5.3 STATUS OF RADIATION BIOLOGY RESEARCH USING HEAVY IONS

There are five new projects from the Universities taken up that would lead towards PhD degree. Two projects from WBUT, Kolkata is aimed at the study of a few naturally occurring microbes where metal accumulation takes place inside, to repair DNA DSB induced on its genome following ionizing radiation. Both the users utilized beam time by taking 85 MeV 12C beam for irradiating the bacteria. The DSB induction and its repair was studied by the research scholars using the Asymmetric Field Inversion Gel Electrophoresis system at the Radiation Biology laboratory of IUAC.

Another project from Kalyani University is focusing on the high LET radiation induced gene expression studies. The aim of the project is to measure several apoptotic parameters and monitor the expression of few genes in human cervical epithelial carcinoma (HeLa) cells irradiated with carbon beam and compare the effects in presence and in absence of PARP inhibitor in HeLa cells. Towards this goal, the user has taken beam time and initial analysis job is being carried out.

A project from MMC College Ghaziabad would study high LET radiation action on breast cancer cells MCF-7. The student is being trained in cell culture and other molecular biology techniques related to the project at the laboratory at IUAC.

Beam time has been availed of in order to study the effects on the signalling parameters upon irradiation of Lung Cancer cell lines by C beam. This has been a collaborative project with the Radiation Biology group at BARC under a project supported by BRNS, Mumbai.

5.3.1 Studies on heavy ion irradiated metal induced radiation sensitization in Bacillus subtilis

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Ionizing radiation has severe effect on organisms, e.g. on the genetic material of the cells, possibly leading to cell death and permanent changes within daughter cells [1,2,3]. The various types of DNA damage experienced may be (a) double strand breaks (DSBs) which in turn lead to chromosomal aberrations [4] due to the the direct physical effects of ionizing radiation forming primary free radicals and the indirect biochemical effects from reactive oxygen species (ROS) (b) single strand breaks (SSBs) and c) base pair substitution mutations

[5,6,7,8]. It is well established that DNA DSBs are the most significant lesions induced in DNA molecules by heavy ion irradiation and are the least efficiently repaired [9].

DNA damage induced by heavy ions in bacterial cells, such as Bacillus subtilis, has already been investigated [10]. To analyze double strand breaks in long molecules of DNA up to some Mbp in length--the technique of pulse field agarose gel electrophoresis has long been used. Moreover metal ions, like iron, calcium, zinc, copper, in growth media has been found to act as radiosensitizers [11,12].

Using ^{12}C heavy ion source Bacillus subtilis cells were irradiated at a dose of 2, 5, 10 and 20 Gray. The cells (control and metal treated) were irradiated in separate petriplates at different doses and were immediately washed out with fresh LB media. Cells were grown in presence of 1mM Lead. Cell aliquots containing 4 X 10^8 cells were plated in separate petriplates, washed with media and pelleted. Next, the cells were re-suspended in 20µl of PBS and 80µl of 1.5% low gelling agarose in PBS to form plugs. Plugs were formed for each dose of damage (2, 5, 10 and 20 Gy) and its subsequent repair after 4hrs of incubation under growth condition. Time course of repair was studied (1hr, 2hr and 4hr incubation post damage) at 20 Gy dose. For repair studies the plugs were formed after incubation of cells at 37°C for different time intervals.

The plugs were then subjected to AFIGE (BIORAD, Hercules, CA, U.S.A.; FIGE Mapper) on 1% agarose gel with 0.5 X TBE buffer at 10°C. AFIGE is used to monitor only the DSBs present in the sample. The pulse programme was set with ramping progression of 0.1 s – 2.0 s, at 180V forward voltage and 120V reverse voltage for 12 hours. The gels were stained with ethidium bromide (0.01µg/ml), observed over the UV transilluminator and photographed.

The gel images were scanned using Molecular analyst (BIORAD) and the percentage fraction of activity released (%FAR) was calculated as %FAR = [(Amount of DNA in the lane)/(Amount of DNA in the lane+groove)] x 100. Each experiment has been repeated in triplicate and the results are plotted as mean±SE.

Y-axis in each case represent % FAR, while the details for X-axis provided separately for each graph. In the X axis D and R stands for damage and repair respectively. Doses in Gray is represented as G.

Bacillus subtilis used in this study were irradiated with different heavy ion dose rates. Figure 1a and 1c reflect the % FAR plotted along the Y-axis as calculated after densitometric scanning to determine the nature of damage and repair in the presence and absence of metal. Fig 1b and 1d depict the time course of repair in case of Bacillus cells without and with metal respectively. It is evident from fig 1a with increasing dose of radiation (from 2 to 5 Gy) there is a subsequent increase in damage, but, there is a sudden decrease at higher doses (10 and 20 Gy); which might mean that the damage is so intensive that the

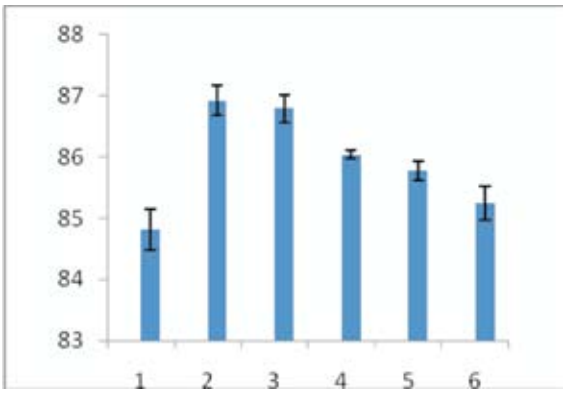

Fig. 1a. DOSE PROFILE F3
1 – Control 1; 2 – 2G D; 3 – 2G R; 4 – 5G D; 5 – 5G R; 6 – 10G D; 7 – 10G R; 8 – 20G D; 9 – 20G R; 10 – Control 2 (Unirradiated cells after 4hrs incubation)

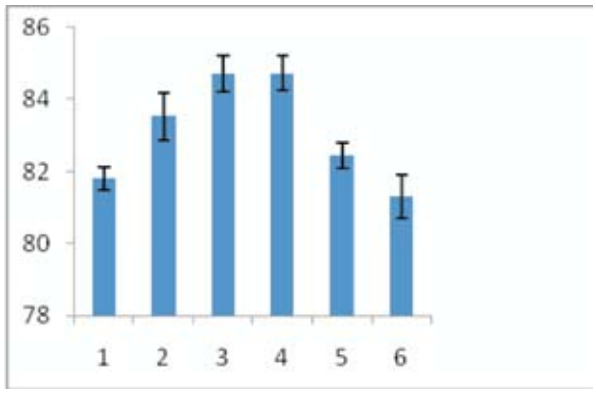

Fig. 1b. TIME COURSE F3
1 – Control 1; 2 –20G D; 3 – 1Hr R; 4 – 2Hr R; 5 – 4Hr
R; 6 – Control 2 (Unirradiated cells after 4hrs incubation).

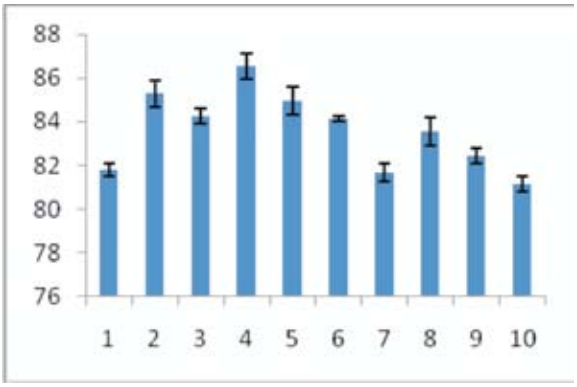

Fig. 1c. DOSE PROFILE F3 Pb
1 – Control 1; 2 – 2G D; 3 – 2G R; 4 – 5G D; 5 – 5G R; 6 – 10G D; 7 – 10G R; 8 – 20G D; 9 – 20G R; 10 – Control 2 (Unirradiated cells after 4hrs incubation).

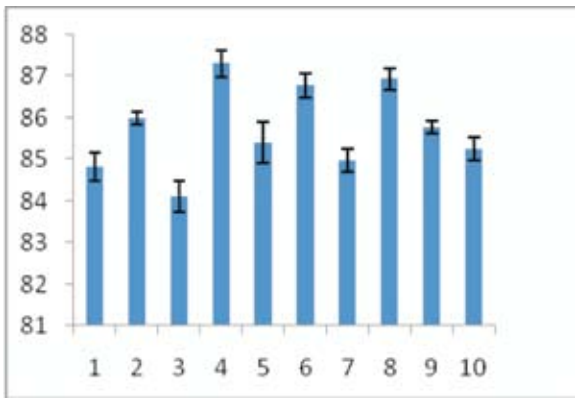

Fig. 1d. TIME COURSE F3 Pb
1 – Control 1; 2 –20G D; 3 – 1Hr R; 4 – 2Hr R; 5 – 4Hr R; 6 – Control 2 (Unirradiated cells after 4hrs incubation).

DNA strands are broken into very small fragments which probably leave the gel during the course of the run, as a result of which there is a decrease in % FAR with increasing dose. In presence of metal there is distinct radiosensitization effect and the nature of graph is similar to Fig 1a. The time course of repair in presence of metal (Fig 1d) shows that repair is gradually taking place after 4 hours of incubation at 37˚C. But the picture is slightly different for time course in absence of metal as shown in Fig 1b. As already mentioned since there is a intense damage at 20 Gy and the smaller fragments might be leaving the gel during the run, therefore on incubation for 1hr the smaller fragments are ligated into larger size that in turn results in an increase of % FAR during the 1 and 2nd hr of incubation. However the % FAR after 4 hours of incubation is almost comparable to Control 2, indicating DSB repair.

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5.3.2 Role of PARP in repair after DNA damage by carbon ion beam in Human Cervical Epithelial Carcinoma (HeLa) cells

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Poly (ADP-ribose) polymerase (PARP) is one of the major DNA repair enzyme involved in base excision repair and also known as DNA nick sensor [1]. After DNA damage PARP-1 gets activated and start to poly(ADP-ribosyl)ate its target proteins. The poly(ADP-ribosyl)ation in damaged condition is qualitatively as well as quantitatively different from that in normal condition. Earlier we observed that inhibition of poly(ADP-ribosyl)ation by PARP inhibitor induces apoptosis [2,3]. We are interested to find out role of PARP isoforms except PARP-1 in repair and also searching for novel PARP interactor after DNA damage by heavy ion beam which produces cluster damage.

Our preliminary data showed that DNA damage by $^{12}C^{6+}$ (62 MeV on cell surface, average LET 287 kev/µm, fluence 0-20×10^6/cm^2) in PARP-1 knocked down HeLa cells (HsiI) are higher than that in parental HeLa as detected by comet assay (Fig 1C). Typical picture of comet are shown in Fig 1A (normal) and Fig 1B (irradiated with $12C^{6+}$ ions). This result established that PARP-1 is involved in repair after DNA damage by $12C^{6+}$ ions. %survival in HsiI was observed to be lower than that in HeLa after treatment with $12C^{6+}$ ions at fluence 4×106/cm2. But we need to repeat all this experiments to elucidate statistically significant difference, if any, between HeLa and HsiI after irradiating with $12C^{6+}$ ions.

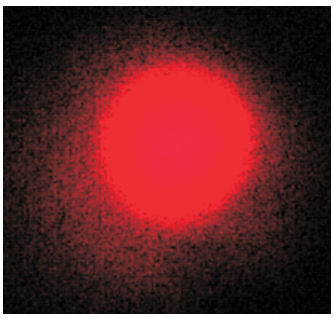

Fig. 1A.

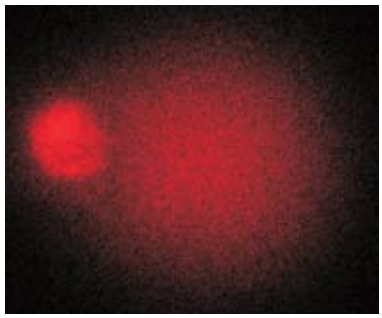

Fig. 1B.

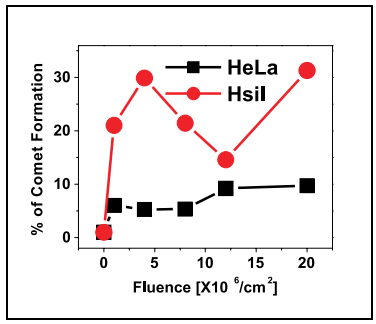

Fig. 1C.

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5.3.3 Activation of crucial signaling components involved in DNA damage sensing, repair, cell cycle arrest and apoptosis following carbon irradiation of A549 cells.

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In this study, lung carcinoma cell line A549 were irradiated with carbon ions and ensuing activation of few important components involved in DNA repair, cell cycle arrest, apoptosis and survival pathways were followed to elucidate the mechanisms behind observed cellular response.

Materials and Methods :

Human lung carcinoma cell line A 549 were irradiated by different doses of 85 MeV Carbon ions from accelerator. After treatment, cells are seeded out in appropriate dilutions (500 cells were seeded in 60 mm petriplate) to form colonies in 1–3 weeks. Survival fraction was evaluated.

After different time points of 6 hours, 15 hours and 24 hours of Irradiation at 1 Gy the cells were subjected to cell cycle analysis using the flow cytometer (BD biosciences).

A set of samples of cells were grown on cover slips and irradiated after fixing them on 35mm petri dishes. They were later subjected to immunofluorescence (IF) staining. The cells were rinsed and mounted with ProLong Gold antifede with DAPI mounting media. Images were captured using Carl Zeiss confocal microscope.

The irradiated or unirradiated cells were subjected to Western Blot analysis protocol [SDS-PAGE]. The membranes were probed with anti-phosphoERK, anti-phospho JNK , and anti Poly ADP. This was followed by incubation with horseradish peroxidase (HRP) conjugated secondary anti mouse antibody. The secondary antibody was detected using BM Chemiluminiscence Western Blotting Kit.

Results:

Only 24.52 % of A549 cells survived which had been exposed to 1 Gy of Carbon beam and while only 4.8 % cells survived at 2 Gy of Carbon beam as observed with clonogenic assay (Figure 1).

Cell cycle analysis results showed that A549 cells were not arrested at any stage of cell cycle, which is indicated by similar percentage of cells in S and G2/M in both control and irradiated cells (Figure 2).

The phosphorylation of H2Ax was found to significantly increase 4 hours after carbon ion irradiation as compared to unirradiated controls (Figure 3), whereas by 4 hours after gamma irradiation most of the repair was complete. This indicates the presence of large number of double strand breaks. There was significantly higher phosphorylation of ATM at serine 1981 position in irradiated A549 cells as compared to controls (Figure 3). Activation/ phosphorylation of ERK and JNK was examined at different time points (0.1, 0.5, 1, 2, 3, 4 and 6 h) after exposure. ERK was not found to be activated at all after carbon irradiation

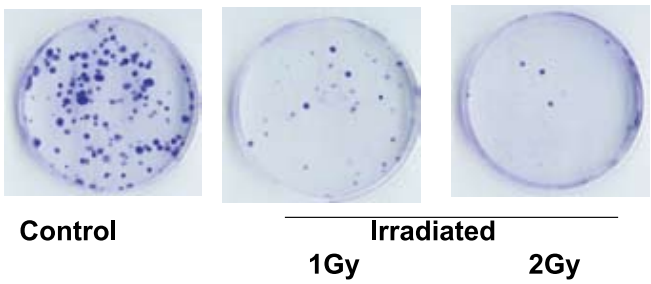

Fig. 1. Clonogenic Survival assay

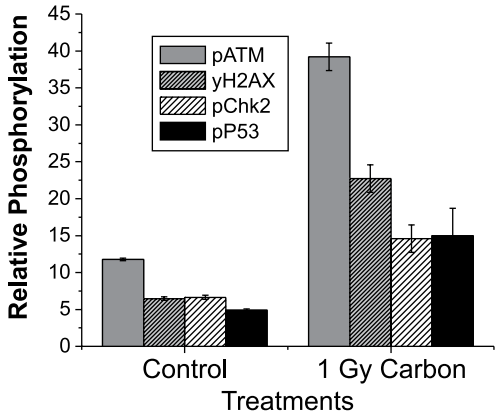

Fig. 2. Cell cycle analysis by FACS

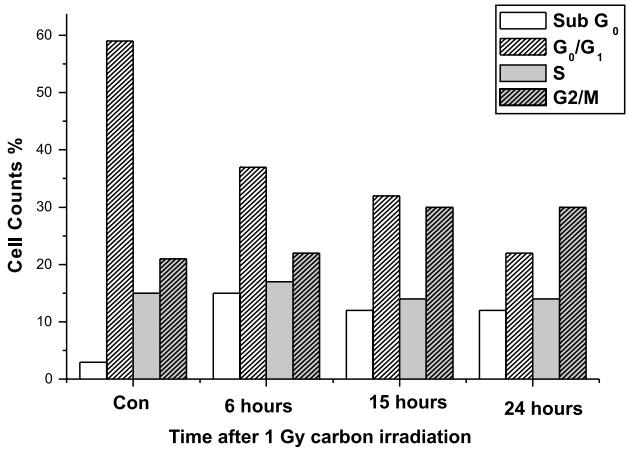

Fig. 3. Relative intensities of various signaling proteins using antibodies against them and observed by confocal microscopy

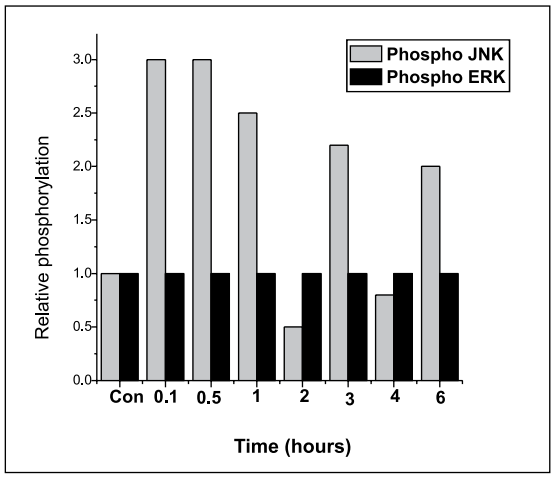

Fig. 4. Relative intensities of phospho ERK and phospho JNK as observed by western blot

(Figure 4). ERK, a signaling kinase plays an important role in deciding the fate of the cell and is mainly involved in survival of the cells. JNK, which is a stress responsive kinase, showed significant activation within 6 minutes (0.1 hour) of carbon irradiation and the activation was persistent till two hours (Figure 4).

In conclusion, our study indicates that after high LET irradiation, the DNA damage sensors such as ATM and H2Ax remain activated till 4 hours after irradiation, indicating very slow repair unlike gamma irradiation where by 4 hours most of the repair takes place. Since the activation of ATM was not efficiently translated into repair, this prolonged ATM activation might be channeled to activation of apoptotic pathways. This in strongly indicated by significant activation of p53 and JNK.

5.4 ATOMIC PHYSICS RESEARCH

We have continued to understand more and more the unusual features of beam-foil experimental data. Such features indicate new phenomena having important relevance to various branches of physics. Last year we have reported atomic astrophysics experiments using highly charged MeV ions, this year we report the measurement of wake field intensity and ion energy loss at the solid surface.

Atomic and molecular physics experiments in the low energy ion beam laboratory have been yielding interesting results as previous years. Position sensitive multi-hit time-of-flight measurement system is used to study the fragmentation dynamics of complete and incomplete fragmentation process as presented below.

5.4.1 Atomic Physics Experiments with Interest in Interdisciplinary Science

T. Nandi

In the recent past we have been succeeded in obtaining important results from atomic experiments having noble implications in interdisciplinary science. A few are mentioned below.

(i) Measurement of wake field intensity

Effect of wake fields is known since a few decades, however, there is no direct measurement on the magnitude of wake field for any ion-surface combinations. Very recently we have succeeded in measuring the wake field intensity of an ion moving through a solid. No direct experimental method would be possible in measuring this. An indirect experimental method, the standard beam-foil time-of-flight technique, is used to determine the Stark mixing parameter of H-like 2s $^2S_{1/2}$ state with 2p $^2P_{1/2}$ state, which is further exploited to quantify the wake field intensity during the passage of ~3 MeV/A $^{51}V_{23}$ ion through a thin C-foil as well as an Au-foil. Current result will help scientific community in a big way as quantification of the wake field is very important for both fundamental research and applications in modern science.

(ii) Can ion energy loss at the solid surface supersede that at the bulk?

General consensus on ion energy loss at solid is that ion looses its energy at the bulk of the solid material. However, our recent study shows that about 1% of 160 MeV $^{51}V_{23}$ ion is lost at the exit surface of the carbon foil. Ion energy loss at solid surface is much more for slow highly charged ions. Using earlier experimental results we confirm that surface-stopping power of the entry-surface can supersede the bulk stopping power for low energy ions in solids. It is found that the surface-stopping power is directly proportional to the charge state of incident ions. Further, it is shown that the surface-stopping power originates from a dynamical image potential set up by a swift ion moving through a thin foil and the various unsolved problems in literature indeed requires its consideration.

5.4.2 Multiple ionization of SF_6 and its dependence on projectile charge

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We have performed an experiment to understand multiple ionization and subsequent fragmentation of SF_6 under the impact of highly charged ions. Keeping the beam velocity fixed at 5 Mev/u, carbon and oxygen projectiles of charge states 4–8 were used in the experiment. Fragment ions from the target were separated on the basis of their m/q ratios by the time-of-flight method using the ejected electron as the start trigger. The velocity of the ions was simultaneously recorded, event-by-event, from the position of arrival and time-of-flight of ions, using a large area, fast, position resolving detector. The target was in the form of an effusive gas beam. Up to four ions arising from the same event could be recorded in coincidence with complete mass and velocity information. From these data, not only the yields of various fragment ions, but also the complete kinematics of several fragmentation channels could be derived. Our focus is on the fragmentation of doubly and triply charged molecular ions.

A surprising result of this experiment is that prima facie, the yields of fragment ions show hardly any dependence on the projectile charge state (q). In the case of two-electron systems, such as He and H_2, it is well-known that the single ionization cross-sections follows approximately a q^2 dependence, and the ratio of higher order ionization cross-sections to single-ionization cross section also has a q dependence. For multi-electron targets the situation is unclear due the opening up of several channels. Since doubly charged molecular ions are often unstable (which is borne out in the case of SF_6), the double ionization yield cannot be directly inferred. To estimate the double ionization yield, it becomes necessary to sum the yield in all fragmentation channels of a particular precursor molecular ion. This analysis is in progress. We are also looking into the fragmentation kinematics of triple and higher charged SF_6, in which the strong and similar momentum correlation between the fluorine ions gives a clear indication that the fragmentation of such highly charged precursors is predominantly concerted, not sequential.

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