analysed the data of this study. All the authors have critically reviewed the content of this manuscript. SS and NS have performed the various experiments of the study.

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