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Studies on electron beam induced DNA damage and repair kinetics in lymphocytes by alkaline comet assay

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ABSTRACT

***Background*:** Exposure to ionizing radiation is known to induce oxidative stress followed by damage to critical biomolecules like lipids, proteins and DNA through radiolysis of cellular water. Since radiation has been widely used as an important tool in therapy of cancer, the detailed investigation regarding the DNA damage and repair kinetics would help to predict the radiation sensitivity of cells. The present study is focused on quantification of DNA damage and repair kinetics of human peripheral blood lymphocytes after 8 MeV pulsed electron beam irradiation *in vitro*. ***Materials and Methods*:** DNA damage and repair kinetics in human blood cells were studied using alkaline comet assay. The dose-response curves for the dose range of 0-5 Gy were established using 8 MeV electron beam. Repair kinetics was studied by incubating the cells from 0 to 90 min at 37°C after irradiation. For quantification of DNA damage, percentage Tail DNA, Tail length, Tail moment and Olive Tail moment were used as a comet parameter. ***Results and Conclusion*:** The study reveals that, induction of DNA damage by 8 MeV pulsed electron beam is near linear with a negligible negative quadratic component at high doses. This small quadratic component is attributed to high dose rate during the pulsed irradiation. The DNA repair halftime and mean repair time for human blood lymphocytes were found to be varying between 9.29 - 23.78 min and 13.41 - 34.31 min respectively. The repair rate is found to be maximum in initial 15 minutes and almost constant after 60 min.

Keywords: *DNA damage, DNA repair kinetics, alkaline comet assay, Microtron accelerator, pulsed electron beam.*

INTRODUCTION

Exposure to ionizing radiation is known to induce oxidative stress followed by damage to critical biomolecules like lipids, proteins and DNA through radiolysis of cellular water [(1)]. It is well known radiobiologically that DNA of cells is the primary critical target for the origin of radiation effects in biological systems [(2)]. In sparsely ionizing radiation like γ-rays, X-rays and electron beams, the energy is carried through tissues by secondary electrons and hence serves as useful tool for the investigation of radiation damage mechanisms. Physico-chemical interaction of radiation with cellular DNA produces a variety of primary lesions, such as single strand breaks (SSBs), double- strand breaks (DSBs), Crosslinks and Base damage. The SSBs, Base damage and Basic site damage are generally repaired by excision repair, whereas DSBs are the principal lesion [(3)] which if un-repaired or mis-repaired may lead to a gene mutation or chromosomal aberrations [(4)] and potentially leading to mitotic or clonogenic cell death, apoptosis and cancer [(5-7)].

Radiation has been widely used as an

important tool in therapy of cancer. It has also been extensively used in medical diagnosis of many other medical complications and treatments. Therefore detailed investigation regarding the DNA damage and repair kinetics would help to predict the radiation sensitivity of cells. At present, the use of electron beam is constantly gaining importance in therapeutic applications. It offers many advantages over conventional ^{60}Co teletherapy, such as better dose profile and drastic reduction in dose to the normal tissues beyond the tumor. The most common clinical uses of electron beams include the treatment of skin lesions, such as basal cell carcinomas and boosting of areas that have previously received photon irradiation. Electron beam with 12-MeV energy will deliver 80% of the dose at a depth of 4 cm, hence studies in the energy range of about 5-20 MeV have relevance in radiation therapy application.

The comet assay is a simple and sensitive cytogenetic tool, which has been extensively used to assess DNA damage and DNA repair quantitatively and qualitatively in single cells [8]. Cell to cell heterogeneity of this assay has given and edge of advantage over assays such as gel electrophoresis techniques, which are based on gross damage. The alkaline version of this assay is being widely used in various research areas, including biomonitoring, routine genotoxicity assessment and studies of DNA repair processes [9-13]. Ostling and Johanson (1984) [3] are the first to quantify DNA damage in cells using a microgel electrophoresis technique for the detection of double-stranded DNA breaks. Later Singh *et al.* (1988) [14] adapted the protocol to detect DSBs, SSBs and alkali-labile sites, which are expressed as frank strand breaks in DNA under alkaline conditions. Since its initial development, the assay has been tweaked at various steps (lysis and electrophoretic conditions) to make it suitable for assessing different types of DNA damage in different cell types [9]. Usually a comet consists of a head; this being the residual nucleus, represents the undamaged DNA, a halo and a tail, formed by the damaged DNA. For the quantification of DNA damage, generally %Tail DNA (% TD), Tail Length (TL), Tail Moment (TM), and Olive Tail Moment (OTM) are used as comet parameters. The % TD gives amount of DNA migrated towards comet tail, which can be obtained using (100 - %Head DNA) [15]. The TL is the distance of DNA migration from the head DNA, TM is the product of the tail length and the fraction of total DNA in the tail. The OTM can be calculated by taking product, between mean difference DNA content of tail DNA and head DNA to the fraction of total DNA in the tail i.e OTM = [(Tail DNA mean – Head DNA mean) X (% Tail DNA/100)] [15].

The present study is focused on quantification of DNA damage and repair kinetics of human peripheral blood lymphocytes after 8 MeV pulsed electron beam irradiation *in vitro*. The alkaline comet assay method was used to assess 8 MeV Pulsed electron beam induced DNA damage and repair kinetics. Dose-response curves were established and repair kinetics was studied by incubating cells from 0 to 90 minutes after irradiation. For quantification of DNA damage, % TD, TL, TM and OTM were used using Comet Assay Software Project (CASP).

MATERIALA AND METHODS

Blood samples

Peripheral blood samples were collected by venipuncture in heparinized vials from a 25 year healthy male donor having no pre-history of radiation. Immediately after the collection of blood, the vials were gently agitated to mix the blood with heparin to avoid coagulation. Blood samples were exposed to 8 MeV pulsed electron beam.

Dosimetry

The Variable Energy Microtron with 14 electron acceleration orbits and corresponding electron energy of 8 MeV was calibrated using chemical dosimeter. The accelerator is indigenously developed at Raja Ramanna Centre for Advanced Technology, India and offers electron beam with excellent beam parameters suitable for radiobiological research applications. The machine operates in pulsed

mode with maximum pulse duration of 2.5 µs and pulse current of 50 mA [16]. A radiation field size of 4 × 4 cm is made available for irradiation with uniform dose distribution at 30 cm from the titanium window. Integrated electron count measured by current integrator (CI) was used as signal for quantification. Fricke chemical dosimeter was used to calibrate and optimize the absorbed dose as described elsewhere [17]. Briefly, Fricke dosimetric solution was prepared using 0.8 N sulphuric acid (H_2SO_4) and 1 mM ferrous ammonium sulphate [$FeSO_4(NH_4)_2(6H_2O)$]. Optical absorbance measurements of the dosimeter were done at 304 nm wavelength using a UV-Visible spectrophotometer (SHIMADZU-1800). A pre-cleaned quartz cuvette of 1 cm optical path length was used for spectrophotometric measurement of the Fricke solution. After setting the spectrophotometer to zero absorbance with unirradiated Fricke solution, optical density of the irradiated dosimetric solution was measured starting first from low dose to high doses. The calibration curve for absorbed dose against integrated electron fluency in units of CI counts is shown in figure 1. It is clear from the curve that, there is a very good linearity (R^2 =0. 999) between absorbed dose and CI counts. The dose rate of the electron irradiator was determined by the same method and was found to be 100 Gy min^{-1}.

Irradiations

Samples were exposed to 8 MeV pulsed electron beam using Microtron accelerator at Mangalore University. The absorbed dose, dose rate was measured using the Fricke dosimeter and the dose rate at the time of irradiation was 100 Gy min^{-1}. The blood samples were aliquoted to 1 ml each in eppendorf tube and kept at room temperature. For dose response studies, these vials were irradiated for different doses from 0 to 5 Gy and for repair kinetics study, different vials were irradiated for a fixed dose of 2 Gy. After irradiation cells were cultured using complete medium (RPMI with 10% FCS) in the ratio 1: 9 and this cultured cells were taken directly for dose response studies. The repair kinetics studies were conducted by incubating the irradiated cells after mixing with the complete medium and 0 to 90 minutes was selected as an incubation time.

Alkaline comet assay

In the present study, the Alkaline comet assay methods outlined by Singh *et al.* (1988) [14] were used. The irradiated whole blood cells were mixed 1:1 ratio with 0.75 % low-melting point agarose (LMPA) in PBS (pH 7.4, Sigma) at 37 °C and 200 µl aliquots of this suspension was pipette and layered on frosted slides pre-coated with 1.5% Normal Melting Point Agarose in PBS (pH 7.4, Sigma). Cover slips were immediately placed and the slides were kept at 4 °C for 5 min. After gel formation, coverslip was removed and a layer of 200µl of LMPA (0.75 % in PBS) was added to the slides and allowed to solidify. The cells were lysed overnight at 4 °C in a chilled lysis buffer (2.5 M NaCL, 100 mM di-sodium EDTA, 10 mM Tris base, 1% Triton X-100 and 10 % DMSO, pH 10). After lysis, slides were washed

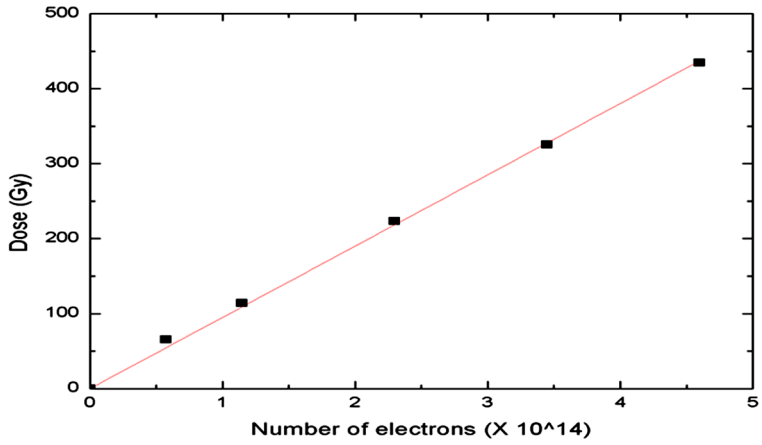

Figure 1. Dose calibration curve for Microtron Accelerator.

with alkaline electrophoresis buffer (300 mM NaOH, 1mM Na_2-EDTA in de-ionized water, pH>13) and placed on a horizontal electrophoresis tank filled with freshly prepared chilled alkaline electrophoresis buffer. The slides were placed in the same buffer for 30 min for alkaline unwinding, and electrophoresis was carried out at 0.74 V cm^{-1}, 300 mA for 25 min. After electrophoresis, the slides were washed gently with neutralization buffer (0.4 M Tris base, pH 7.2) to remove alkali. Then samples were stained with ethidium bromide and visualized at 40X magnification using an Olympus BX 51 fluorescent microscope. To capture comet images, Jenoptik C5 cooled CCD camera with ProgRes Capture Pro 2.8 software was attached to the microscope was used. For quantification of DNA damage, the quantitative measurement of % TD, TL, TM and OTM was made using CASP.

Mathematical formulations and statistics

The dose response of DNA damage, has been fitted by an error-weighted minimum Chi^2 method to a linear quadratic model, represented by Comet parameter $_{(\%\,TD,\,TL,\,TM\,and\,OTM)}$ = C + αD + βD^2. Where D represents the radiation dose in Gy, C is the background DNA damage and α, β are linear and quadratic coefficients respectively. For the repair kinetics study, the nature of the graph follows fist order exponential decay curve, i.e. $Y \times A½B\ e^{(-x/t)}$. Where *A* is offset value, *B* is the amplitude and *t* is the decay constant [18]. Hence, the experimental data's were fit to fist order exponential decay curve to estimate mean repair time *(T)* and repair half time $(t_{1/2})$. The $t_{1/2}$ is time required to repair half of the initial DNA damage and is calculated as $t_{1/2} = ln\,2/\alpha$, where $\alpha = 1/t$. The theoretical repair time *(T)* is the time required to completely repair induced DNA damage theoretically and is directly calculated from decay constant as $T \times 7/t$. The repair rate (R) was calculated using the in-house made formula:

$$R = \frac{(D_{t_1} - D_{t_2})}{(t_2 - t_1)}$$

Where D_{t1} is the damage at t_1 incubation time and D_{t2} is the damage at t_2 incubation time.

Each experiment was repeated at least three times at similar condition and %TD, TL, TM and OTM values were used to assess the level of DNA damage. The mean of the samples compared using Student's *t*-test. Differences where $P<0.05$ considered to be statistically significant [19].

RESULTS AND DISCUSSION

Experimental results were analyzed to quantify DNA damage and to study repair kinetics using alkaline comet assay method when human peripheral blood lymphocytes were irradiated to 8 MeV pulsed electron beam from Microtron Accelerator. To assess DNA damage, the comet parameters like %TD, TL, TM and OTM values were estimated, using CASP (figures 2-5). In the figures 2-5, the presented values were normalized to control value to avoid initial variables in different experiments and data were fitted to linear quadratic equation as explained in materials and methods section. A significant increase in DNA strand breaks as indicated by an increase in all comet parameter values for all doses were observed (figures 2-5). The results have been summarized in table 1. The dose response relation can be written as % TD = 1 + (4.88 ± 0.42) D – (0.33 ± 0.09) D^2, for TL = 1 + (35.79 ± 1.86) D – (3.49 ± 0.50) D^2, for TM = 1 + (3.66 ± 0.38) D – (0.09 ± 0.11) D^2, and for OTM = 1 + (3.97 ± 0.24) D – (0.26 ± 0.077) D^2.

The α-coefficient from the above dose response relation is, for %TD (4.88 ± 0.42 Gy^{-1} > - 0.33 ± 0.09 Gy^{-2}), TL (35.79 ± 1.86 Gy^{-1} > - 3.49 ± 0.50 Gy^{-2}), TM (3.66 ± 0.38 Gy^{-1} > - 0.09 ± 0.11 Gy^{-2}), and OTM (3.97 ± 0.24 Gy^{-1} > -0.26 ± 0.077 Gy^{-2}), it clearly shows that, the response is linear with a small quadratic component. The small negative quadratic (β) component indicates saturation of induction of DNA damage and lack of DNA damage repair process. This quadratic component may be attributed to the high dose rate of irradiation (100 Gy min^{-1}).

Experimental DNA repair half time and mean repair times were estimated. Incubation after irradiation allowed the repair of induced DNA damage. The rejoining of strand breaks was

examined by incubating the cells at 37 °C for different time intervals ranging from 0 to 90 min after irradiation (2 Gy) prior to performing the comet assay. Kinetics of comet parameters is shown in figures 6-9. The results are summarized in table 2. The P-value for the figures 6-9 are found to be 6.99E-6 (R^2=0. 984), 2.06E-8 (R^2=0. 997), 7.93E-4 (R^2 = 0. 966), 2.82E -7 (R^2= 0.991) respectively, indicating goodness of fit. The estimated mean repair times for %TD, TL, TM and OTM are found to be 30.68 ± 3.63, 20.21 ± 1.03, 15.70 ± 2. 29, 26.06 ± 1.92 minutes respectively. The corresponding estimated repair half times are 21.26±2.52, 14.01±0.71, and 10.88 ± 1.59, 18.06 ± 1.33 minutes respectively. As indicated by these results, $t_{1/2}$ values varied between 9.29 min to 23.78 min and T values varied between 13.41 min to 34.31 min. The data obtained in the present study agrees with earlier reported studies that, the bulk of DNA repair takes place within 15 min and rest will be completed within 120 min after exposure [14, 20]. Similar results have also been reported in lymphocytes from patients who are treated for different type's cancers [21]. Also, from the comparison of the repair kinetics between human peripheral blood lymphocytes and granulocytes, it is evident that the repair of single strand breaks was demonstrable after 15 min incubation period for both types of cells. However, there was a trend for lymphocytes to repair faster and a greater extent than granulocytes [22]. Similar findings were observed using cryopreserved lymphocytes, and no significant difference in repair capacities was detected between cryopreserved and freshly

Table 1. Summary of DNA damage study.

Comet parameter	C	α	β	R^2	P
%TD	2.78 ± 0.01	4.88 ± 0.42	-0.33 ± 0.09	0.993	1.25E-7
TL	0.2 ± 0.010	35.79 ± 1.86	-3.49 ± 0.50	0.987	9.09E-7
TM	1.0 ± 0.12	3.66 ± 0.38	-0.09 ± 0.11	0.992	1.97E-7
OTM	1.0 ± 0	3.97 ± 0.24	-0.26 ± 0.077	0.991	2.82E-8

Table 2. Summary of DNA damage repair kinetics study.

Comet parameter	Yo	A	Theoretical repair time *(T)* (min)	DNA repair half time *($t_{½}$)* (min)	R^2	P-value
%TD	0.70 ± 0.21	7.33 ± 0.36	30.68 ± 3.6	21.26 ± 2.5	0.984	6.99E-6
TL	3.02 ± 0.37	63.73 ± 1. 7	20.21 ± 1.0	14.01 ± 0.7	0.997	2.06E-8
TM	0.75 ± 0.13	7.88 ± 0.77	15.70 ± 2.3	10.88 ± 1.6	0.966	7.93E-4
OTM	1.15 ± 0.11	6.46 ± 0.23	26.06 ±1.92	18.06 ± 1.3	0.991	2.82E-7

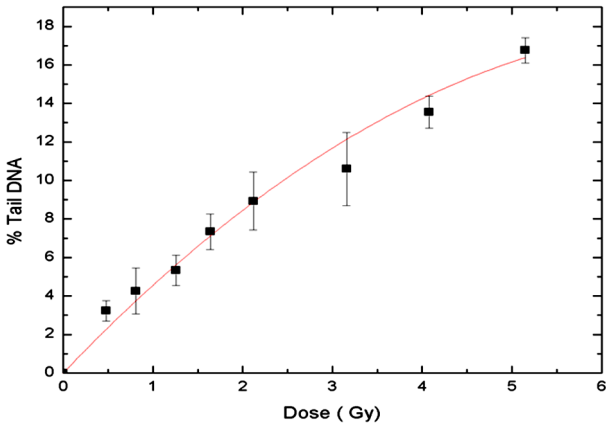

Figure 2. Variation of % Tail DNA with dose for human peripheral blood cells after 8 M eV Plused electron beam irradiation.

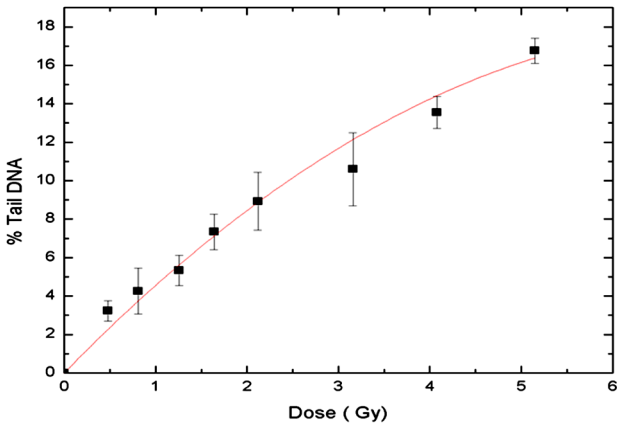

Figure 3. Variation of Tail length with dose for human peripheral blood cells after 8 M eV pulsed electron beam irradiation.

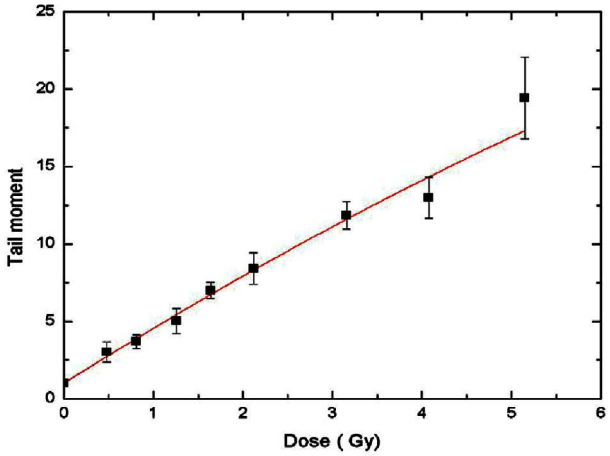

Figure 4. Variation of Tail Moment with dose for human peripheral blood cells after 8 M eV Plused electron beam irradiation.

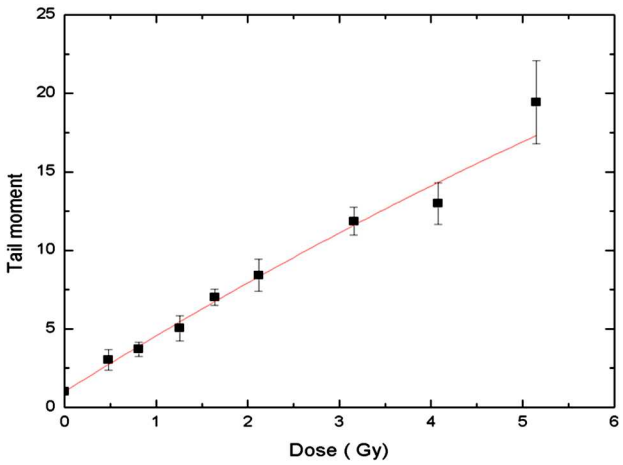

Figure 5. Variation of OTM with dose for human peripheral blood cells after 8 M eV Plused electron beam irradiation.

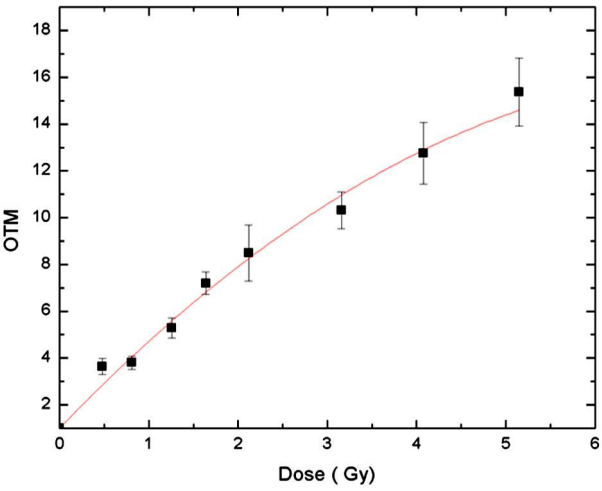

Figure 6. DNA repair kinetics study-Variation of % Tail DNA with incubation time.

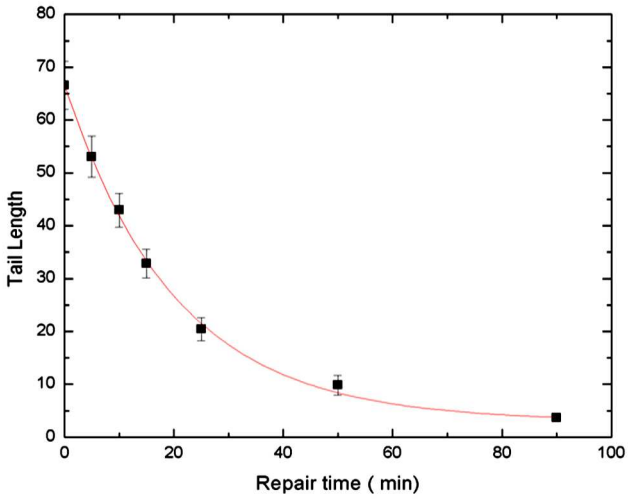

Figure 7. DNA repair kinetics study-Variation of Tail length with incubation time.

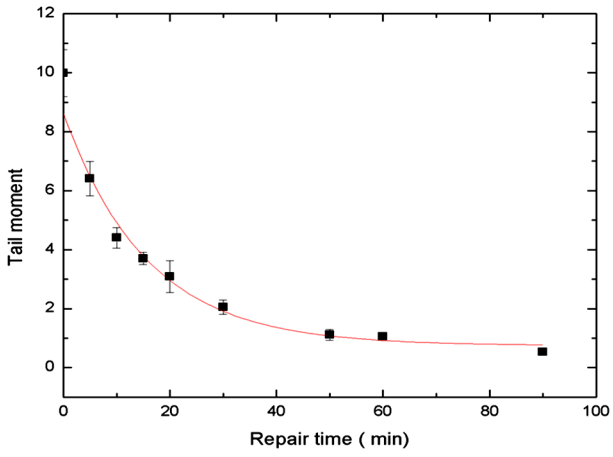

Figure 8. DNA repair kinetics study-Variation of Tail Moment with incubation time.

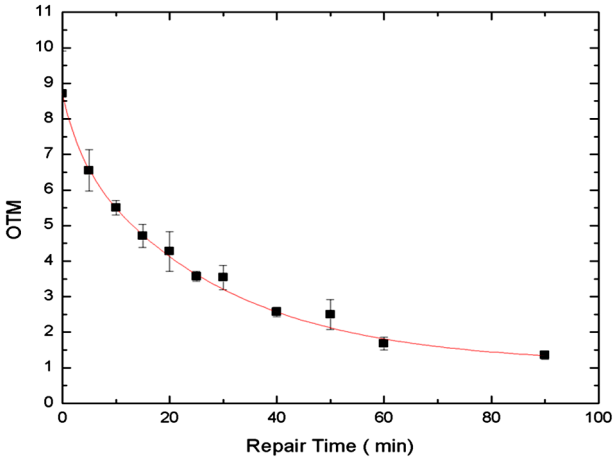

Figure 9. DNA repair kinetics study-Variation of OTM with incubation time.

isolated lymphocytes [23].

The repair rates for different incubation times were estimated and are presented in table 3. Four comet parameters are being studied. In all parameters, the rate of repair in the first 15 min is found to be very high compared to 30, 60 and 90 min. After 30 min, the repair rate is almost constant, which agrees with literature reported data [3, 21,22]. Using gamma radiation similar kind of data were also presented for human lymphocytes [19] and murine leukocytes [22]. Malcolmson *et al.* [21] has reported near completion of slower repair component in a span of 120 minutes. The DNA damages remained after 120 min of incubation are likely to be residual damages that cannot be repaired.

Table 3. Summary of DNA repair rate study.

Comet parameter	Repair rate			
	0-15 min	15-30 min	30-60 min	60-90 min
%TD	0.29 ± 0.003	0.09 ± 0.048	0.06 ± 0.001	0.02 ± 0.0006
TL	2.25 ± 0.12	0.72 ± 0.11	0.42 ± 0.03	0.18 ± 0.11
TM	0.42 ± 0.04	0.11 ± 0.002	0.03 ± 0.005	0.02 ± 0.001
OTM	0.27 ± 0.06	0.08 ± 0.009	0.06 ± 0.005	0.01 ± 0.002

CONCLUSION

Induction of DNA damages by pulsed electron beam as estimated in the present study using alkaline comet assay parameters were found to be near linear with a negligible negative quadratic components. The observed small quadratic components may be attributed to possibility of complex damages leading to apoptosis due to high dose rate of pulsed electron beam. The experimental DNA repair halftime and theoretical repair time for human blood lymphocytes were estimated and found to vary between 9.29 - 23.78 min and 13.41 - 34.31 min respectively for various comet parameters studied. The repair rate was found to be maximum during first 15 minutes of incubation and also found to be almost constant after 60 minutes of incubation.

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Conflicts of interest: *none to declare.*

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