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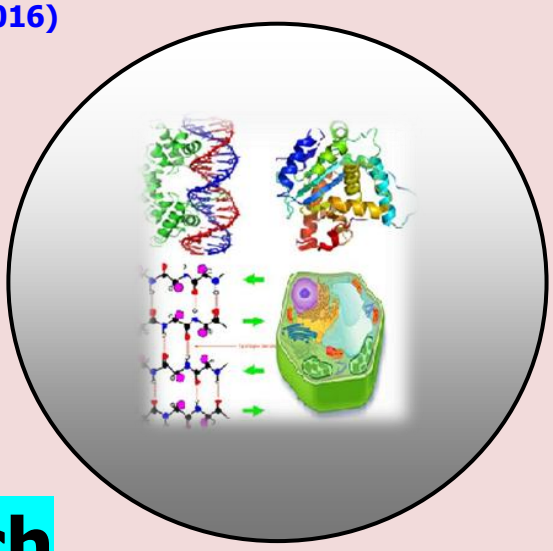

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Micronucleus Assay based Genotoxicity Assessment in Human Peripheral Blood Lymphocytes of Patients undergoing Diagnostic CT Imaging and Radiotherapy

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ABSTRACT

Ionising radiation induce genomic instability and other biological manifestations which are dependent on physical property, ionising nature, dose and duration of radiation exposure. The diagnostic radiation exposures are planned within safer limits and are considered harmless. Undoubtedly CT scanning is a wonderful modality designed for better diagnosis and treatment of patients. However the magnitude of health risk associated with its usage remains controversial due to divergent reports. The present study assesses the immediate genotoxic/DNA damaging effects of low dose diagnostic radiations, in lymphocytes of patients undergoing the CT scanning procedure. Blood from the patients undergoing CT and radiotherapy was collected and cultured for genotoxicity assessment by micronucleus assay. Slides were prepared, stained and micronuclei formation was counted in 1000 bi-nucleated cells per sample. Abnormalities like nuclear buds and nucleoplasmic bridges were also scored. The values of micronuclei formation in low dose radiation samples was compared with controls with no known radiation exposure and those with high exposure, radiotherapy. A significantly increased number of micronuclei were observed in patients undergoing CT procedures as compared to the Controls. Other abnormalities Nuclear Buds and Nuclear Bridges were also significantly higher in the CT and radiotherapy cases. The finding suggests CT scanning procedures induce immediate genotoxic effect in lymphocytes of exposed patients and further investigations are needed to evaluate the delayed effects of low doses.

Key words: CBMN, Low dose radiation, DNA damage and Genotoxicity.

INTRODUCTION

Ionising radiations (IR) cause DNA damage by either disrupting the DNA backbone directly or by induction of free radicals which subsequently causes indirect damage (Kavanagh et al., 2013, Khanna and Jackson, 2007, Alloni et al., 2012, Mariotti et al., 2010). In response to radiation exposure many complex set of biochemical signalling pathways are activated, which attempt to repair and maintain genomic stability (Kavanagh et al.,

2013, Khanna and Jackson, 2007, Alloni et al., 2012,). Once these pathways are inefficient to repair the radiation damage the two types of cellular effects namely deterministic and stochastic effects are observed. Deterministic effects are known to occur above a threshold dose and are considered to increase risk with dose and associated severity of outcome. Stochastic effects are the effects caused when genome of the cell is modified, here the viability of cell is maintained and changes are permanent and transferred to next generation. The probability of these effects increases with dose, but severity of outcome may or may not be related with dose. The estimation of cancer risk involving lower doses radiation exposure like diagnostic procedures (Patricia and Joseph, 2011, Berrington de Gonzalez and Darby, 2004, Martha et al., 2012) occupational exposure is still unclear. Diagnostic procedures utilizing ionizing radiation are in frequent use now days as they are beneficial for better diagnosis and treatment of the disease. It is presumed that such modalities pose minimal risk to patients and has larger benefit to risk ratio. However concern arises when the radiation exposures are done without explainable clinical rationale and when the alternative safer modalities are available, and also when they are repetitively done (Aaron Sodickson et al., 2011, Amy Berrington de González et al., 2007, Brenner et al., 2003). It has been shown that there is inadequate awareness about radiation dose, radiobiology and their environment impact which often leads to unnecessary exposure of radio diagnostic procedures (Saberi et al., 2013, Soye et al., 2008). Amount of damage produced by a given dose depends on type of radiation as there are differences in Linear energy transfer (LET). It has been found that high LET radiations (alpha particles and neutrons) generally produce more damage per rad of dose than do low LET radiations (x-rays, gamma and beta rays) (Alloni et al., 2013, Mariotti et al., 2012). The damage caused by exposure to LET radiation is still cause of concern as radiation exposure leads to formation of DNA double strand breaks which are very difficult to repair. On the contrary some studies suggest that repair mechanism acts best at lower dose of radiation exposure where repair centers are formed with better efficiency of damage repair (Feinendegen et al., 2005, Mark et al., 2012). The risk from the radiation are reported only when a significant number of scans have been performed and when a large population is already exposed to an unknown number of CT Scans (Joao Carreia et al., 2005, Bedetti et al., 2008). The amount of biological damage increases as the radiation dose increases, but it may or may not be comparative to the exposure dose (Joao Carreia et al., 2005). Here in the present study we evaluate the immediate effects of low dose diagnostic radiation exposure (CT Scan) on human peripheral blood lymphocytes using micronucleus assay. The assay evaluates the genomic instability induced in lymphocytes of patients undergoing diagnostic CT on different body regions for the first time. The micronuclei frequencies in normal unexposed individual are used as control and in radiotherapy patients are used as positive control of radiation damage. The micronuclei formation was compared with the amount of radiation patient received in the procedures. The study will elicit radiation awareness among the patents and providers and estimates the radiobiological risk.

MATERIAL AND METHOD

Sample collection: The patients who met the inclusion criteria of the study based on the standard questionnaire were included in the study. The study includes 30 cases out of which 10 were normal with no known exposure to radiations. Normal group included volunteers who were not exposed to any known geographical, occupational, diagnostic radiation exposure. The low dose group included those patients who were undergoing CT scan at Dr Ram Manohar Lohia Institute for reasons other than malignancy and genetic disorders. Ten patients undergoing treatment of radiotherapy at the radiation oncology department were recruited in high dose group. Radiation exposure history was recorded and informed consent from all the study subjects was taken prior to sample collection and enrolment in the study. Approval from institutional ethical committee of Dr. Ram Manohar Lohia Institute of Medical Sciences was taken prior to the commencement of study. The blood was collected from patients undergoing radiotherapy during the routine blood profiling for radiation therapy. The total radiation dose given to the patients till the blood sampling day was estimated as per the records on linear accelerator (LINAC) of the radiation oncology department of Ram Manohar Lohia Institute of Medical Sciences. For low dose sample collection, the CT radiation dose given to patient was recorded and blood sample collected within 1 hour of scan. All blood samples from patient were collected in sterilized heparinised tubes and kept on ice till further use. The sample was processed to lymphocyte culture within 2 hours of collection.

Cytokinesis-block micronucleus (CBMN): The samples were processed for CBMN assay as described by Fenech et al 2003.

The protocol followed was as same as used to assess the genotoxicity in peripheral blood lymphcyted of *in-vitro* exposed cultures (20) Briefly 0.5ml of blood was cultured in PB-MAX medium (GIBCO-12557); and incubated at 37ºC in 5% CO2 incubator (ESCO) for 72 hours. Cytochalasin-B (Himedia-RM7683) at a concentration of 500ug/ml was added at 44 hrs. Cultures were harvested at 72 hr following a short hypotonic treatment (10 min) in 0.56% KCl (GIBCO-10575-090) at room temperature. Cells were fixed 3–4 times in fresh, chilled Carnoy's fixative (methanol: acetic acid, 3:1) and left for overnight fixation. Cells were dropped over clean, chilled slides, were air dried, and stained with Giemsa (GIBCO -10092-013); (5%, prepared in Gurr's buffer, pH 6.8). The photographs were taken and scoring was carried out at 40x magnification in Ziess, microscope. Micronuclei (MN) were identified and binucleated cells were scored.

Micronuclei scoring

The scoring criteria of Michael Fenech (Michael Fenech, 2000), was followed for manual scoring of micronuclei (MNi) in approx 1000 binucleated cells. The presence of MNi in single binucleated (BN) cell was scored and considered as indicator of radiation induced damage. Only binucleated cell were scored to make sure the cell division has occurred and micronuclei were genuine. The standard criterion defined was as follows: Cells with cytoplasmic boundaries stained with Giemsa containing one nuclei (Mon), two nuclei with or without micronuclei were counted. Nuclei circular in shape, with intact nuclear membranes (Mon, BN, and MNi) visibly separate from each other were scored. Micronuclei with size 1/9th to 1/256th of nuclear size, within cytoplasmic boundaries of binucleated cell were counted. Tri-nucleated and multinucleated cells were not scored. The frequency of BN, BN with MNi, and the number of MNi in the BN cells was counted by two persons separately.

Statistical Analysis

The values are presented in frequencies, percentages and mean ± SD, the mean of the micronuclei present was calculated and the findings were based on these calculated values across all groups. Statistical Difference ($p < 0.05$) between no exposure controls and radiation exposed samples were determined by a two tailed paired t test. All the analysis was carried out on SPSS 16.0 version (Chicago, Inc., USA).

RESULTS

The mean ages of Normal controls, Low dose group and high dose group were 30 (± 4.3), 35 (± 12) and (45 ± 12.4) respectively (Table 1). The mean age of normal control was not statistically different from the low dose group and mean age of high dose group was also not different from that of low dose group. The male to female (M/F) ratio distribution was 3/7 in Normal control, 6/4 in low dose group and 8/2 in high dose group. On an average the low dose group received 1386 (± 422.55) milli Gray (mGy) of radiation whereas the radiation dose was about 15 folds higher 20788 (± 12423.83) in high dose group (Table 1). The dosage received by the patients during the scan, clinical features and diagnosis are summarized in Table 1. An approximate number of 1000-1200 binucleated cells per sample were analysed for the micronucleus assay (Figure 1) and the frequency of micronucleus was then calculated. In the No Exposure Control group, the observed frequency of micronucleus was found to be minimal i.e., 0.30 (± 0.12) whereas in the high radiation control group a significant increase in micronucleus formation was observed i.e., 15.67 (±2.53), Figure 2(a). It should be taken into account that the mitotic index of the cells decreases if the radiation exposure is high as compared to normal. However in the group of interest i.e. the group of patients exposed to diagnostic procedures the number of binucleate cells counted was approximately same as normal but the micronucleus frequency was increased to 8.52 (±3.33), which was significantly higher when compared to normal. The other related abnormalities which were observed were the presence of nuclear buds and nuclear bridges which also are markers of genotoxic damage. In the no exposure group the frequency of nuclear buds and that of nuclear bridges was found to be same i.e., 0.06 (± 0.08), however the number of nuclear buds increased to 3.40 (± 1.76)in the low dose group and 4.36 (± 1.89) in the high dose group (Figure 2(b)), which is approximately four times of the normal group also the number of nuclear bridges increased to (0.45 ± 0.70) in low dose and (1.47 ± 1.51) in high dose group (Figure 2(c), however it was not found to be significant.

DISCUSSION

In the current study we have employed CBMN assay to estimate the genotoxic effects of diagnostic low dose radiation in individuals undergoing CT scan. Aim of the study was to assess whether the diagnostic CT scan has any immediate cytotoxic effects on humans and whether this damage can be detected in blood. The study finds its importance in detecting significant increase in micronucleus formation at lower doses of radiation exposure, which otherwise is considered safe for humans. We can easily study the genotoxic effects in the peripheral blood of the patients. This study is noteworthy as it detects the immediate effects of diagnostic radiations after partial exposure of body in blood, which is an easy source of pathological and clinical investigations. Analysis of micronuclei in cytokinesis-blocked binucleated cells is an easy and fast procedure and is an alternative method to detect chromosome alterations induced by ionizing radiation, even at very low levels (Tucker et al., 2013, Ramalho et al., 1988). The MN assay can be applied in biological dosimetry and to evaluate nuclear damage after radiation exposure.

Table 1. Table illustrating the epidemiological data of the patients showing gender, age, mean age; type of diagnostic procedure and amount of radiation exposure across the groups.

S. No	Sample	Sex	Age	Exposure type in last one year	Diagnosis	Radiation Dose (mGy)
1	NEC1	F	38	None	none	none
2	NEC2	F	27	None	none	None
3	NEC3	M	28	None	none	None
4	NEC4	M	29	None	none	None
5	NEC5	F	25	None	none	None
6	NEC6	F	29	None	none	None
7	NEC7	F	30	None	none	None
8	NEC8	F	27	None	none	None
9	NEC9	F	36	None	none	None
10	NEC10	M	28	None	none	none
11	LD1	F	34	CT Head	Pain in neck, head	750.0
12	LD2	M	17	CECT Head	Seizure (1episode)	1596.6
13	LD3	M	17	Angio (Brain)	Haemorrhage suspected	1963.1
14	LD4	F	32	CT Head	Headache	1585.7
15	LD5	M	47	CT Head	Headache	809.9
16	LD6	M	37	CECT Head	Head Injury	1692.5
17	LD7	M	38	CECT Head	Headache	1581.1
18	LD8	F	43	CECT Thorax-Head	Outgrowth on head	867.2
19	LD9	F	30	CECT Head	Pain in chest	1400.0
20	LD10	M	55	CECT Head	Fits	1613.9
22	HDC1	M	54	Radiotherapy	CA Prostate	10000
21	HDC2	M	52	Radiotherapy	CA L. Parietal	12000
23	HDC3	M	36	Radiotherapy	Brain Tumor	12000
24	HDC4	M	37	Radiotherapy	CA PFS	23000
25	HDC5	M	54	Radiotherapy	CA Breast	12000
26	HDC6	F	30	Radiotherapy	CA GB sulcus	30880
27	HDC7	M	36	Radiotherapy	CA lower alveolus	36000
28	HDC8	M	72	Radiotherapy	CA Buccal Cavity	28000
29	HDC9	F	45	Radiotherapy	CA Urinary Bladder	40000
30	HDC10	M	37	Radiotherapy	CA Prostate	4000

It has been shown that workers engaged in operational radiology (Nowak, 1991) and nuclear medicine (Bouchter and Haas, 1985) are chronically exposed to low-level ionizing radiation. Cytogenetic studies demonstrated that a low radiation dose or a low dose rate lead to increased frequencies of chromosome damage (24). Thus, it becomes very important to monitor the patients after scanning through radiation based scanner be it CT Scan or angiography, for permanent changes. The objective of the present investigation was to study a group of patients undergoing diagnostic CT scan and radiotherapy in comparison with matched normal controls in immediate response to radiation with in 2 hours. The micronuclei frequencies in normal humans have been studied at large to generate a baseline data on radiation exposures (Tucker et al., 2013).
In order to avoid the age based variation in micronucleus assay the patients recruited in the present work were around the same age, as the numbers of micronuclei tend to change within the age and gender of the subject (Tucker et al., 2013). As per the guidelines of the International Commission on Radiological Protection (IRCP) and Atomic Energy Regulatory Board (AERB) the recommended safe dose for occupational exposure is 5cGy . Much higher doses (≥2000cGy) are delivered to patients during radiotherapy and a broad range of doses (100cGy-500cGy) of radiation is used in while commencing a diagnostic scan (Linet et al., 2010). The total dose given to the patients depends on number of sequences read in a scan, procedure coupled with contrast imaging also adds to radiation exposure. In the present study diagnostic radiation doses were about 15 times less as compared to the radiotherapy doses. The radiotherapy group depicted more deleterious effects on lymphocytes and there were less viable cells observed at the high radiation doses and increased the micronuclei frequencies (Tewari et al., 2016). However in case of low dose group the micronuclei formation was twenty eight folds higher compared to normal patients. We observed that the radiation did not alter the mitotic indices of the cells on exposure to lower doses of radiations; however the micronuclei frequency was increased. This shows the induction of cytoxicity was not adequate to cause the cell death however the vital cells harbouring the damage were capable enough to divide along with the damage caused. Thus the micronuclei estimation seems to be a promising method to detect the immediate cytogenetic effect of low doses. It is contentious whether this effect may or may not be detectable after a period of one week or so as we have evaluated the damage within one hour when the repair mechanism has not yet started and taken over the cells. It has been reported that the DNA repair foci are formed within two hours of the radiation exposure and repair mechanism takes care of the damage (26). The fact that any of the cell escaping the repair, and having genomic instability if remains viable may divide to trigger carcinogenesis.

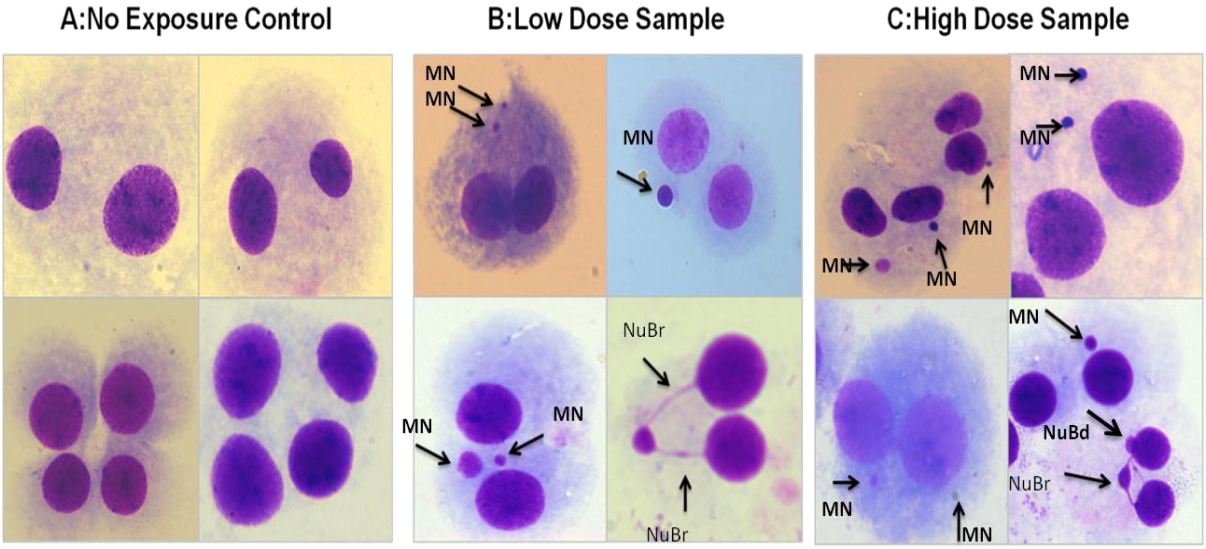

Figure 1 A. Normal binucleate cells, B:Micronucleus and other abnormality observed in the low dose exposure group, C:Micronucleus,Nuclear Buds and Nuclear Bridges observed in the high radiation exposure group (MN-Micronucleus, NuBd-Nuclear Bud, NuBr -Nuclear Bridge).

Ionizing radiation is used commonly in medical diagnostics, and there is concern about the potential damage and risk induced. The assessment of radiation exposure and damage is complex as many indirect effects are generated along with the direct ones hence the effects may be farfetched than the immediate.

After the radiation exposure the lymphocytes die and replaced within three months of duration but during and after the exposure they may release cytokines causing bystander effects, which may signal non targeted cells to undergo alterations. The risk of cancer from diagnostic x-ray exposure and CT scans has been debated widely (Rothkamm et al., 2007). A major focus of radiation biologists is to understand the cellular responses to low doses of radiation that mimic human exposure during diagnostic radiography or occupational activities and to relate these to risk from exposures. This study demonstrates that after exposure to low doses of ionizing radiation; DNA damage occurs and that has the potential to stimulate downstream mechanisms.

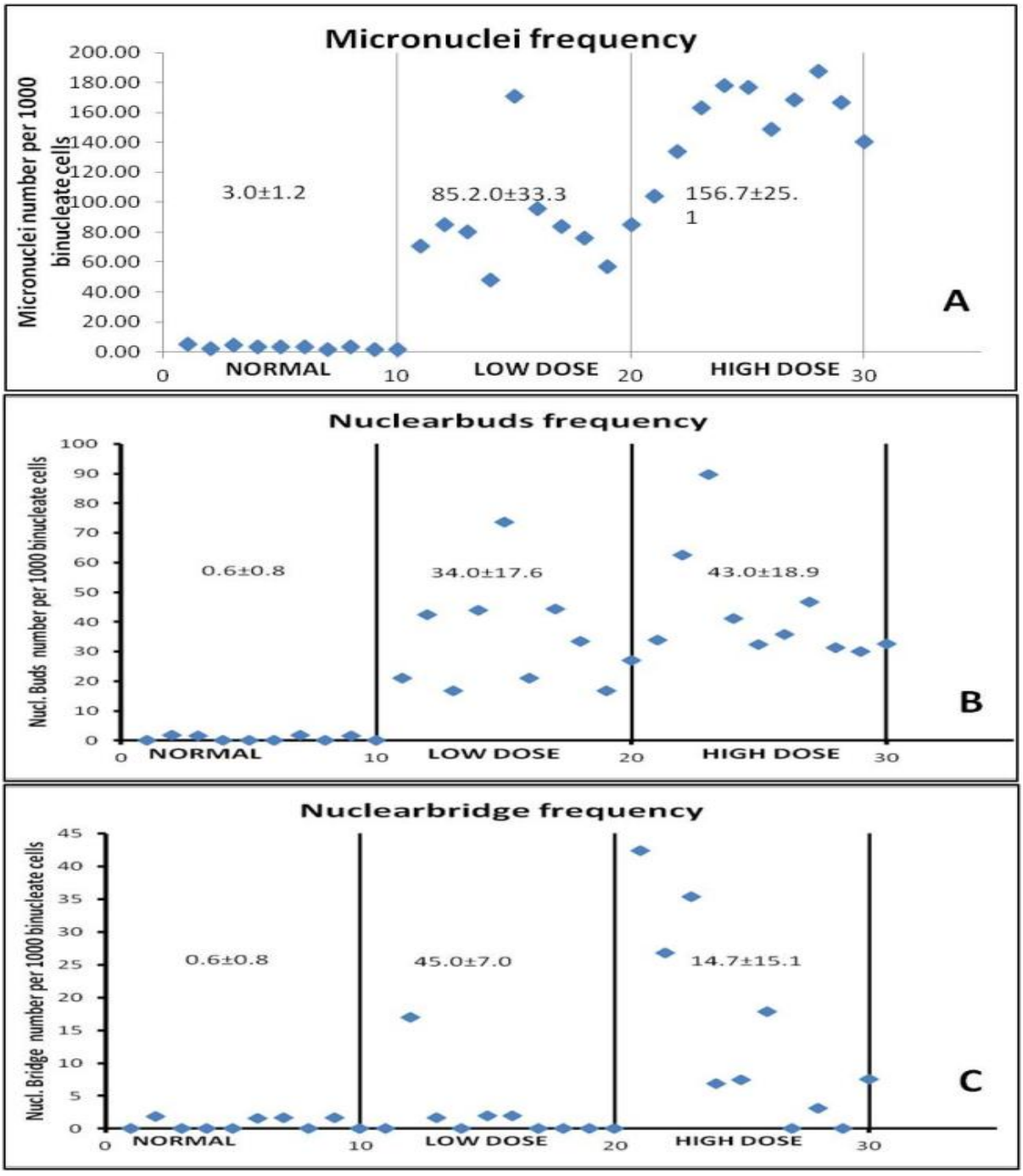

Figure 2 A. Micronucleus frequency across all groups B: Nuclear Buds (NuBd) frequency across the three groups, C: Nuclear Bridges (NuBr) observed in the groups.

The effect being reported need to be tested for the lasting of effects and can be crossed validated in the same patients after one week or so when the repair mechanism is initiated and taken over the damage. The effect may not be seen after two hours hence we have checked the assay in two hours when repair mechanism has not started it takes two hours for the repair mechanism and proteins to be expressed. It has also been reported that after exposure to low doses of radiation the resistivity of body increases towards radiotherapy (Delaney et al., 2005) also there is increased probability of the person to develop diabetes and retinopathy (Uzun et al., 2016). The track of radiation is also known to generate the reactive oxygen and nitrogen species. The radiation causes the cognitive problems because it speeds up the brain's aging process and recent research suggests that the cause may be chronic inflammation or oxidative stress after radiation exposure. Oxidative stress occurs when cells cannot remove free radicals causing and a vicious cycle of oxidative stress and related events may be triggered leading to death of endothelial cells causing retinopathy and neuropathy (Uzun et al., 2016, Tewari et al., 2012). Present work is limited to the lymphocytes which are known to be very sensitive towards radiation damage are incapable to divide. The blood being the body fluid is circulating everywhere in human body; hence the damage we are detecting is representing a fraction of the total changes occurring in blood. The evaluation of effect may have been diluted as the blood being exposed represents the partial; exposure occurred during the scan. The total damage occurring may be more than the reported as the tissues which came direct in contact with the radiation have not been tested. Although we cannot test the tissue which directly observed the radiation exposure but the blood can be used as substitute to evaluate and express the damage being induced due to radiation exposure. We have reported that lymphocytes when exposed to in-vitro radiation exposure give a linear dose response relationship with micronuclei frequencies. However the chromosomal aberrations tend to be non specific at lower doses (Brenner, 2004).

CONCLUSION

For many years, radiation dose-related cancer risks at low doses were generally estimated from results of the follow-up studies of the atomic bomb survivors and of patients treated with moderate- to high-dose radiation(31). Although it is now well accepted that many different processes are involved in the development of radiation induced cancer (such as epigenetic alterations and microenvironment modification). This work is crucial as it highlights the immediate genotoxic evens which can be easily studied in lymphocytes of low dose diagnostic radiation exposed patients and we need to fully elucidate the role of DNA damage and its repair following exposure.

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