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RESEARCH PAPER

Response of Normal Cells Following Multiple Radiation Exposure under Radiotherapy Setting

M.H. Yashavarddhan[#,@], Sandeep Kumar Shukla[#,*], Ajay Kumar Sharma[#], and Mrutyunjay Suar[@]

[#]*Division of Radioprotective Drug Development Research, Institute of Nuclear Medicine and Allied Sciences, Delhi - 110 054, India*

[@]*School of Biotechnology, KIIT University, Bhubaneswar, Odisha-751 024, India*

**E-mail: sandeepshukla@inmas.drdo.in*

ABSTRACT

Radiotherapy is an established approach for killing of tumour cells. During the process, most of the normal cells also get affected due to direct exposure or by bystander effects. To measure the damage pattern in healthy cells, a pilot study was designed under radiotherapy settings. Right leg region of Strain 'A' male mice was locally exposed to Cobalt^{60} gamma radiation with a dose of 2 Gy/ day for 5 consecutive days. After completion of each fraction, blood haematology and γH2AX studies were performed at 1 h time point in blood and bone marrow cells. Chromosomal aberration study in bone marrow was carried out at 24 h post irradiation of each fraction for evaluation of DNA damage. γH2AX and chromosomal aberration were found significantly ($p<0.001$) increased with each consecutive dose upto 4th fractions. Blood hematology showed a linear reduction in total WBC counts which included the reduction in lymphocytes and increased granulocytes with each passing dose up to 4th fraction. However, non significant damage ($p>0.05$) for all parameters have been observed for 4th and 5th split doses. The study indicated that repeated exposure leads to damage fixation in normal cells, possibly indicating a state of adaptation.

Keywords: Radiotherapy; Fractionated radiation; Normal cells; γH2AX; Chromosomal aberration; Blood haematology

NOMENCLATURE

WBC	White blood cell
SSB	Single strand break
DSB	Double strand break
Ser	Serine
CBC	Complete blood count
PBS	Phosphate buffer saline
RBC	Red blood cells
BSA	Bovine serum albumin
FITC	Fluorescin isothiocyanate
SD	Standard deviation
MFI	Mean fluorescence intensity
Del	Terminal deletion
DM	Double minute
RT	Robertsonian translocation
EEA	End to end association

1. INTRODUCTION

Cancer is a symptom in which abnormal cells divide in an uncontrolled manner and destroy body tissue[1]. Radiotherapy has been commonly used in clinics for treatment of cancer[2]. The total dose estimation for patient plays an important role in maximizing the effectiveness of treatment which is decided by various factors like type of tumour, size, and proliferation[3]. The main side effect of the radiotherapy is damage to the surrounding healthy cells due to direct exposure or by the bystander effect[4,5]. The main focus of the treatment is to kill maximum number of cancer cells with minimum damage to the normal cells[6,7]. To achieve this objective, splitting dose technique is used in which clinically relevant radiation dose is delivered at multiple times to the patients over a period of time to provide time gap which allows the healthy cell to repair the radiation mediated damage[8].

There are various reports conveying the measurement of DNA damage biomarkers for evaluation of the effectiveness of cancer therapy[9,10]. These biomolecules also help in measuring the damage level in normal healthy cells[11]. Radiation-induced DNA damage has various outcome like single strand breaks (SSBs), double strand breaks (DSBs), base modifications, etc[12-14]. DSBs are regarded as lethal lesions due to difficulty in their repair[15]. Recently, γH2AX has been used as biomarker for the measurement of DNA double strand breaks[12,16]. This protein belongs to H2A family and gets phosphorylated at ser 139 position on generation of double strand break[17,18]. Published literature cites the availability of γH2AX in cells with-in minutes of damage which can stay upto hours and months depending upon the type of system and radiation exposure scenarios[19]. The phosphorylation event of H2AX is

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mediated by kinases of the PI3 family which includes ATM, ATR, and DNA-PKcs[20]. This biomarker protein has reportedly been used to monitor the effectiveness of radiation doses to the human subjects in clinics[21,22]. The level of γH2AX has been linked to absorbed dose and serves as a predictor of radiosensitivity[23].

After DNA damage, the body repair mechanism gets activated and repairs the broken end of the DNA[24]. The broken ends may rejoin in different patterns from their original arrangement and cause abnormalities[25]. These abnormalities referred as chromosomal aberrations which include fragments, rings, dicentrics, gaps, terminal deletion (del), centromeric attenuation, end to end association (EEA) and robertsonian translocations (RT)[26,27]. Assessment of chromosomal aberration can give an idea about the severity of damages and can also give a clear picture about the functioning of the repair mechanism.

During radiotherapy treatment, blood haematology plays an important role in indicating the overall body status of the patient[28]. Several reports show severe fall in blood cell count after radiotherapy treatment following which the patient may need blood transfusion depending on the CBC status[29].

Tumour cells are killed by each consecutive dose of radiation during radiotherapy treatment[7,30]. However, information is not available about response of normal cells after repeated exposure. The radiosensitivity of normal and tumour cells differ significantly. The current investigation measures the damage level in healthy cells exposed to the repeated exposure under radiotherapy setting using latest γH2AX technique and conventional chromosomal and blood haematology assays.

2. MATERIALS AND METHODS

2.1 Chemicals

Sodium chloride (S3014), potassium chloride (P9541), sodium phosphate dibasic (S3264), potassium phosphate monobasic (P9791), potassium bicarbonate (12602), ammonium chloride (A9434), ethylenediaminetetracetic acid di-sodium salt (EDTA) (E6635), paraformaldehyde (158127), Triton X-100 (T8787), bovine serum albumin (S5482), colchicines (SC9754) and glycerine (44892) were obtained from Sigma-Aldrich. Anti-phospho-histone γH2AX Ser 139 monoclonal antibody (05-636) and goat anti-mouse IgG (H+ L) fluorescein-conjugated antibody (02-506) were obtained from Merck-Millipore. Methanol (Himedia, MB113), acetic acid (Merck, 100056), Giemsa acid (Fisher scientific, G146) were purchased from specific manufacturer for this work.

1L 1X PBS (8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of sodium phosphate dibasic, 0.25 g of potassium phosphate monobasic to 1 L at pH 7.4), 1L 1X RBC lysis buffer (1 g of potassium bicarbonate, 8 g of ammonium chloride and 0.03 g of di-sodium EDTA), the paraformaldehyde (3%), 50 mM ammonium chloride, 0.5% Triton X-100/PBS, 10% BSA/PBS and 0.1% Triton X-100/PBS solutions were prepared in the laboratory. For chromosomal aberration assay 0.075 M of potassium chloride solution, fixative solution (methanol and acetic acid solution in 3:1 ratio) and 5% Giemsa solution (3.8 g of Giemsa in 250 ml of methanol and 250 ml of glycerine) were prepared in the laboratory.

2.2 Animals

Strain 'A' male mice (10-12 weeks old and 25 g - 30 g weight) were used for the experiments. Mice were housed with 6 animals per cage with standard pellet diet and water. In the animal house, 12 h dark/ light cycle was maintained with the temperature of 25 °C - 30 °C.

2.3 Ethics Statement

All the experiments performed on mice were approved by the committee on the Ethics of Animal Experiments of the Institute of Nuclear Medicine and Allied Sciences (INMAS) and the Defence Research and Development Organisation (DRDO) of Delhi, India (Institute Animal Ethics Committee number: INM/IAEC/2016/21 valid until 23/02/2017). The experiments were conducted according to the general national guidelines that were set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of the Government of India.

2.4 Treatment Groups and Time Points

Mice were divided into six groups as follows:

(i) Untreated: Non-irradiated mice.
(ii) 1st fraction: Localised radiation of 2 Gy to right leg region of the mice and the radiation was given in a single go.
(iii) 2nd fraction: Localised radiation of 4 Gy to right leg region of the mice and the radiation given as 2 Gy per day for 2 consecutive days.
(iv) 3rd fraction: Localised radiation of 6 Gy to right leg region of the mice and the radiation given as 2 Gy per day for 3 consecutive days.
(v) 4th fraction: Localised radiation of 8 Gy to right leg region of the mice and the radiation given as 2 Gy per day for 4 consecutive days.
(vi) 5th fraction: Localised radiation of 10 Gy to right leg region of the mice and the radiation given as 2 Gy per day for 5 consecutive days.

All the three studies performed in the current study, i.e. blood haematology, γH2AX measurement and chromosomal aberration study, were performed in all the treatment groups as mentioned above. However, blood haematology and γH2AX measurement studies were performed in a set of animals with 6 animals/ group at 1 h and the chromosomal aberration study was performed in a separate set of animals which also contains 6 animals/ group on 24 h.

2.5 Radiation

Mice were kept in a restrainer and right leg was exposed to 2 Gy/day radiations for 5 consecutive days at a fixed dose rate of 0.699 Gy/min in Bhabatron II Cobalt-60 tele therapy unit in a field size of 2x2 cm^2. A 5 mm lead metal shielding was done to cover all the other body parts of the mice except the right leg region to avoid exposure to other body parts.

2.6 Blood and Bone Marrow Cells Collection

For blood haematology and γH2AX experiment, mice from all the treatment groups were euthanised at 1 h post irradiation time point using cervical dislocation. Approximately 1 ml Blood was collected from the heart by cardiac puncture

in EDTA tubes to avoid clotting. Total bone marrow cells were flushed out in PBS from both legs of the euthanised mice. Similarly, for chromosomal aberration study mice were euthanised at 24 h using cervical dislocation, However, 2 h prior to sacrifice 5 mg/kg body weight of colchicine was injected intraperitoneally for the arrest of the chromosome at metaphase. Total bone marrow cells were flushed out in 0.075 M potassium chloride solution for analysis.

2.7 Blood Haematology

20 µl of collected blood from each experimental animal was analysed by Nihon Kohden fully automatic haematology analyser.

2.8 Flow Cytometric Measurement of γH2AX

The γH2AX measurement was done as per the protocol mentioned in Yashavarddhan[12], *et al.* The RBCs from isolated blood and total bone marrow cells were removed by RBC lysis buffer. RBC free cells were washed twice with PBS (centrifugation at 1200 rpm for 10 min) and fixed in 100 µl of 3 per cent paraformaldehyde for 30 min on ice. The fixed cells were washed twice with 1 ml of PBS followed by washing with 1 ml of 50 mM ammonium chloride. After washing twice with PBS, the cells were permeabilised in 200 µl of 0.1 per cent Triton X-100 in PBS for 20 min at 4 °C. The blocking step was performed in 10 per cent BSA in PBS for 100 min at room temperature, followed by an overnight incubation with anti-phospho-histone γH2AX Ser-139 monoclonal antibody (1:50). The cells were then washed with PBS and incubated with polyclonal goat anti-mouse FITC-conjugated secondary antibodies (1:400) at 4 °C. The fluorescence intensity of γH2AX in 10,000 cells/sample was analysed using a BD flow cytometer.

2.9 Chromosome Aberration Assay

Bone marrow cells were incubated at 37 °C for 25 min. The samples were then centrifuged at 400 g for 10 min. Cells were fixed by adding 1 ml of a fixative solution while vortexing at a moderate speed. The fixed cells were washed twice with the fixative solution. The supernatant was discarded and the pelleted cells were resuspended in 200 µl of fixative solution. Three drops of cell suspension were dropped from a distance of about 1 meter on a chilled glass slide at an angle of 45°. Slides were then air-dried and stained with 5 per cent Giemsa on the following day[31-33]. A total of 50 well spread plates were scored from each animal. The total number of aberrant cells and different aberrations like dicentrics, rings, fragments, gaps, terminal deletion, centromeric attenuation, double minute (DM), end to end association and robertsonian translocations as well as severely damaged cells were scored from each group.

2.10 Statistical Analysis

The data are presented as the mean ± SD of 6 variants from each group. Comparisons were made between the groups. The data was analysed using one-way analysis of variance, and multiple comparisons among different groups were performed by applying the Bonferroni t-test. A probability of <5% was considered significant.

3. RESULTS

3.1 Blood Haematology

Blood from euthanised mice of different treatment groups was analysed for whole blood counts (WBC), lymphocytes and granulocytes percentage at 1 h time point (Fig. 1). In the untreated samples, the WBC count was 9.2±0.5 million cells/ml with 70.2±1.2 % of lymphocytes and 29.8±1.2% granulocytes populations. Animals irradiated with each fraction had significant ($p<0.001$) fall in total WBC counts. However, 1st fraction of dose showed comparable ($p>0.05$) counts with control. The WBC counts in 1st to 5th fraction were found to be 8.5±0.5, 4.7±0.4, 3.5±0.2, 2.4±0.3 and 2.3±0.3 million cells/ml respectively. In the case of differential counts, the lymphocytes *vs* granulocytes in 1st to 5th fractions were 66.2±2.8 *vs* 33.8±2.8, 59.2±2.1 *vs* 40.8±2.1, 51.8±1.7 *vs* 48.2±1.7, 43.0±1.7 *vs* 57.0±1.7 and 43.0±2.3 *vs* 57.0±2.3 respectively. The reduced

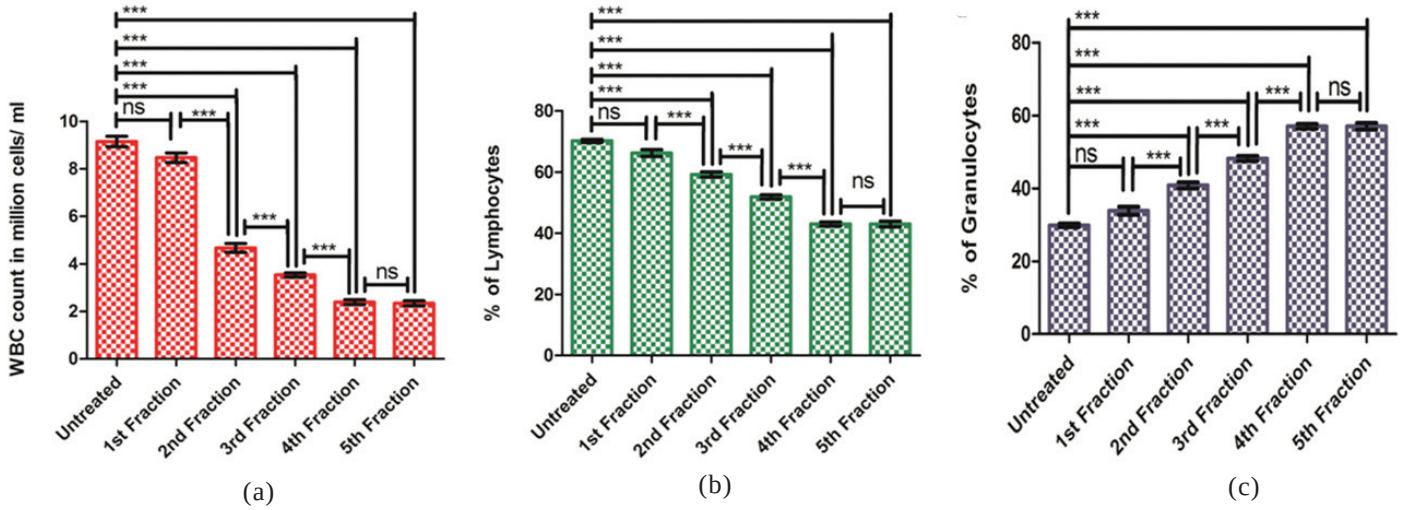


Figure 1. Graph represents WBC and differential cell count of different treatment groups mice: (a) WBC counts at 1 h time point after giving different fractions of radiation, (b) Percentage of lymphocytes of different treatment groups, (c) Percentage of granulocytes of different treatment groups. The bars represent the mean ± SD of 6 animals. A value of $p<0.05$ is considered statistically significant. *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ns= not significant ($p>0.05$).

lymphocyte count corroborated with increased granulocyte was found from 2nd fraction of irradiation up to 5th fraction. 1st fraction had non-significant (p>0.05) changes when compared with the controls. The dose-dependent changes were observed in blood haematology parameters between different fractions of irradiated groups. A linear fall was observed up to the 4th fraction. However, 4th and 5th fraction doses showed comparable (p>0.05) cell counts (Fig. 1).

3.2 Assessment of γH2AX using Flow-cytometry

Flow cytometry is a rapid and quantitative method to measure the fluorescence intensity of a particle. For quantitative measurement of H2AX phosphorylation, this technique was used. Isolated blood and bone marrow cells were stained with anti-γH2AX ser-139 antibody and measured after the clinically relevant dose of irradiation ranging from 0-10 Gy (2 Gy per day for five consecutive days) using flow cytometry (Fig. 2). In untreated (0 Gy) samples 117±23 Mean fluorescence intensity (MFI) in blood and 114±14 MFI in bone marrow cells were measured with 5±2 and 9±1 percentage of the positive population in blood and bone marrow cells respectively. After exposure to 1st fraction (2 Gy), both γH2AX positive cells and MFI increased significantly (p<0.001) up to the 4th fraction (8 Gy). Although a significant increase in both MFI and percent positive cells were observed after 5th fraction (10 Gy) when compared with the non-irradiated group, there were no significant (p>0.05) differences between 4th fraction and 5th fraction. In 1st fraction, 1048±120 MFI in blood and 1859±88 MFI in bone marrow cells with 21±3 and 32±2 percentages of positive cells were observed respectively. In the case of 2nd fraction (4 Gy) 1659±85 MFI in blood and 2438±44 MFI in bone marrow cells with 35±3 and 47±2 percentages of positive cells were observed respectively. Similarly, percent positive cells in 3rd fraction (6 Gy) and 4th fraction were 46±3 and 60±4 in blood and 60±2 and 71±2 in bone marrow cells respectively. In corroboration with γH2AX positive cells, MFI of 4th fraction was higher than 3rd fraction (3rd *vs* 4th fraction: 2673±75 *vs* 3083±72 in blood and 2755±43 *vs* 3406 ± 44 in bone marrow cells). 4th and 5th fractionated dose did not differ significantly for both MFI and percentage of γH2AX positive cells. At 1 h time point after 5th fraction blood cells showed 3096±75 MFI with 60 ± 5 of positive cells and similarly, bone marrow showed 3424±50 MFI with 71±2 % of positive cells were observed (Fig. 2).

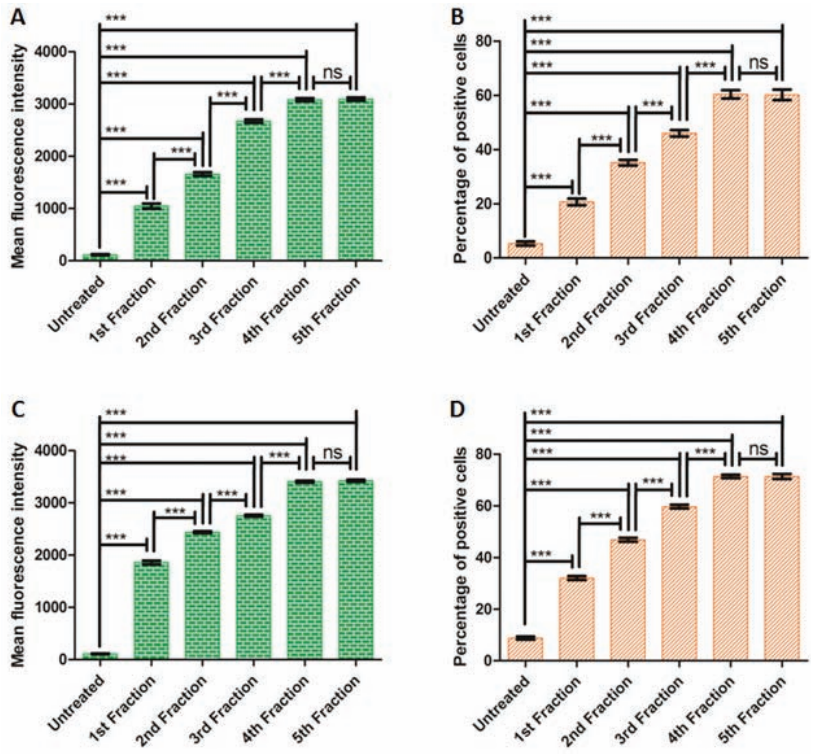


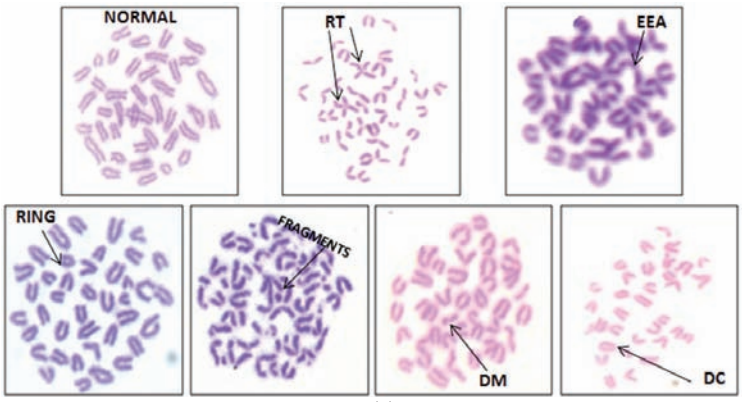
Figure 2. Measurement of γH2AX in mice blood and bone marrow cells of different treatment group at 1 h time point using Flow cytometry: (A) and (B) represents the MFI and percentage of γH2AX positive cells in the blood, (C) and (D) represents the MFI and percentage of γH2AX positive cells in bone marrow. The bars represent the mean ± SD of 6 animals. A value of p<0.05 is considered statistically significant. *=p<0.05, **=p<0.01, ***=p<0.001, ns=not significant (p>0.05).

3.3 Cytogenetic Aberration

The frequency of aberrations in control animals is markedly low or even absent for some types of aberrations (Fig. 3). The total number of aberrations in different fractions of irradiated groups was significantly increased (p<0.001) when compared with the untreated (42.5±5.0, 63.3±4.2, 82.3±5.6, 94.5±5.2 and 98.4±5.4 numbers of aberration from 1st to 5th fraction respectively). However, it was found similar for 4th and 5th fraction of radiation. The RT, EEA, rings and fragments were amongst the prominent types of aberrations. The RT and EEA number have shown a non-linear increase from 1st to 5th fractions, however, the frequency of RT and EEA enhanced when compared with control in case of all the fractions (RT *vs* EEA were 0.4±0.2 *vs* 1.0±0.5 in control, 3.2±0.4 *vs* 14.5±1.2 in 1st, 37.7±0.6 *vs* 10.0±0.9 2nd, 30.2±1.2 *vs* 20.8±1.0 in 3rd, 41.8±1.2 *vs* 39.7±0.9 in 4th and 17.3±0.8 *vs* 52.3±1.1 in 5th fractions). The number of fragments first increased in 1st to 3rd fraction but later on reduced and reached the untreated values (Fig. 3). The frequency of rings was also enhanced in different fractions of radiation exposure compared to untreated (Fig. 3), whereas the aberrations like gaps, deletion and DM did not show any significant trend although they were observed in exposed animals only at various fractions of exposure (Fig. 3).

4. DISCUSSION

Use of radiation for the treatment of cancer is a well approved, non-surgical method[34]. Radiotherapy is a commonly used practice where multiple fractions of a clinical dose are delivered over a period to kill cancer cells[2,8]. The success lies with the maximum effect of radiation on the cancer cells along with minimal damage to surrounding normal cells[6,7]. There are various factors governing the radio-sensitivity of a cell. The difference in radiation response of tumour and normal cells provides an opportunity to use interventional approaches in clinics.



(a)

Groups	Untreated (0 Gy)	Fraction 1 (2 Gy)	Fraction 2 (4 Gy)	Fraction 3 (6 Gy)	Fraction 4 (8 Gy)	Fraction 5 (10 Gy)
Fragments	1.3 ±0.3	10.3±1.6	11.2±1.2	15.0±1.4	2.0±0.9	0.5±0.3
Rings	0.5±0.3	10.2±0.8	0.5±0.5	12 .3±0.5	10.0±0.6	10.3±1.0
Gap	0.4±0.2	0.5±0.5	3.0±0.6	0.8±0.5	0.5±0.5	7.5±0.8
RT	0.4±0.2	3.2±0.4	37.7±0.6	30.2±1.2	41.8±1.2	17.3±0.8
EEA	1.0±0.5	14.5±1.2	10.0±0.9	20.8±1.0	39.7±0.9	52.3±1.1
Del	0.5±0.3	0±0	0.7±0.6	0.2±0.4	0.3±0.7	3.0±0.6
DM	0.2±0.4	3.3±0.5	0.2±0.4	3.0±0.6	0.2±0.4	7.5±0.8
Total	4.3±2.2	42±5	63.3±4.2	82.3±5.6	94.5±5.2	98.4±5.4

(b)

Figure 3. Chromosomal aberration study in mouse bone marrow after different fractions of radiation: (a) Representative image of the different type of aberration found in mouse bone marrow and (b) Different type of aberration and the total number of aberration of different treatment group. The bars represent the mean ± SD of 6 animals.

Numerous reports clearly indicate killing of the tumour cells during split dose regimen of radiotherapy[30]. The fractionation of radiation leads to an effective killing of the tumour with each consecutive dose which is shown to be more effective than earlier delivered dose[7,30]. The response of normal cells to the repeated dose of radiation is not available. The present investigation aims to evaluate DNA damage induction in normal cells exposed to the repeated dose of radiation. The localised 2 Gy radiation was delivered to the right leg of mice repeatedly for 5 days. Directly exposed bone marrow and indirectly exposed blood cells of mice were measured for DNA damage. The latest biomarker γH2AX has been used for the quantification of DNA damage. Similarly, a well established conventional cytogenetic assay was used to confirm the DNA damage. The overall haematology system of the animal was observed through blood haematology.

The present study showed that significant level of γH2AX was found in blood and bone marrow cells of mice after 1st dose of radiation delivered to right leg. The linear increase in γH2AX formation was observed up to 4th split dose of radiation in comparison to the untreated controls. The 5th fractionated dose to animals leads to similar γH2AX level as observed in 4th fractions. In our chromosomal aberrations study, we have observed different types of alterations such as fragments, rings, gaps, robertsonian translocations (RT), end to end associations (EEA), deletion, and double minute (DM). The frequency of chromosomal aberrations was increased with increasing fractions and duration of radiation. Aberrations like fragments and rings were almost constant in the fractions that followed the 1st split dose but the frequency of RT and EEA increased significantly during successive doses. This finding is in resonance with earlier reports conveying RT and EEA have been measured at small doses of radiation[31-33]. After giving 5th fractioned dose the damage level was found comparable to 4th dose. The stabilization of chromosomal damage from 4th to 5th split dose corroborates with the finding of γH2AX study. Blood haematology indicated fall in WBC count in a dose-dependent manner. However, the total counts in 4th split treated groups were almost equal to the 5th fraction. Decrease in lymphocytes with increased granulocytes was observed in all irradiated groups. The comparable WBC count of the 4th and 5th split dose treated groups clearly supports damage stabilization, as observed in γH2AX and chromosomal aberration studies.

The above study explicitly shows adaptation of normal cells to the radiation stress after repeated exposure. The differential response of normal and tumour cells exposed to the repeated exposure may be based on repair mechanisms and type of cells[35]. In the case of fractionated radiation doses, the damage to DNA is not as severe due to the low dose and repair of damaged DNA occurs between exposures[12]. In brief, after giving 1st split dose of radiation the body repair mechanism gets activated and damage would be repaired if it was not too extensive. However, after 2nd and subsequent doses, the pre-activated repair mechanism can easily counter the damage. In other words, the fractionated low-dose exposure induced a balance between the damage induction and repair which lead to stabilization of DNA damage[36]. In the present study, similar balance in damage induction and repair was observed in non-dividing blood and proliferative bone marrow cells. The possible reason is based on the fact that the bone marrow cells divide and form blood cells based on the system requirement against any stress. The balance between damage and repair against repeated exposure in normal cells might involve the regulated response of repair mechanism, although, this type of control is not available in tumour cells where division occurs in uncontrolled fashion[37,38].

Besides the above findings, we also found damage in the blood cells on 1 h after 1st split dose of radiation. This could be due to the direct exposure of blood which was passing through the exposure area at the time of radiation and possibly due to bystander effect. Similar observations have been previously proved under *in vitro* and *ex vivo* conditions[39-42].

Considering all the results, our study indicates that the normal cells get adapted to damage due to multiple low dose exposure.

5. CONCLUSIONS

The present work has greater application in studying the response of normal cells against repeated radiation exposure. Since in radiotherapy, both normal and tumour cells are present the actual outcome of the current study may differ due to the interaction among cells. Further studies are needed in this direction.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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CONTRIBUTORS

Mr M.H. Yashavarddhan obtained his MSc in Biotechnology from KIIT University, Bhubaneswar. Currently working as a Research fellow at INMAS, Delhi. He is working on regulatory network controlling the radiation induced DNA damage signalling pathway.

In the current study, he conceived, designed, performed the experiments.

Dr Sandeep Kumar Shukla received his PhD (Biotechnology) from Jamia Humdard University-INMAS, DRDO, Delhi. Presently working as a Scientist 'D' in INMAS, Delhi. His research interests are : Radiation countermeasure and DNA damage and repair.
In the current study, he conceived, designed, performed the experiments.

Dr Ajay Kumar Sharma received his PhD (Zoology) from School of Studies in Zoology, Jiwaji University, Gwalior. Presently working as a Scientist 'D' in INMAS, Delhi. His research interests are : Molecular biology and cytogenetics which includes chromosomal aberrations, micro-nuclei assay.
In the current study, he performed the cytogenetic studies.

Dr Mrutyunjay Suar received his PhD (Molecular Biology) from University of Delhi. Currently he is holding the position of Director in school of biotechnology, KIIT University, Bhubaneswar. His research interests are : Molecular mechanism of Salmonella enterica serovar Enteritidis based enterocolitis in mouse model, development of a safe live recombinant Salmonella vaccine against enterocolitic diseases and development of early diagnostic kits for diseases like typhoid and diarrhea in rural health set-up.
In the current study, he provided guidance.