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Quantitation of genome damage and transcriptional profile of DNA damage response genes in human peripheral blood mononuclear cells exposed *in vitro* to low doses of neutron radiation

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► ***Original article***

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ABSTRACT

Background*:** Humans are exposed to ionizing radiation from different sources that include natural, occupational, medical, accidental exposures. Evaluation of the effect of low level of neutron exposure to human cells *in vitro* has important implications to human health. Attempts were made to measure genome damage, transcriptional profile of DNA damage response and repair genes in peripheral blood mono-nuclear cells (PBMCs) exposed to different doses of neutron irradiation (^{241}Am-^{9}Be source) *in vitro*. ***Materials and Methods*:** Blood samples were collected from six random, healthy individuals with written informed consent. The frequency of micronuclei (MN), nucleoplasmic bridges (NPB), DNA strand breaks and gamma-H2AX foci intensity were measured in PBMCs exposed to low doses of neutron (3.0 to 12mGy). Transcription profile of *ATM, P53, CDKN1A, GADD45A, TRF1, TRF2, PARP1, NEIL1, MUTYH, APE1, XRCC1, LIG3, FEN1* and *LIG1* were analysed in PBMCs at 30 min and 4h post-irradiation using real time quantitative PCR. ***Results and Discussion: Our results revealed a significant increase (P≤0.05) in the frequency of MN at 9.0 and 12.0mGy as compared to control. A dose dependent increase in the percentage of DNA in tail and an increased intensity of gamma-H2AX foci were observed. CDKN1A and GADD45A showed marginal up-regulation at 30 min, whereas PARP1 showed increased expression at 4h post-irradiation across the doses studied. ***Conclusion:*** The present study revealed that GADD45A, CDKN1A and PARP1 can be used as early signatures for low-dose neutron exposure. However, further in vitro and *in vivo* studies are required to establish its implications in radiation protection science.

Keywords: *Neutron irradiation, human peripheral blood lymphocytes, micronuclei, nucleoplasmic bridges, gene expression, comet assay, gamma-H2AX, Real time q-PCR, fluorescence microscopy.*

INTRODUCTION

Ionizing radiation (IR) induces a variety of DNA lesions in human cells depending upon the type and quality of radiation. High LET radiation exposure induces more complex damages, including double-strand breaks (DSB) and clustered DNA damages [(1)]. At high-dose exposures, it has been unequivocally demonstrated that IR may lead to deleterious effects in human cells. IR induced DNA damage response may accumulate increased frequencies of chromosome aberrations, micronuclei formation and mutations in human cells. As a consequence, cells might activate various cellular and molecular processes such as activation of DNA damage response (DDR), DNA repair pathways, cell cycle check points and

apoptosis. However, various DNA repair pathways efficiently repair these lesions to maintain the genome integrity.

Humans are exposed to a variety of genotoxic stress including IR (low and high LET) that comes from natural background, cosmic, cosmogenic, occupational, medical (diagnostics and radio-therapeutic) and accidental exposures [2]. Although, plenty of data is available in human cells exposed to IR both *in vitro* and *in vivo*, biological effect of low dose radiation exposure in human cells/tissues is still inconsistent and inconclusive [3]. Limited data is available on biological effects of low doses of neutron exposure in human cells/tissues using multiple DNA damage end points and their response at cellular and molecular level.

The relative biological effectiveness (RBE) of neutron varies from 1 to 10 [4] depending on the type of cells/tissues or neutron dose or biological endpoints studied. Neutron doses may vary depending upon the type or duration of occupational exposure [5]. This includes exposures to airline crew members or astronauts, neutron therapy or accidental exposures [6] (Wilson and Townsend 1988).

Chromosome aberrations (dicentrics, translocations etc.) and micronuclei have been extensively used in biological dosimetry and population monitoring [7-12]. Chromosome aberration analysis is extremely reliable for evaluating genome damage in human lymphocytes exposed to low and high LET radiation [7,8]. Cytochalasin-blocked micronuclei (CBMN) assay is also another preferred method for assessing genome damage as it is faster, cost-effective and reproducible [13,14]. Hence, CBMN assay has been used as an alternative to the chromosome aberration assay as it measures simultaneously chromosome breakage, chromosome loss, non-disjunction, necrosis, apoptosis and cytostasis [14]. Enumeration of nucleo-plasmic bridges (NPB) in CBMN assay is essential as these are the dicentric chromosomes resulting from DSB mis-repair. NPBs are formed due to telomere end-to-end fusion caused by telomere shortening, loss of telomere capping proteins or defects in telomere cohesion.

Quantitation of DNA damage using alkaline single cell gel electrophoresis (comet assay) is one of the most commonly used methods for radiation-induced DNA damage [15]. In this method, DNA strand breaks are measured in terms of mean percentage of DNA in the tail (%T). Similarly gamma-H2AX assay is another sensitive technique to detect DNA damage especially double strand breaks (DSB) in single cells. One of the earliest events of cellular responses to DSB is the phosphorylation of the histone H2AX at Ser139, which is known as gamma-H2AX [16]. In recent years, gamma-H2AX foci analysis has been extensively used for natural background, occupational and medical exposures [17-21].

Several studies have demonstrated that IR induced DNA damage may lead to alterations of transcription profile of genes involved in DNA Damage Response (DDR) and DNA repair pathways in human PBMCs [19, 22-26]. Some of the DDR and DNA repair genes have the potential to be used as radiation signatures. For instance, the products of DDR genes such as *ATM, P53, GADD45A* and *CDKN1A* are recruited at the site of DNA damage in response to ionizing radiation by the phosphorylation of specific target proteins on serine or threonine residues [27-29]. In proliferating cells, activated ATM/ATR proteins phosphorylate p53, which upon activation induces GADD45A and CDKN1A, thus leading to G_0/G1 arrest. Similarly, cyclin-dependent kinase inhibitor1a (CDKN1A) inhibits cyclin-kinase activity, which is regulated at the transcriptional level by p53 [30]. IR induced *GADD45A* and p97 are involved in DNA repair, maintenance of genomic stability, cell cycle control and apoptosis [31-34]. Telomere-specific proteins such as TRF1 and TRF2 also play important roles in maintaining genome integrity [35-36]. Similarly, base excision repair pathway is known to play an important role in the processing of oxidative DNA damages [37]. Studies have shown role of APE1 in response to oxidative stress induced due to low and high LET radiation [26, 38].

Studies have shown linear dose response of micronuclei induction in human lymphocytes exposed to high doses of fission neutrons in the dose range of 250-1500 mGy [39] and 10 to 100mGy [40]. However, no study is available in

human lymphocytes that are exposed *in vitro* to very low doses of neutron as low as 3-12mGy. In the present study, attempt has been made to analyze the frequency of MN, NPB and DNA damages including DSBs in neutron-irradiated cells *in vitro*. Transcription specific of DDR (*ATM, P53, GADD45A, CDKN1A*), telomere specific (*TRF1, TRF2*) and BER (*APE1, FEN1, LIG1, LIG3, MUTYH, NEIL1, PARP1,* and *XRCC1*) genes were studied to find out molecular response, if any, in resting human PBMCs exposed to low doses of neutron irradiation *in vitro.*

MATERIALS AND METHODS

Sample collection

Approximately 12 ml of venous blood samples were collected in heparinized vials from six random healthy individuals (Age range: 25-45 years). All the samples were collected with written informed consent approved by the Medical Ethics Committee, Bhabha Atomic Research Centre, Trombay, Mumbai. All the individuals included in the study are non-smokers and without having any chronic illness.

Neutron Irradiation

Whole blood samples were exposed to various neutron doses (3.0, 6.0, 9.0 and 12.0 mGy) using a ^{241}Am-^{9}Be source at a dose rate of 3.0 mGy/h. The total activity of the source was 12 curie with a neutron flux of 28.8 x 10^6 neutron/s. The average energy of the neutrons emitted from the source was 4.3MeV. The ^{241}Am-^{9}Be source, which is in a stainless-steel capsule, yields approximately 60% of dose due to neutron and rest were from 60keV of gamma radiation. The source was standardized by a manganese sulphate bath system and the dose was evaluated from the fluence rate using conversion factors as described in ICRP (1996) [41]. The blood samples were irradiated at room temperature in 1.5 ml sterile polypropylene tubes at a distance of 10 cm to achieve a dose rate of 3.0mGy/h.

Blood samples were divided into two aliquots and irradiated at room temperature with different neutron doses (3.0, 6.0, 9.0 and 12.0 mGy). An aliquot of blood sample (4 ml) was used for the CBMN CYTOME assay. The rest of the blood sample (8 ml) was further aliquoted and used for comet assay, gamma-H2AX assay and gene expression analysis wherein the PBMCs were separated and divided into multiple aliquots of 1×10^6cells/ml each and irradiated at room temperature. PBMCs were re-suspended in RPMI-1640 medium containing 10% heat inactivated fetal calf serum. Sham-irradiated controls were kept at room temperature and processed along with the irradiated samples at each dose point.

CBMN CYTOME assay

For CBMN CYTOME assay, whole blood culture was set up using the micro-culture technique [42]. Each blood culture was set up with 0.4 ml of whole blood and 4.5 ml of RPMI-1640 medium (Sigma-Aldrich) containing 10% heat inactivated fetal calf serum (Sigma-Aldrich), 1% of L-Glutamine (Sigma-Aldrich), 100 IU/ml benzyl penicillin, 100 µg/ml streptomycin (Sigma-Aldrich). Lymphocytes were stimulated by phytohaemagglutinin (PHA) (10µg/ml, Sigma Aldrich). The cultures were incubated at 37°C, 5% CO_2 in a humidified atmosphere and after 44h of PHA stimulation, cytochalasin B (5 µg/ml, Sigma Aldrich) was added to each culture tube. After 72h, the cultures were terminated and centrifuged at 1000 rpm for 6 min. The supernatant was discarded and the pellet was treated with chilled hypotonic solution; 0.075 M potassium chloride (Qualigens, Mumbai, India) for 2 min followed by methanol and acetic acid (3:1) fixation. Fixed cell suspensions were dropped onto pre-cleaned slides and air dried. About four slides were prepared for each dose point, blindly coded and stained with 2% Giemsa (Sigma Aldrich). The cover slip was mounted carefully with DPX (Merck, India Ltd.) without any air bubble. Stained slides were analyzed using a CX-31 Olympus microscope at 400X magnification. An average of 2000 binucleated (BN) cells were scored and verified by two independent scientists. Likewise, at least two to three slides were scored randomly for each dose

point. Binucleated cells with well-preserved cytoplasm were scored for both MN and NPB.

The scoring criteria of MN and NPB were as described by Fenech (2007) [14]. In brief, the criteria for scoring MN were as follows: the size of the micronuclei (MN) should not be greater than 1/3 volume of main nuclei, staining should be uniform and the morphology of the MN should be like the main nuclei (round shape with no overlapping). Similarly, the criteria for NPB is as follows: the bridge of the NPB should be a continuous link between the nuclei of both the main nuclei with uniform staining and the size of NPB may vary from narrow to broad but the width should not exceed $1/4^{th}$ of the diameter of the main nuclei.

Isolation of PBMCs from blood

PBMCs were separated from whole blood using Histopaque® 1077 (Sigma Aldrich, St Louis, USA) solution by density gradient centrifugation at 400X g for 30 min at room temperature. The buffy layer was washed with ice-cold 1X phosphate buffer saline twice at 110 X g for 10 min and further divided into different sub-aliquots. Each sub-aliquot had a density of approximately 1×10^6cells/ml, which was measured using a trypan-blue exclusion cell viability assay. PBMCs were irradiated at room temperature with different neutron doses (3.0, 6.0, 9.0 and 12.0 mGy) with sham-irradiated controls.

Quantitation of DNA strand breaks using alkaline comet assay

Total DNA strand breaks were measured in terms of percentage of DNA in tail (%T) using the alkaline comet assay. Prior to irradiation, PBMCs (10^5 cells per dose point) were suspended in RPMI1640 medium (Hi-Media Pvt. Ltd., Mumbai) containing 10% fetal bovine serum (FBS) and 2mM L-glutamine at 37°C (with 95% humidity and 5% CO_2 concentration). Frosted slides (bio Plus microscopic slides, India) were overlaid with 1% melting agarose (Sigma Aldrich, USA), which was prepared in 1X Tris borate saline buffer. Irradiated PBMCs (1×10^5 cells/ml) were mixed with molten low melting agarose (0.5 %) at 37°C and the mixture was overlaid onto the 1% base layer and the cover slip was laid onto it. For each dose point, the un-irradiated and irradiated samples were processed after 30 min. Duplicate slides were prepared for each sample and were stored at 4°C in the dark. The slides were then immersed in pre-chilled lysis solution (2.5M NaCl, 100mM EDTA, 10mM Trizma base, 10% DMSO, and 1% Triton X-100) at 4°C for 60 min. After lysis, the slides were immersed in freshly prepared alkaline solution (300mM of NaOH and 20mM EDTA, pH>13) for 30 min at room temperature in the dark. Electrophoresis was carried out in alkaline electrophoresis solution (pH >13) containing 300 mM NaOH and 500 mM EDTA (pH 8.0) at 25V, 300 mA for 20 min. The slides were then rinsed in neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 5 min and fixed using 70% ethanol for 5 min[43,44]. The cells were stained with SYBR Green I and observations were made using fluorescent microscope (Nikon Eclipse Ti-U inverted microscope, Japan). An average of 100 cells (50 cells from each slide) was scored from two slides. Analysis was done using TriTek Comet Score FreeWare™version 1.5, where %T was calculated as follows :

Percentage of DNA in Tail = (Total Tail Intensity / Total comet Intensity) × 100.

Quantitation of DSBs in neutron irradiated PBMCs using gamma-H2AX marker

DSBs were visualized as gamma-H2AX foci using fluorescence microscopy. Cells were fixed after incubation of 30 minutes post-irradiation of each dose point. Cells were fixed with 1% formaldehyde for 15 min on ice and after fixation cells were suspended in 70% ethanol. For permeabilization, cells were briefly incubated in 1% BSA-Triton-X-100-PBS solution. PBMCs were re-suspended in 1:100 (1µg/100µl) dilution of anti-phospho-Histone H2AX (Ser 139) antibody (Millipore Cat no. 05-636, USA) and incubated at 4ºC for overnight. After overnight incubation, cells were washed with 1% BSA-T-PBS solution containing 0.05 % Tween 20 and incubated with Alexa Fluor 488 labeled rabbit anti-mouse antibody (Molecular probes A-11059, Invitrogen, USA). Labeled PBMCs were counter-stained with propidium iodide (5µg/ml)

and coated onto Poly-L-Lysine coated cover-slips (BD BioCoat™ 354085, BD Biosciences, USA). The cover-slips were mounted on glass slides and visualized in a fluorescence microscope (Carl Zeiss, Model - LSM 510 Meta, Germany) at 100x magnification for gamma-H2AX intensity analysis. Two slides were prepared for each sample and 20-25 random images were captured from both the slides. About 100 cells were scored from each slide to analyse the intensity per nucleus. The analysis was carried out by using Image J software. Scoring was done blindly without knowing the sample information.

Transcription profile of DDR, telomere specific and BER genes

RNA isolation* and *cDNA synthesis:

For gene expression analysis, PBMCs were separated from blood samples, irradiated and total RNA was isolated at 30 minutes and 4h post-irradiation using Hipura RNA isolation Kit (Himedia Laboratory Pvt. Ltd., India). Total RNA was quantified using Picodrop Microlitre spectrophotometry and the purity was checked by taking 260/280 nm ratio. RNA integrity was checked on 0.8% agarose gel stained with ethidium bromide. The bands of 28S and 18S were clearly observed. For each sample, total RNA (500ng) was reverse transcribed to cDNA using transcriptor high fidelity cDNA synthesis kit (Roche Diagnostics, GmbH, Germany).

Real Time quantitative PCR (RT-qPCR)

RT-qPCR was performed to quantitate the mRNA expression of DNA damage response (*ATM, P53, GADD45A, CDKN1A*), telomere specific (*TRF1* and *TRF2*) and BER genes (*APE1, FEN1, LIG1, LIG3, MUTYH, PARP1, NEIL1*and *XRCC1)* using a SYBR green based assay in LC480 (Roche Diagnostics, GmbH, Germany). All the reactions were carried out in duplicates and were normalized with ACTB (b-actin). Primer sequences used in the study are given in Table 1 and were purchased from Sigma Aldrich, USA. Each RT-qPCR reaction was performed in a total volume of 12.5 µl PCR master mixture containing 1X reaction buffer, 0.25 mM of each dNTP, 0.5U of Fast Start Taq DNA polymerase, 5.0 pmols each of both forward and reverse primers for all the genes. All the PCR components were purchased from Roche Diagnostics, Gmbh, Germany. A total of 45 cycles of PCR reactions was carried out for all the genes. The PCR temperature conditions were as follows: a pre-incubation step at 95^0C for 5 min followed by denaturation at 95^0C for 10 sec, annealing at 59^0C for 45 sec and extension at 72^0C for 15 sec. Melting-curve analysis was done to confirm the amplified product. The reaction was conducted in three steps: melting at 95°C for 5 min followed by an annealing step at 58°C for 1 min and an extension at 72°C followed by a final step at 40°C for 10 sec. The quantification was performed by using the LC480 software version 1.1 and the results are expressed in normalized ratio as described by Pfaffle (2001) [45]. The calculations are as follows: Normalized ratio = (Concentration of Target/ Concentration of Reference)$_{sample}$**:** (Concentration of Target/ Concentration of Reference) $_{Calibrator}$. The relative expression with respect to control was calculated.

Statistical analysis

Statistical analysis was performed using the SPSS software (17.0.1 version, 2008, IBM Corporation, USA). The level of significance was set at $p \leq 0.05$ for all statistical analysis. Frequency of MN and NPB were calculated per 1000 BN cells. Paired "t" test was performed to find out the significance of MN, NPB, gene expression pattern and gamma-H2AX intensity between control and irradiated samples. Coefficient of variation (CV), which represents the variability in relation to the mean was calculated by taking the ratio of standard deviation with the mean (CV=standard deviation/mean). The CV was calculated for MN, NPB and DNA stand breaks.

Table 1. Primer sequence of genes studied.

Sr. No.	Gene Name	Primer sequence	Base Pair (bp)
1	β – actin PR1	5'-CCAGAGGCGTACAGGGATAG-3'	20
	β – actin PR2	5'-CCAACCGCGAGAAGATGA-3'	18
2	ATM PR1	5'-GGGAACATAAAATTCAGACAAACA-3'	24
	ATM PR2	5'-CACGCAGGGCTAATTCATC-3'	19
3	CDKN1A PR1	5'-CGAAGTCAGTTCCTTGTGGAG-3'	21
	CDKN1A PR2	5'-CATGGGTTCTGACGGACAT-3'	19
4	p53 PR1	5'-CTTTCCACGACGGTGACA-3'	18
	p53 PR2	5'-TCCTCCATGGCAGTGACC-3'	18
5	GADD45A PR1	5'-GAGAGCAGAAGACCGAAAGG-3'	20
	GADD45A PR2	5'-TGACTCAGGGCTTTGCTGA-3'	19
6	TRF1 PR1	5'-TGCTAAGTGAAAAATCATCAACCTT-3'	25
	TRF1 PR2	5'-TTGTTCTTGTCCTTTTGCTTTCT-3'	23
7	TRF2 PR1	5'-CCCACCGTTCTCAACCAA-3'	18
	TRF2 PR2	5'-GTTCCACTTGCCTTTGGGTA-3'	20
8	APEX1 PR1	5’-CGAGCCTGGATTAAGAAGAAAG-3’	22
	APEX1 PR2	5’-TTTGGTCTCTTGAAGGCACA-3’	20
9	PARP1 PR1	5’-CCAGGATTCCGCATGACT-3’	18
	PARP1 PR2	5’-AGTTCCTTCTGTGTCCGGATT-3’	21
10	NEIL1 PR1	5’-GCAGTGGGAAGTCAGGTTCT-3’	20
	NEIL1 PR2	5’-GGCCTCATTCACAAACTGG-3’	19
11	XRCC1 PR1	5'-CTGGGACCGGGTCAAAAT-3'	18
	XRCC1 PR2	5'-CAAGCCAAAGGGGGAGTC-3'	18
12	LIGASE3 PR1	5'-GATCACGTGCCACCTACCTTGT-3'	22
	LIGASE3 PR2	5'-GGCATAGTCCACACAGAACCGT-3'	22
13	MUTYH PR1	5’-ATGACACCGCTCGTCTCC-3’	18
	MUTYH PR2	5’-GCTTCTGCCTCCCTTCCT-3’	18
14	FEN1 PR1	5’-CTGTGGACCTCATCCAGAAGCA-3'	22
	FEN1 PR2	5'-CCAGCACCTCAGGTTCCAAGA-3'	21
15	LIGASE1 PR1	5'-GAATTCTGACGCCAACATGCA-3'	21
	LIGASE1 PR2	5'-CCGTCTCTGCTGCTATTGGA-3'	20

RESULTS

In the present study, analysis of MN and NPB was done using the CBMN assay. DNA damage was quantified using single cell gel electrophoresis (comet assay) and gamma H2AX assay. In addition, the transcriptional profile in PBMCs was analysed for 14 genes at various neutron doses between 3.0 to 12 mGy.

CBMN CYTOME assay

Blood samples of six individuals were irradiated with neutron doses between 3.0 to 12.0 mGy along with un-irradiated control for each donor. A whole blood culture was set up for CBMN assay. The frequencies of MN and NPB at various doses of neutron exposure are shown in table 2. The frequency of MN was observed to be 7.09 ± 3.53, 11.64 ± 5.04, 12.27 ± 6.65, 16.54 ± 9.04 and 19.68 ± 10.12 per 1000 BN cells in control (un-irradiated), 3.0, 6.0, 9.0 and 12.0 mGy, respectively. The frequency of MN at 9.0 mGy and 12.0 mGy was significantly higher ($p<0.05$) as compared to control. The frequency of NPB was observed to be 0.61 ± 0.48, 2.07 ± 1.37, 2.58 ± 1.68, 3.20 ± 1.99 and 3.78 ± 2.36 per 1000 BN cells in control (un-irradiated), 3.0, 6.0, 9.0 and 12.0 mGy, respectively. The frequency of NPB were significantly ($p<0.05$) increased at 6.0, 9.0 and 12.0 mGy. Inter-individual variation was

clearly observed. The coefficient of variation (CV) among the individuals with respect to MN ranged from 43.3 % to 54.7 %. Similarly the CV value varied from 62.2% to 78.7 % for NPB. The distribution of percentage of BN cell with MN and NPB is given in table 2. The percentage of BN cells with MN was significantly higher ($P<0.05$) at 9.0 and 12.0 mGy. However, only 0.06% to 0.4% of BN cells with NPB were observed across the doses studied.

Table 2. Frequency distribution of micronuclei and nucleoplasmic bridges per 1000 binucleated cells in neutron-irradiated lymphocytes.

Dose (mGy)	No. of BN cells analysed (N=6)	Freq of MN/1000 BN cells ± SD	No. of BN cells with MN			%of BN cells with MN	CV (%)	Freq of NPB /1000 BN cells ± SD	CV (%)	No. BN cells with NPB			% of BN cells with NPB
			0	1	≥ 2					0	1	2	
Control	11432	7.09 ± 3.53 (81)	11367	59	6	0.57	49.8	0.61 ± 0.48 (7)	78.7	11425	7	0	0.06
3.0	11596	11.64 ± 5.04 (135)	11474	109	13	1.05	43.3	2.07 ± 1.37* (24)	66.2	11572	24	0	0.21
6.0	11244	12.27 ± 6.65 (138)	11129	103	12	1.02	54.2	2.58 ± 1.68* (29)	65.1	11215	29	0	0.26
9.0	10635	16.55 ± 9.04* (176)	10484	129	22	1.42*	54.7	3.20 ± 1.99* (34)	62.2	10591	34	0	0.32
12.0	10311	19.69 ± 10.12* (203)	10139	144	28	1.67*	51.4	3.78 ± 2.36* (39)	62.4	10272	39	0	0.38

C.V: co-efficient of variation, SD: standard Deviation, BN: binucleated, MN: micronuclei, NPB: Nucleoplasmic bridge, N=Number of individuals

Measurement of DNA strand breaks using alkaline Comet assay:

Mean percentage of DNA in tail (%T) in neutron irradiated PBMCs was calculated for all the individuals along with control. A significant dose dependent increase in %T was observed. A representative image showing the dose response of DNA in tail (%T) in resting human PBMCs exposed to neutron radiation at different doses between 3.0 to 12.0 mGy is shown in figure 1a. The mean percentage of DNA damage in tail was observed to be 2.10 ± 0.62 in control (un-irradiated) samples and 3.89 ± 0.92, 6.35 ± 0.59, 7.91 ± 0.49 and 9.52 ± 0.42 in 3.0, 6.0, 9.0 and 12.0 mGy, respectively (table 3 and figure 1b). The coefficient of variation was found to be maximally in the range between 29.7% (as shown in table 3).

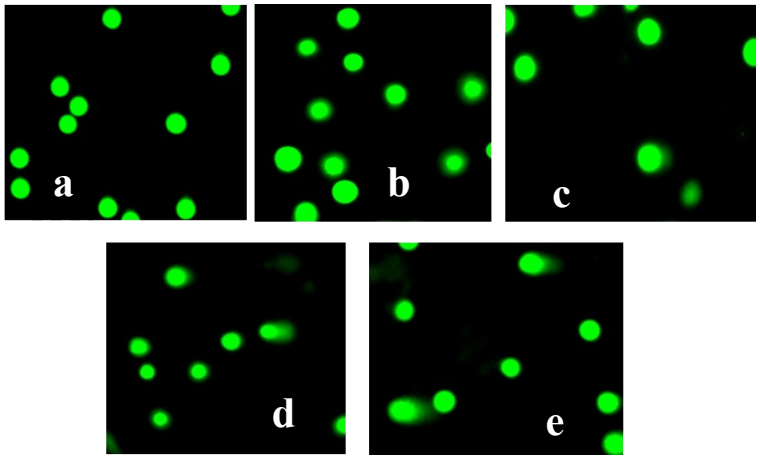

Figure 1. Representative image showing percentage DNA in tail (%T) in resting human PBMCs exposed to various doses of neutron irradiation using alkaline comet assay. **(a)** unirradiated, **(b)** 3.0 mGy, **(c)** 6.0 mGy, **(d)** 9.0 mGy, **(e)** 12.0 mGy.

Table 3. The average percentage of DNA in tail (%T), and the coefficient of variation (CV) in human lymphocytes exposed to various doses of neutron irradiation.

Dose (mGy)	Mean percentage of DNA in tail (%T) ± SD	Coefficient of variation (%)
Control	2.10 ± 0.62	29.52
3.0	3.89 ± 0.92	23.65
6.0	6.35 ± 0.59	9.29
9.0	7.91 ± 0.49	6.19
12.0	9.52 ± 0.42	4.41

% T=Mean percentage of DNA in tail, CV= Co-efficient of variation, CV=SD/ average mean %T *100

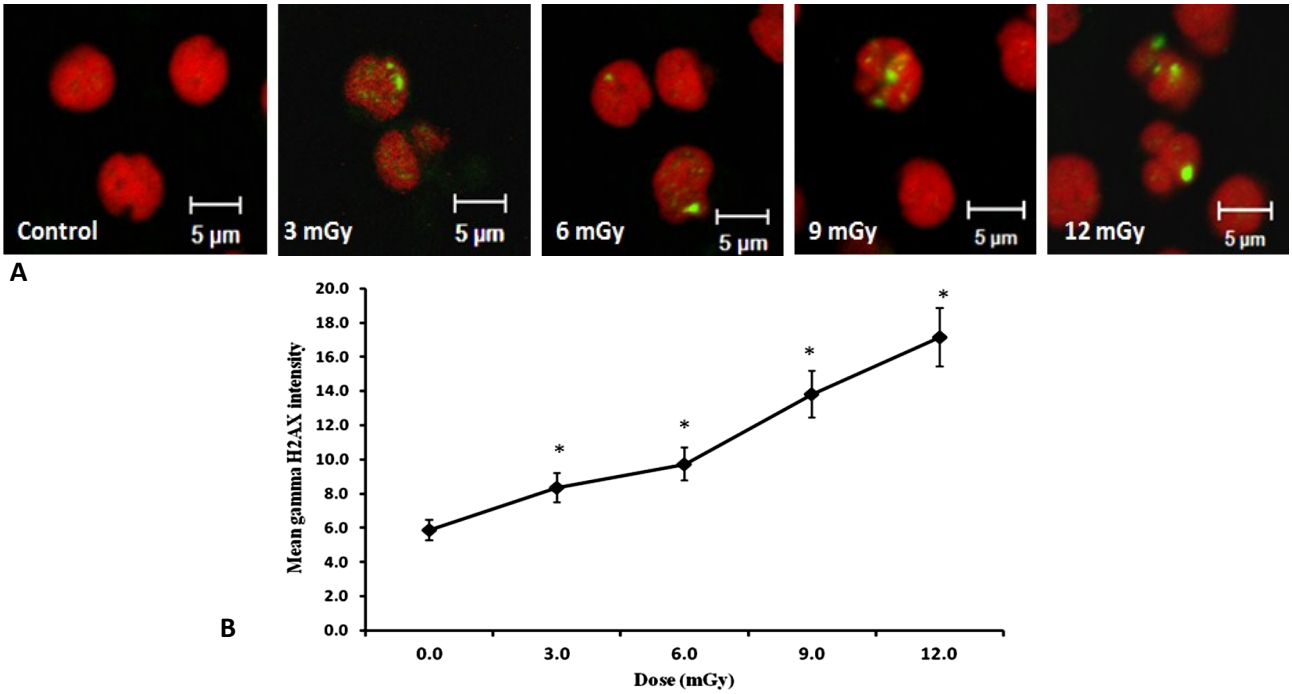

Figure 2. Gamma H2AX foci intensity per nucleus in human PBMCs exposed to various doses of neutron irradiation (3.0, 6.0, 9.0 and 12 mGy) along with sham- irradiated control. **(A)** Representative image showing gamma-H2AX foci intensity per nucleus in human PBMCs exposed to neutron **(B)** Mean intensity of gamma-H2AX per nucleus in human PBMCs exposed to various doses of neutron irradiation (3.0, 6.0, 9.0 and 12 mGy) along with sham-irradiated control. Indicated are means and SE from 6 donors . (*) indicates significant ($p \leq 0.05$) as compared to control.

Transcription profile of DDR, telomere specific and BER genes in neutron irradiated PBMC:

The transcriptional profile of DDR, telomere specific and BER pathway genes was studied in PBMCs at different neutron doses (3.0, 6.0, 9.0 and 12.0 mGy) along with un-irradiated control at 30 min and 4h of post irradiation. The relative expression of all the genes was analysed with respect to *b-actin*. The relative expression of the target genes was normalized against the expression values of the un-irradiated controls and the fold-change values obtained at 30 min and 4h post irradiation across all the doses (figures 3 and 4).

As shown in figure 3, both *CDKN7A* and *GADD45A* have shown increased expression at 30 min across the doses as compared to 4h post irradiation. At 30 mins, *CDKN1A* showed an increased mRNA expression upto 12 mGy as compared to control but at 4h post-irradiation, it showed a dose dependent increase. At 30 mins post irradiation, *GADD45A* showed dose-dependent up-regulation up to 12 mGy. No change in expression profile was observed for

ATM, P53, TRF1 and *TRF2* at both the time points studied (30 min and 4h). As shown in figure 4, *PARP1* showed dose-dependent increase in expression at 4h post irradiation as compared to 30 min across all the doses. In contrast, *NEIL1, MUTYH, APE1, XRCC1, LIGASE3, FEN1* and *LIGASE1* showed similar expression patterns across the doses at 30 min and 4h.

In summary, *CDKN7A* and *GADD45A* showed dose dependent up-regulation though not significant at 30 min as compared to 4h post irradiation across all the doses up to 12mGy, whereas PARP1 showed a dose dependent increase in expression at 4h post irradiation as compared to 30 min.

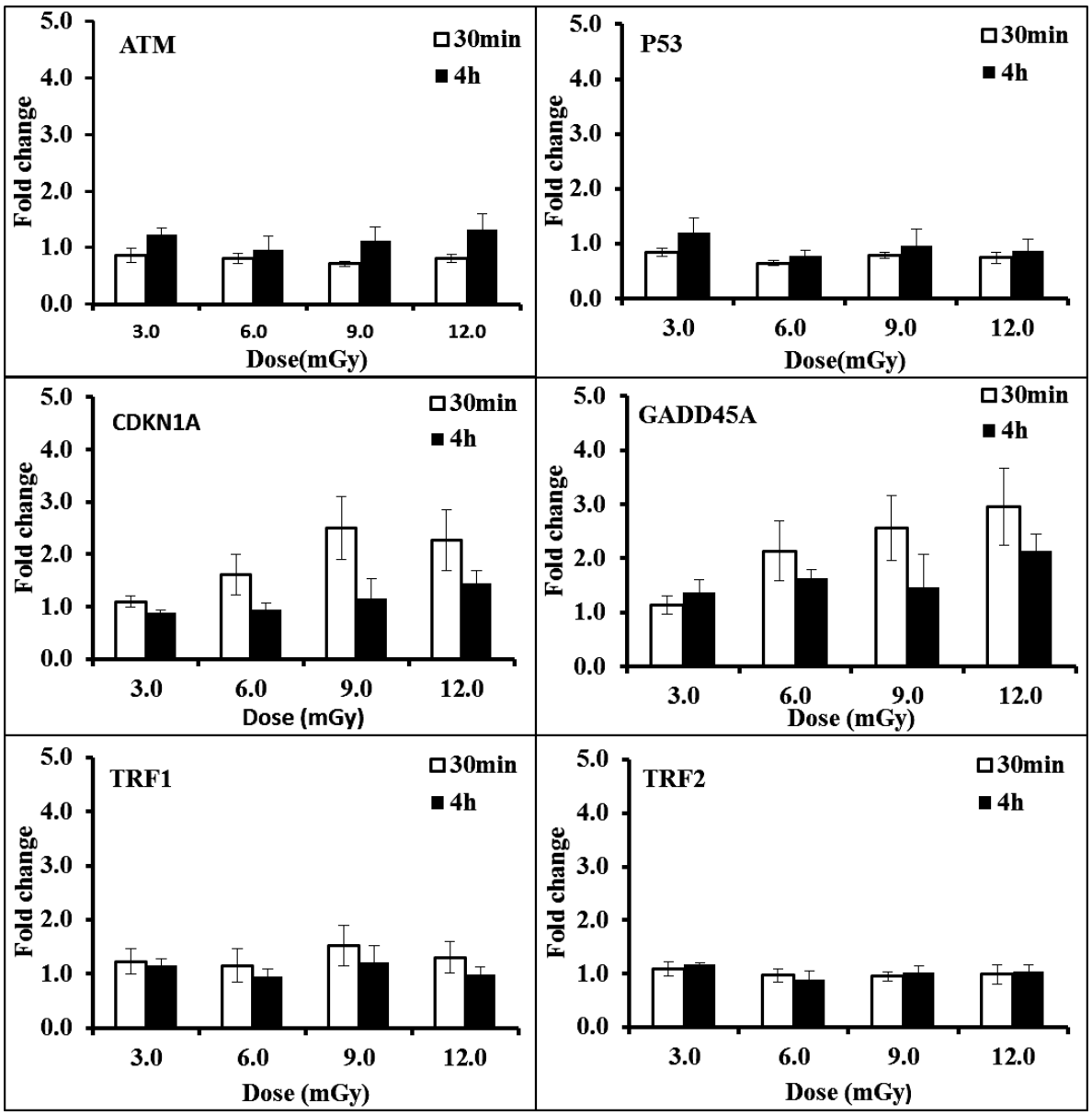

Figure 3. Gene expression profile of ATM, P53, CDKN1A, GADD45A, TRF1 and TRF2 in human PBMCs exposed to various doses of neutron irradiation (3.0, 6.0, 9.0 and 12.0 mGy) at 30 min and at 4h along with sham-irradiated control in six individuals. Data were normalized with respect to control. Indicated are means and SE from 6 donors . (*) indicates significant ($p \leq 0.05$) as compared to control.

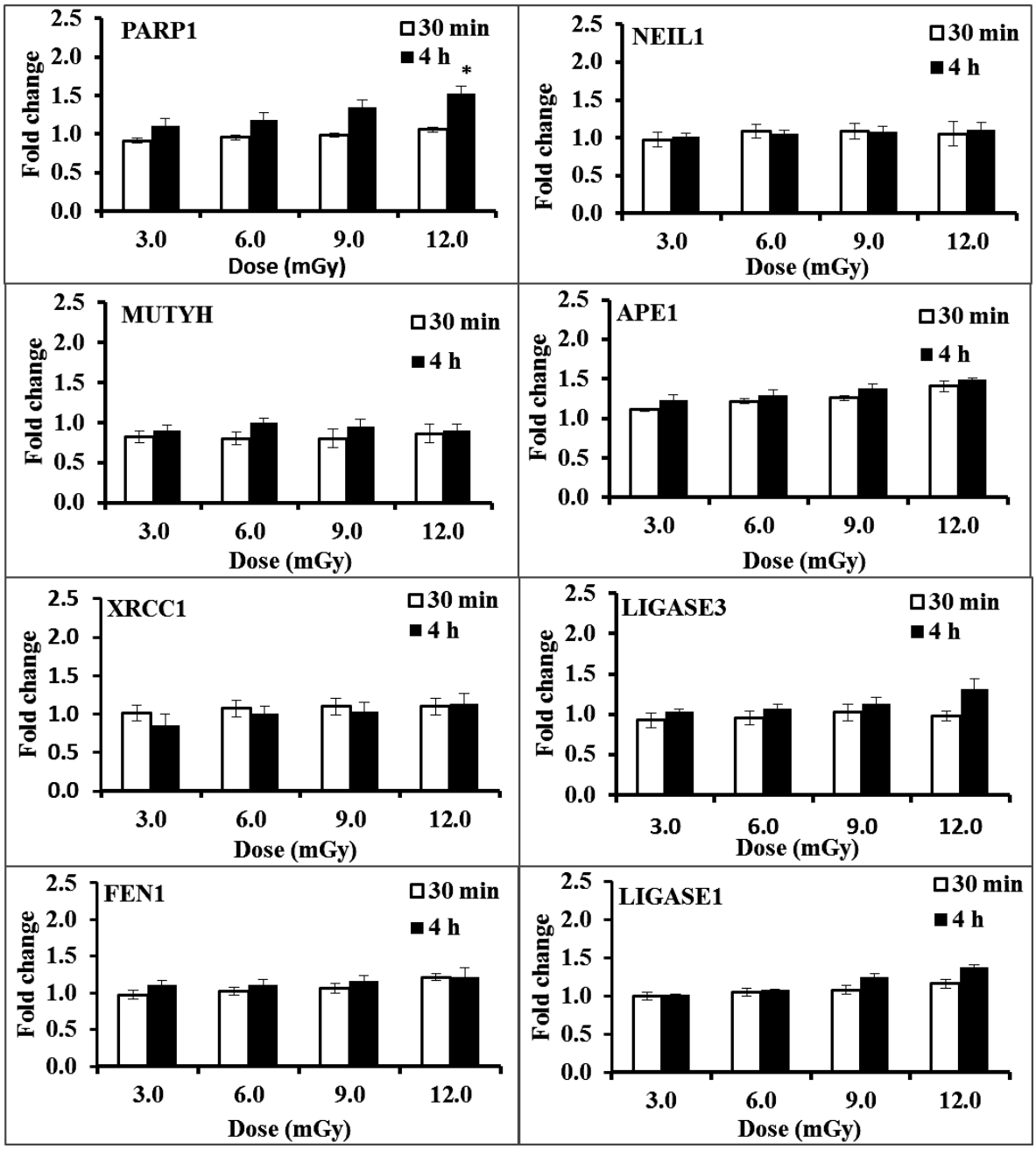

Figure 4. Gene expression profile of PARP1, NEIL1, MUTYH, APE1, XRCC1, LIG3, FEN1 and LIG1 in human PBMCs to various doses of neutron irradiation (3.0, 6.0, 9.0 and 12.0 mGy) at 30 min and at 4h along with sham-irradiated control. Data were normalized with respect to control. Indicated are means and SE from 6 donors. (*) indicates significant ($p \leq 0.05$) as compared to control.

DISCUSSION

In the present study, *in vitro* effect of low dose neutron irradiation was assessed in resting human PBMCs using different biological endpoints. Several studies have been carried out to study biological effects of neutrons in human cells along with other types of radiation exposures. Data from Hiroshima and Nagasaki atomic bomb studies have shown biological effect of significant neutron dose to exposed population [46]. Therapeutic use of neutron is also very effective in treating human tumor / cancerous tissue along with proton [47].

Human PBMCs are in the resting stage (G_0/G_1) of the cell cycle and are highly sensitive to IR exposure. Dicentrics, NPB and MN are reliable end-points to study radiation induced DNA damage in human cells and provide reliable dose estimates after acute whole-body radiation exposures in peripheral blood lymphocytes [48].

Low doses of fast neutrons have shown to induce increasing frequency of micronuclei in polychromatic erythrocytes and lymphocytes [49,50]. Dose-dependent increase in the frequency of micronuclei has also been reported in V79 cell lines [51,52]. Studies have shown linear dose response of micronuclei induction in human lymphocytes exposed to high doses of fission neutrons in the dose range of 250-1500 mGy [39] and 10 to 100mGy [40]. In the present study, dose dependent increase in the frequency of MN and NPB was also observed which is similar to other studies. However, this is the only *in vitro* study in human lymphocytes exposed to very low doses of neutron as low as 3-12mGy. The distribution of MN and NPB analysis showed that a very small percentage of cells have more than two MN per BN cell (binucleated) and reason is not clear yet.

Alkaline comet assay is a sensitive method to measure radiation induced DNA damage in terms of percentage of DNA in the tail (%T) in human lymphocytes. Several studies have shown dose dependent increase in DNA damage using comet assay [26,54]. In the present study, we have also observed similar results. Approximately 10% of DNA in the tail was observed at 12mGy of neutron irradiation.

Quantitation of Gamma-H2AX foci intensity per nucleus is used as a biomarker in population monitoring and biological dosimetry studies for both whole body and partial body exposure [54]. There are studies which have demonstrated neutron-induced clusters of DSBs in cell lines having a low capacity to repair DSB after irradiation with a neutron mixed beam [55-57]. In the present study, mean fluorescence intensity of the gamma-H2AX per nucleus revealed a significant increase with respect to increasing neutron dose. The densely-stained foci observed may be due to complex/clustered DNA damage in neutron irradiated cells. The findings in the present study are similar to the findings reported by Vandersickel *et al.,* 2014 [58]. Studies carried out by Zhang et al., have shown that Gamma-H2AX foci intensity was induced in response to long term exposure to low doses of neutron combined with gamma-radiation [59].

IR induced lesions may lead to alteration in the transcriptional profile of DDR and DNA repair genes. Several studies have shown alteration of gene and protein expression in response to IR [24-26, 60-62]. There are few studies, where gene expression analysis has been carried out in neutron-irradiated cells. For instance, it was reported that Rp-8 (a gene associated with apoptosis, PDCD2) and the long terminal repeat (LTR) of the human immunodeficiency virus (HIV-LTR) gene show a 2-3 fold increase in the expression pattern in Syrian hamster embryo (SHE) cells exposed to low dose of neutron, while SHE cells exposed to low doses of fission-spectrum neutrons showed no change in the expression of genes encoding c-jun, Rb, H4histone, p53, and *cmyc* [63, 64].

DNA damage response genes such as ATM, *p53, PARP1, CDKN1A, GADD45A* play an important role in activating repair mechanisms [63, 65]. *GADD45A* expression profile is used as an early radiation signature for gamma-radiation in resting lymphocytes [66]. *TRF1* and *TRF2* are telomere specific genes and responsible for maintaining genome integrity. Base Excision Repair (BER) plays an important role in repairing spontaneous reactive oxygen species [67]. In the present study, alteration of early expression of *GADD45A* and *CDKN1A* was observed at 30 min across the doses whereas PARP1 showed increased expression at 4h. These are indicative of DDR response in G_0/G_1 human lymphocytes exposed to low doses of neutron. However, further *in vitro* and *in vivo* studies are required to find out low-dose neutron radiation signatures at transcript and protein level, if any. It is also important to understand the repair kinetics and its interaction with DDR and DNA repair genes/ proteins in maintaining genomic stability. The impact of low dose neutron irradiation on genome damage is highly relevant for human health. Molecular and/or cellular studies in

human cells/tissues with multiple end points in a larger scale might throw new insights to low dose radiation biology especially neutron irradiation.

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Contribution by each author

D C Soren (DCS), Sneh M Toprani (SMT): Vinay Jain (VJ): Divyalakshmi Saini (DS), Birajalaxmi Das (BD): DCS, CBMN CYTOME assay, SMT: comet assay and transcription profiling of BER genes, VJ: gamma-H2AX foci, DS: transcription profiling of DDR genes, BD: Conceived the Project, designed the experiments, analysed the data, writing and reviewing the manuscript.

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