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Article *in* Journal of Environmental Biology · July 2020

DOI: 10.22438/jeb/4(SI)/MS_1915

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Journal of Environmental Biology

JEB™ p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP

DOI : http://doi.org/10.22438/jeb/41(SI)/MS_1915

Effects of low-dose irradiation on chromosomal damage and oxidative stress in cultured human peripheral blood

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Paper received: 10.02.2020 *Revised received*: 25.06.2020 *Accepted*: 10.07.2020

Abstract

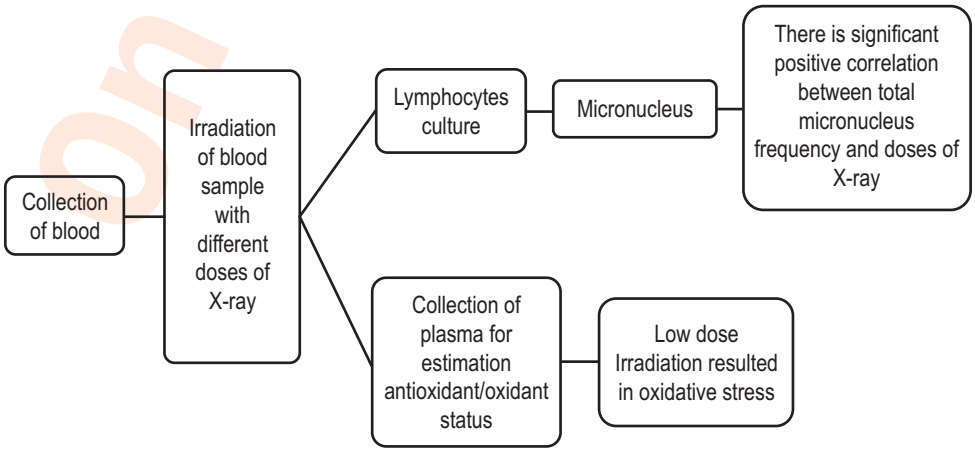
Aim: To investigate the effects of low doses of X-ray exposure on chromosomal damage and antioxidants level in cultured human peripheral blood.

Methodology: Blood samples collected from healthy young volunteers (male and female) in sterile heparinized tubes were irradiated at 97 kVp with 300 mAs using X-rays machine. *In-vitro* irradiation of whole blood was performed at different doses (0, 25, 50, 100, 200 and 300 mGy) with an average dose rate of 19.6 $mGysec^{-1}$. After irradiation, the lymphocytes were collected and cultured for 72 hr. Micronuclei (MN) assay was carried out following the standard protocol for the assessment of chromosomal damage. The level of glutathione (GSH), activities of glutathione-s-transferase (GST) and superoxide dismutase (SOD) were estimated from the blood plasma. Malondialdehyde (MDA) content was also estimated to determine oxidative stress after low-dose irradiation.

Results: A significant positive correlation (r^2=0.98, p <0.001) was observed between total MN frequency and irradiation dose in human peripheral blood lymphocytes. Irradiation of blood also caused significant decrease in GSH level and GST activities with increased in irradiation dose. Significant reduction in SOD activity was observed only at doses ≥ 100 mGy. Induction of oxidative stress in human blood due to irradiation was clearly evident from enhanced MDA content.

Interpretation: This study indicates that exposure to ionizing radiation less than 100 mGy can cause genetic damage and induce oxidative stress. Furthermore, the results suggested that detection of genetic damage using MN assay is sensitive enough at a lower dose in contrast to IAEA manual where the detection limit is only 0.2-0.3 Gy.

Keywords: Antioxidants, Human lymphocytes, Lipid peroxidation, Micronucleus, X-ray



How to cite : Zosangzuali, M., L. Pachuau, K.S. Pau and Zothansiama: Effects of low-dose irradiation on chromosomal damage and oxidative stress in cultured human peripheral blood. *J. Environ. Biol.*, **41**, 908-914 (2020).

Introduction

Ionizing radiation have the ability to directly interact with the cellular components and causes ionization of biomolecules and initiate sequence of events leading to patho-physiological changes (Lehnert, 2007). The increasing use of ionizing radiation in medical diagnosis and treatment has raised concern about its potential long-term effects on human health as prolonged exposure to low-dose radiation has been found to cause adverse health effects including cancer (Gilbert, 2009; Richardson *et al*., 2015). The use of X-rays has become a popular medical diagnosis and presents a major source of radiation exposure for the general population. Due to their high energy content, X-ray exposure can injure normal cells, alter genetic materials and induce multiple changes in biomolecules (Hall, 2000; Azzam *et al*., 2012). Although high doses of ionizing radiation are clearly known to produce deleterious consequences such as cardiovascular diseases and cataracts in human (UNSCEAR, 2006; Baselet *et al*., 2016), the potential risk of low-dose exposure is still a matter of debate (Feinendegen *et al*., 2011; OECD, 2016). The biological effects of ionizing radiation such as X-rays are mainly related to damages they cause to DNA, by inducing single-strand DNA breaks, double-strand DNA breaks, DNA base alterations and DNA–DNA cross-links (Ward, 1998; Hall, 2009). The potential genotoxicity of ionizing radiation can be determined by micronuclei assay, which is an effective tool to evaluate the genotoxic or clastogenic effects of physical and chemical agents (Fenech *et al.,* 1999). Measurement of micronuclei frequency is extensively used in molecular epidemiology and cytogenetics to evaluate the presence and the extent of chromosomal damage in human population exposed to genotoxic agents (Maffei *et al.,* 2002; Kirsch-Volders *et al.,* 2011; Vellingiri *et al.,* 2014; Zothansiama *et al*., 2017; Zothansiama *et al.,* 2019).

Ionizing radiation directly interacts with atoms or molecules and generates highly reactive oxygen species (ROS) such as hydroxyl radicals (˙OH) that inflict oxidative damage in cell (Adams, 1986; Le Caër, 2011). Previous studies have reported an increase in the production of endogenous ROS due to exposure to high dose of ionizing radiations (Zhou *et al*., 2011; Kohanoff and Artacho, 2017). Excessive generation of ROS results in oxidative stress and attacks molecules in the membranes and tissues resulting in various diseases such as cardiovascular diseases, lens opacity of the eyes, lung disease and cancer (Yoshikawa and Naito, 2002; Wang *et al*., 2016). Although cells are equipped with an impressive repertoire of antioxidants to counterbalance oxidative stress by neutralizing ROS, excess generation of ROS may sometimes overwhelm the endogenous antioxidants and cause oxidative cellular damage (Kono and Fridovich, 1982; Jagetia and Shetty, 2016). Reactive oxygen species, due to their extreme reactivity are not amenable to direct measurement in biological material. Therefore, altered levels of antioxidative enzymes such as glutathione-s-transferase, superoxide dismutase, catalase, glutathione peroxidase, and small-molecule antioxidants such as glutathione are routinely used to assess oxidative stress. Elevated levels of ROS alter lipids by producing lipid peroxides such as malondialdehyde and 4-hydroxynonemal (Burton and Traber, 1990). Lipid peroxidation has been reported to be associated with various pathological conditions and is used as a marker of oxidative stress (Gutteridge, 1995).

According to the United Nations Scientific Committee on Atomic Radiation, low-dose radiation (LDR) include radiation < 200 mGy for low linear energy transfer (LET) radiation or 50 mGy for high LET radiation (Liu *et al*., 2007, UNSCEAR, 1994). Although several studies have reported increased levels of chromosomal aberrations in human lymphocytes exposed to low-doses of ionizing radiations (Andreassi *et al*., 2009; Zakeri and Hirobe, 2010; Sakly *et al*., 2012), contradictory results have been reported on the genotoxic effects of low-doses of ionizing radiation by various workers (Demirel *et al*., 1997; Thierens *et al*., 2002; Joseph *et al*., 2004). Thus, there is an increasing concern over the magnitude of health risks from exposures to man-made radiation such as diagnostic X-rays that involve low doses (UNSCEAR, 2006). The risk associated with LDR and understanding intracellular responses are of social importance. In views of this, this study was carried out to evaluate *in-vitro* effects of low-dose radiation on chromosomal damage and antioxidant levels in cultured human peripheral blood.

Materials and Methods

Collection of blood sample: Blood samples were collected by venipuncture from healthy male and female of 26 years old, non-smoking volunteers, who did not have any previous history of radiation exposure for diagnostic or treatment purpose, tobacco and alcohol use and intake of medicine for the last one year. Blood samples were collected under sterile conditions in heparinized tubes. The study was approved by the Human Ethics Committee, Mizoram University, Aizawl, India vide approval No. MZU/IHEC/2016/001.

Irradiation of blood: Irradiation of blood was performed in the Department of Radio-diagnosis and Imaging, Civil Hospital, Aizawl, Mizoram. Blood collected from the donors were exposed to a diagnostic X-rays source (Allengers 325 FC, Allengers Medical System Private Ltd., Solan, HP, India) in a polypropylene phantom (dimensions: 30x30 cm^2), placed transversally to the axis of irradiation source. During irradiation process, radiation field was set at 20 x 20 cm^2, and the distance between the surface of the phantom and the source of radiation was 65 cm. Blood samples were irradiated at 97 kVp with 300 mAs. The average dose rate of exposure was measured using RaySafe. An average dose rate of 19.6 $mGysec^{-1}$ was given for 1.3, 2.6, 5.1, 10.2 and 15.3 sec for the total exposure doses of 25, 50, 100, 200 and 300 mGy, respectively, at an interval of 1 min. All samples were

irradiated at room temperature. After exposure, the blood samples were allowed to sediment and the buffy coat was collected in a sterile tube and immediately stored at 4 °C for further use.

Lymphocyte culture and micronucleus assay: Lymphocyte were cultured following the method of Fenech and Morley (1985). Briefly, the blood was allowed to sediment and the buffy coat was collected in individual sterile glass tubes. After counting the viable cells with haemocytometer, 1 x 10^6 nucleated cells were inoculated into sterile glass tubes containing RPMI-1640 medium supplemented with 10% fetal calf serum and phytohemagglutinin as mitogen. The cells were allowed to grow for the next 44 hr in a humidified atmosphere of 5% CO_2 at 37 °C. Cytochalasin B was added at a final concentration of 5 µgml^{-1} to block cytokinesis and cells were allowed to grow for another 28 hr. Cells were harvested at the end of 72 hr after initiating lymphocyte culture by centrifugation. A mild hypotonic solution was added to the cell pellet so as to retain the cell membrane. Cells were then fixed with freshly prepared Carnoy's fixative (methanol: acetic acid, 3:1). Cell suspension was placed in a pre-cleaned coded slides to avoid observer's bias and spread by air blowing. The cells were stained with acridine orange and scored under a fluorescence microscope (DM 2500, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). A total of 1000 binucleate cells (BNC) with well-preserved cytoplasm were scored from each individual for the presence of micronuclei (MN) according to the criteria described by Fenech *et al*. (2003).

Estimation of biochemical parameters: Antioxidants were estimated in the plasma of each individual. Total protein contents were determined by the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

Glutathione (GSH): GSH content was measured using the method described earlier (Moron *et al*., 1979). Briefly, 900 µl of 0.02 M sodium phosphate buffer (pH 8.0) and 20 µl of 10 mM 5,5´-dithio-2-nitroben-zoic acid (DTNB) were mixed with 80 µl of plasma and incubated for 2 min at room temperature. The absorbance of the sample was read against blank at 412 nm on a UV-Visible spectrophotometer (SW 3.5.1.0. Biospectrometer, Eppendorf India Ltd., Chennai), and GSH concentration was calculated from the standard curve and expressed in µmolmg^{-1} of total protein.

Glutathione-s-transferase (GST): GST activity was estimated by the method of Beutler (1984). Briefly, 850 µl of phosphate buffer was mixed with 50µl of 20 mM 1-chloro-2, 4-nitrobenzene (CDNB), incubated for 10 min at 37°C followed by the addition of 50 µl each of plasma and 20 mM GSH. The blank consisted of all the reagents and distil water was added instead of plasma. The absorbance of blank and test was measured at 360 nm and enzyme activity was expressed in unit mg^{-1} of total protein.

Superoxide dismutase (SOD): SOD activity was measured by the method of Fried (1975). Briefly, 100 µl each of 186 µM phenazine methosulphate and plasma were mixed with 200 µl of 780 µM nicotinamide adenosine dinucleotide (NADH) and 300 µl of 3 mM nitrobluetetrazolium. After incubation for 90 sec at 30°C, 1 ml of acetic acid and 4 ml of n-butanol were added to stop the reaction. The blank consisted of all the reagents, and distil water was added instead of plasma. The absorbance of test and blank was measured at 560 nm and the enzyme activity was expressed in unit (1U = 50% inhibition of NBT reduction) mg^{-1} protein.

Lipid peroxidation: Lipid peroxidation was estimated by the method of Beuege and Aust (1978) and expressed in terms of malondialdehyde (MDA) content. Briefly, plasma was added to a mixture containing 10% trichloroacetic acid, 0.8% thiobarbaturic acid and 0.02 N HCl in 1:2 ratio. The mixture was boiled for 10 min in a boiling water bath, cooled immediately and centrifuged at 1000 rpm for 10 min. The supernatant was collected and its absorbance was read at 535 nm against the blank. The blank contained all the reagents and distil water was added instead of plasma. The malondialdehyde (MDA) concentration of the sample was calculated using extinction coefficient of 1.56 X 10^{-6} M^{-1} cm^{-1}.

Statistical analyses: The results are expressed as mean ± SE. One-way ANOVA followed by Tukey's post-hoc test was used for multiple comparisons. SPSS Ver. 20.0 software (SPSS Inc, Chicago, Illinois, USA) and Graph Pad Prism ver. 6.0 were used for statistical analyses. p-value of <0.05 was considered statistically significant.

Results and Discussion

The frequency of micronuclei (MN) in human peripheral blood lymphocytes exposed to low doses of X-ray was significantly higher than control (0 mGy), except at the lowest dose (25 mGy) where no significant (p > 0.05) change was

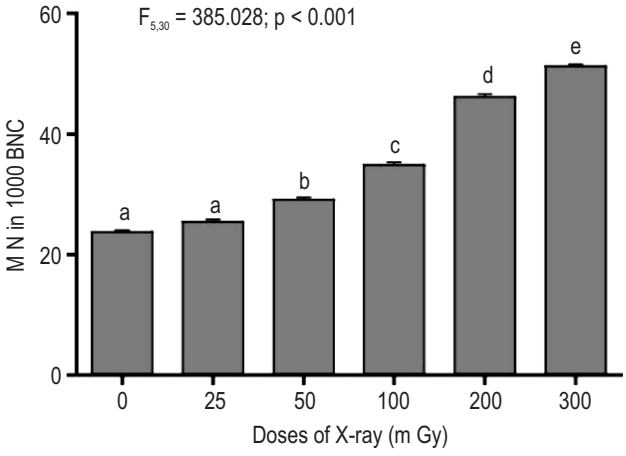


Fig. 1 : Effect of different doses of X-rays on the micronuclei formation in the cultured human peripheral blood lymphocyte of healthy donors. Means not sharing same letter are significantly different.

observed when compared with control (Fig. 1). Significant positive correlation (r^2=0.98, p < 0.001) was also observed between total MN frequency and the doses of X-ray exposure in human peripheral blood lymphocytes. Moreover, formation of two micronuclei was observed only in 200 and 300 mGy exposed lymphocytes.

X-ray irradiation at low doses also resulted in dose dependent alleviation of GSH content in human peripheral blood. However, no significant variation was observed between control and lowest dose (25 mGy) in the GSH content (Fig. 2a). *In-vitro* irradiation of human blood also caused considerable change in GST activities. Increased dose of X-ray significantly reduced the GST activities. Even the lowest dose (25 mGy) of radiation led to significant reduction in the activity of GST when compared with control (Fig. 2b). The activities of SOD did not show significant variation up to 50 mGy exposure. Significant reduction in SOD activity was observed only from the doses ≥ 100 mGy. However, no significant change in SOD activity was observed in lymphocytes exposed to doses between 200 mGy and 300 mGy (Fig. 2c). Irradiation induced oxidative stress in human blood was clearly evident by elevated MDA content which is a common indicator of lipid peroxidation (Fig. 3).

The study of the genotoxic effect of ionizing radiation using micronucleus assay has been a major research area and it has been correlated with cytotoxic effect in various studies (Ramalho *et al.,* 1998; Jagetia and Venkatesha, 2006; Ropolo *et al*., 2012; Koyama *et al*., 2016). Micronuclei assay is an easy and less cumbersome technique for detecting chromosomal damage in human lymphocytes. Despite extensive use of MN assay as a quantitative indicator of X-rays induced chromosome damage in both *in-vitro* and *in-vivo* studies (Ramalho *et al*., 1988; Maffei *et al*., 2002; Joseph *et al*., 2004; Ropolo *et al*., 2012; Koyama *et al*., 2016), reports on the effects of fractionated X-rays irradiation on MN formation are limited. Furthermore, the potential effects of very low doses of radiation on the biological system need to be examined as radiation exposure received by human population are of low doses, which are emitted by natural sources such as cosmic rays, radionuclides present in the earth crust and diagnostic X-rays (UNSCEAR, 2006). DNA double strand breaks (DSBs) are the major cytotoxic damage caused by ionizing radiation and are potent inducers of cell death (Morgan *et al*., 1996). In this study, human peripheral blood lymphocytes exposed to 0 to 300 mGy of X-rays increased the frequency of micronuclei in a linear manner. Cells of human embryo, newborn and children exposed to 0.02 to 2 Gy irradiation have been reported to induce micronuclei in a dose-dependent manner (Koyama *et al.,* 2016). Similar results have been reported for human lymphocytes exposed to X-rays and γ-rays in earlier studies (Jagetia *et al.,* 2003; Miszczyk *et al.,* 2015). However, linear quadratic response for chromosomal damage after low-dose irradiation has been also reported (Lusiyanti *et al.,* 2016). Pearce *et al*. (2012) reported that the risk of developing leukaemia might increase three times among children exposed to CT scans with a cumulative dose of about 50 mGy. In contrast, some studies reported insignificant change in the expression of

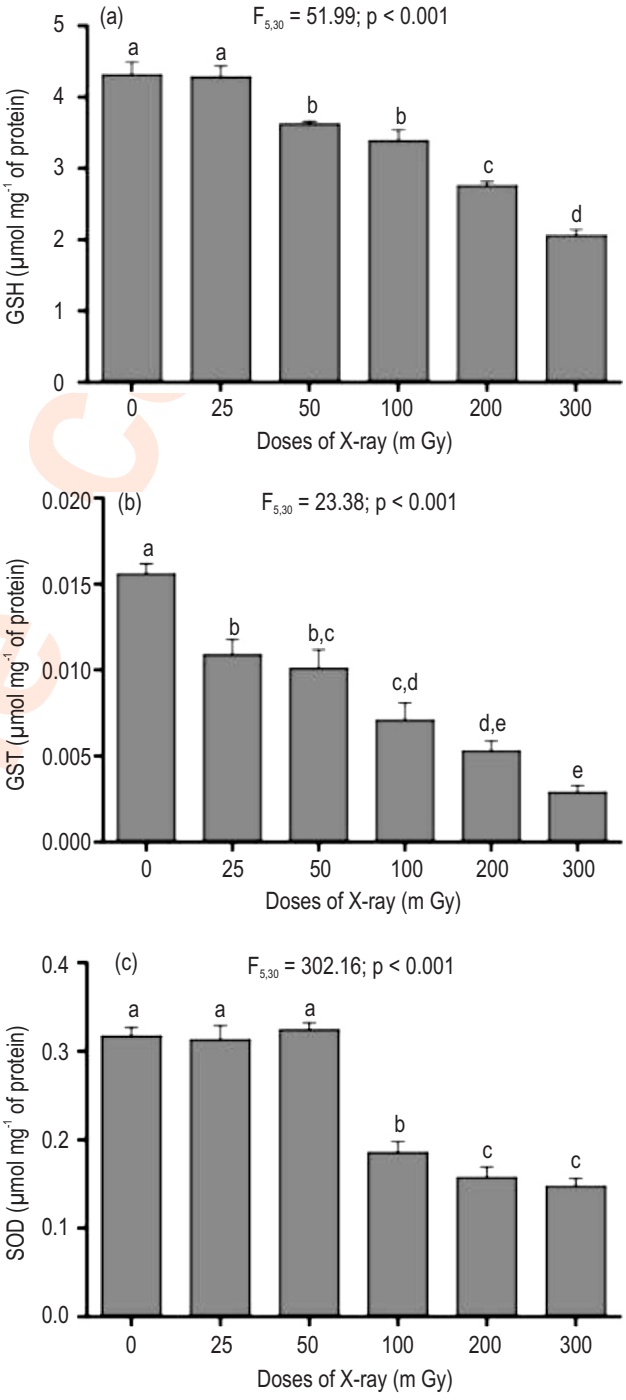


Fig. 2 : Effect of different doses of X-rays on (a) GSH level, (b) GST activity and (c) SOD activity in human peripheral blood lymphocytes of healthy donors. Means not sharing same letter are significantly different.

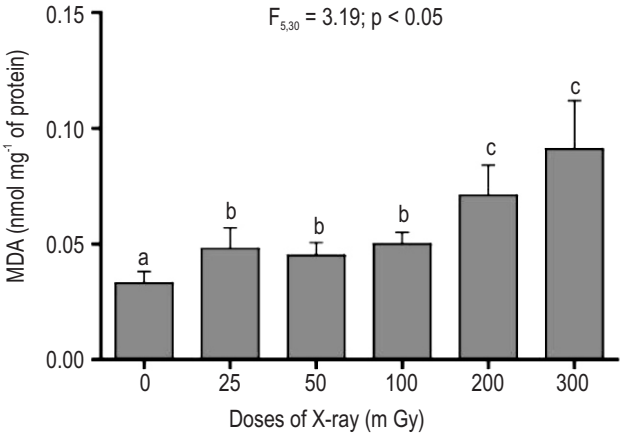


Fig. 3 : Effect of different doses of X-ray on MDA content in human peripheral blood lymphocyte of healthy donors. Means not sharing same letter are significantly different.

radiation responsive genes in irradiated human peripheral white blood cells when compared to data from patients receiving total body irradiation (Amundson *et al.,* 2004, Filiano *et al.,* 2011, Paul *et al.,* 2011). Cervelli *et al.* (2014) also reported insignificant differences in cell viability or DNA repair in vascular endothelial cells exposed to single or fractionated low-dose irradiation. Previous study also documented that the cultured human lymphocytes can acquire resistance to chromosomal aberrations induced by subsequently high dose radiation when cells are pre-exposed to low-dose radiation known as adaptive response (Olivieri *et al*., 1984). Nevertheless, the present study confirms the ability of low doses of X-rays to induce MN formation and the ability of MN assay to estimate DNA damage at a low radiation dose of 50 mGy.

The majority of the energy of ionizing radiation such as X-rays deposited in cells results in the ejection of electrons from water which subsequently causes the formation of several ROS including the highly reactive hydroxyl radicals (OH) that inflict instant oxidative damage in various biomolecules (Adams, 1986; Le Caër, 2011). Ionizing radiations produces these free radicals within micro-seconds, however, their effects persist long after their production due to the cascade of events triggered by these free radicals at molecular and cellular level. This finally leads to an increase in the oxidative stress (OS) and instability of genome in the exposed cells (Einor *et al.,* 2016). Ionizing radiation in the presence of molecular oxygen converts hydroxyl, superoxide, and organic radicals into hydrogen peroxide and organic peroxides which are longer lived ROS that continue to damage the cellular genome and other important biomolecules. Moreover, hydrogen peroxide reacts with redox-active metal ions, such as Fe^{2+} and Cu^{2+}, via Fenton reactions and generates the most deleterious OH radical and thus intensifying cellular oxidative stress leading to patho-physiology (Biaglow *et al.,* 1992). In this study, human blood lymphocytes exposed to low doses of X-rays showed significantly higher malondialdehyde content indicating increased lipid peroxidation and reduced GSH levels and antioxidant enzyme activities of GST and SOD. Human lymphocytes irradiated with 3 Gy γ-radiation significantly reduced GST, CAT and SOD activities as compared to control (Bravard *et al.,* 1999). Irradiation of mice resulted in a dose-dependent decline in the GSH concentration and activities of GPx; SOD and CAT with concomitant increase in the lipid peroxidation in various tissues including liver, intestine and skin indicating oxidative cellular damage (Jagetia and Reddy, 2005; Jagetia and Rajanikant, 2015; Jagetia and Shetty, 2016). An increase in lipid peroxidation and reduction in CAT activity was observed in intestinal cells of mice exposed to 2 Gy of γ-rays and 1.6 Gy of ^{56}Fe radiation (Datta *et al*., 2012). A number of studies have revealed biochemical changes associated with a prolonged exposure to ionizing radiation and their relationship to the antioxidant system (Achudume *et al.,* 2010; Atasoy *et al.,* 2013). The decrease in the GST and SOD activities in human blood lymphocytes exposed to low doses of X-rays may be due to the persistence of oxidative stress. In addition to rapid burst of ROS observed immediately following irradiation, cells can exhibit prolonged increase in ROS (Yoo *et al.,* 2000), which may lead to oxidative cellular damage such as DNA lesions including double-strand breaks.

Conclusion: Exposure of human blood to low-doses of X-rays has shown to increased MN frequencies and lipid peroxidation, and diminished activities of antioxidant enzymes. This *in-vitro* experiment reaffirms the ability of low doses of X-rays to trigger micronuclei formation and the ability of micronucleus assay to estimate DNA damage at a radiation dose as low as 50 mGy indicating its sensitivity to detect DNA damage.

Acknowledgment

We thank the Department of Science and Technology (DST), Government of India, for providing Inspire fellowship to Mary Zosangzuali (DST/INSPIRE Fellowship/[IF170375]).

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