1. INTRODUCTION

In eukaryotic cells, the negatively charged DNA is closely associated with positively charged histone proteins which aids in their packaging to highly compact structure. This DNA-protein complex known as chromatin have different levels of compaction which regulates gene expression in cells. The basic repeating structural unit of chromatin is nucleosome consisting of 146 base pairs of DNA wrapped around 8 histone protein core octamer H2A, H2B, H3 and H4. Nucleosomes along the entire length are connected by linker histone H1. This linker histone plays an important role in stabilizing chromatin higher order structure. The nucleosome loops around itself to form a highly compact chromosomal structure. The downstream activities of DNA such as replication, repair and transcription are regulated by the higher order structure of DNA in chromatin.

Ionizing radiation causes lethal injuries either directly or indirectly inducing damage to the biological macromolecules, most importantly the genetic material or DNA. Cells transiently halt their cell cycle progression to provide sufficient time for repair machinery to act on for the repair of radiation-induced DNA damages. This may include delay in replication or S-phase of cell cycle besides activating other cell cycle checkpoints. Multiple replicons (replicon cluster equivalent to a chromosome band) along the length of a chromosome fire in a definite pattern for the timely completion of replication. Histone deacetylase inhibitors (HDACi) have been shown to alter the pattern of DNA replication origin activity including earlier replication in S-phase of normally late replicating chromosome bands. This may also indicate an acceleration of repair steps since DNA damage acts as an impediment for replication. Trichostatin A (TSA), a well-known HDACi, was studied for its effect on replication initiation using Indian Muntjac cell as a model. It was found that TSA treatment enhanced the replication rate and increased the number of replicon clusters firing at any given time per chromosome. It also compensated the decline of replication rate in irradiated cells, helped in their revival and brought them to the control level.

Keywords: Ionizing radiation; Radiation mitigation; HDAC inhibitor; Chromosome replication; Differential staining

Effects of Ionizing Radiation on Chromosome Replication and its Modification by HDAC Inhibitors

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ABSTRACT

Ionizing radiation causes lethal injuries either directly or indirectly inducing damage to the biological macromolecules, most importantly the genetic material or DNA. Cells transiently halt their cell cycle progression to provide sufficient time for repair machinery to act on for the repair of radiation-induced DNA damages. This may include delay in replication or S-phase of cell cycle besides activating other cell cycle checkpoints. Multiple replicons (replicon cluster equivalent to a chromosome band) along the length of a chromosome fire in a definite pattern for the timely completion of replication. Histone deacetylase inhibitors (HDACi) have been shown to alter the pattern of DNA replication origin activity including earlier replication in S-phase of normally late replicating chromosome bands. This may also indicate an acceleration of repair steps since DNA damage acts as an impediment for replication. Trichostatin A (TSA), a well-known HDACi, was studied for its effect on replication initiation using Indian Muntjac cell as a model. It was found that TSA treatment enhanced the replication rate and increased the number of replicon clusters firing at any given time per chromosome. It also compensated the decline of replication rate in irradiated cells, helped in their revival and brought them to the control level.

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Acetylation of histone takes place on NH$_3^+$ group of lysine amino acid residue of histone protein. It neutralizes the positive charge of histones thus weakening the interaction between DNA and histone protein. This results in increased accessibility of DNA for transcription factors and other proteins involved in DNA metabolism. Acetylation is carried out by a group of enzymes called histone acetyl transferases (HATs). Histone deacetylation has reverse action which results in decreased level of gene expression. It is carried out by enzymes called HDACs. The activity of HDAC is greatly interfered by the class of compounds known as HDAC inhibitors (HDACi). They increase the accessibility of transcription factors to DNA by inhibiting HDAC activity. They have also been shown to alter the pattern of DNA replication origin activity. Some HDACi has been studied to cause earlier replication in normally late replicating imprinted genes in human cells.

Ionizing radiations cause lethal cellular injuries both directly by damaging cellular macromolecules and indirectly by generation of ROS. Nuclear DNA is the major target for ionizing radiation induced damage. To preserve the genomic integrity, the cells initiate a network of DNA damage response system. One of the most important DNA damage responses is cell-cycle checkpoint activation. Checkpoint activation after radiation exposure helps in faithful repair of damaged DNA and thus proper transfer of genetic information to the next generation. The DNA damage checkpoints temporarily block the cells in G1, S, and G2 phase of cell cycle. This may include
delay in chromosomal replication to give sufficient time for repair machinery to repair the damage.

Trichostatin A (TSA), a well-known HDACi has been studied for its role in epigenetic modulation. Its ability to alter the chromatin topology to a more open form enables repair and replication machinery to access DNA more easily.

In this study, we observed that TSA treatment following radiation exposure increased the number of replication initiation sites in normally late replicating regions of Indian muntjac cells. The regions of replication initiation were identified by determining bromo deoxyuridine (BrdU) incorporated and non-incorporated chromosomal segments along the entire length of chromosome. BrdU competes with thymidine during DNA synthesis in S-phase. Giemsa staining helps in differentiating BrdU substituted and non-substituted segments of DNA. Staining chromosomes with Giemsa in high pH conditions result in differential staining. BrdU substituted regions get palely stained compared to intensely stained non-substituted region if the staining is performed at pH 6.8 while the reverse is observed at pH 10.4\textsuperscript{11,12}.

Indian muntjac cells were chosen for the experiment because of their small chromosome number and large chromosomal size which was ideal for convenient analysis.

2. MATERIALS AND METHODS

Cell culture: Indian muntjac (ATCC CCL-157) cells were grown in F10 Ham’s media supplemented with 10% FBS (MS Biologicals; Israel). To prevent bacterial growth, antibiotics (penicillin, Streptomycin, Nystatin) were added to the media. Cells were incubated in 5% CO\textsubscript{2} environment at 37°C temperature.

Irradiation and Drug Treatment: Culture dishes were irradiated with 2 Gy γ-radiation using a cobalt teletherapy unit (Bhabatron, Panacea Biotech) at a dose rate of 1Gy/min (SSD adjusted to the field size of 35x35cm to achieve desired dose rate).

TSA was dissolved in HBSS, filtered with 0.2 µM syringe filter and added to the cultures to attain the required concentration.

Grouping of culture flasks:

(i) Control – Culture containing cells only without any treatment.

(ii) Drug alone – Culture administered with TSA only at a concentration of 50nm.

(iii) Radiation alone – Culture irradiated with 2Gy γ-radiation.

(iv) Drug plus radiation – 2 Gy irradiated cells treated with 50 nm TSA 1 h post irradiation.

BrdU labeling: Muntjac cells were allowed to grow and attain active replication cycle suitable for the experiment. Irradiation of cells with 2 Gy radiation dose was done followed by TSA treatment after 1 h of irradiation. Cultures were divided into three treatment schedule: 1 h of BrdU treatment, 2 h of BrdU treatment and 4 h of BrdU treatment. BrdU (Sigma-Aldrich, USA) dissolved in HBSS (Sigma-Aldrich, USA) was added to the culture at the final concentration of 30µm at each scheduled time. During the entire treatment period, the cultures were kept in dark. Time scale of the treatment schedule is diagrammatically represented in Fig. 1.

Cultures were arrested at metaphase by adding colchicine (Sigma-Aldrich, USA) dissolved in HBSS at the final concentration of 2.5µg/ml to the media. Following 1h of colchicine treatment, cells were processed and fixed for metaphase preparation.

Chromosome preparation: After the scheduled time, cells were harvested and washed in PBS (Hi-media). Metaphase arrested cells were given hypotonic treatment (0.57% potassium chloride, Sigma- Aldrich, USA) for 20 min at 37°C. After 20 min of incubation, cells were centrifuged and pelleted down to remove the KCl solution. Pellets were washed twice in PBS to remove the remaining KCl solution. Cells were then fixed with 10 ml of fresh Cornoy’s fixative (3:1 methanol:acetic acid, (Sigma-Aldrich, USA) added drop wise to the cells with constant stirring. The step was repeated twice each time with fresh fixative for better fixation and then stored at 4°C overnight suspended in fixative. After overnight incubation, the fixative was discarded by centrifugation at 1000rpm for 10 min and few drops of fresh fixative were added to the pellet.

Slide preparation: Before slide preparation, slides were cleaned, immersed in 50% ethanol and kept in refrigerator overnight. Homogenous suspension of cells could drop on the surface of clean grease free chilled slide from a sufficient height using micropipette. Slides were then allowed to air dry for 24 h.

Giemsa staining: Metaphase slides were stained in Giemsa diluted 12 times in freshly prepared 0.3M disodium hydrogen phosphate dihydrate (pH 10.4, adjusted with 1N NaOH) buffer. High pH of buffer helped in differential staining of BrdU incorporated and non-incorporated segments of chromosome\textsuperscript{13}. Slides were flooded with Giemsa, kept for 8-10 min and then rinsed in RO water and air dried.

Scoring: Slides were observed under a light microscope with 100X magnification and fitted USB camera 2.0. BrdU substituted and non-substituted regions showed differential staining pattern where BrdU substituted segments were intensely stained and non-substituted segments were lightly stained. Well spread chromosomes were analysed for the banding patterns. Length of the BrdU incorporated and unincorporated segments were measured by Image J software (National Institute of Health, USA). Only the longest chromosome was considered for scoring, the length of dark and light regions was measured on an arbitrary scale. The whole chromosome was reconstituted based on the average length of dark and light bands measured.
out of at least 25 metaphases per treatment group. Each band was considered a single replicon cluster along the length of the chromosome.

3. STATISTICS
Data were analysed using Graph Pad Prism 5 software. Values presented are mean±SD, one way ANOVA was used to estimate level significance. P<0.05 was considered significant.

4. RESULTS
At least 25 metaphases from each different treatment group of slides corresponding to different duration of BrdU incorporation were analysed. Chromosomes were observed for any change in replication pattern due to the effect of radiation and drug. The total number of replicon cluster in all the treatment groups of the different duration of BrdU treatment was also determined. If the cell is exposed to BrdU for short time period, BrdU will remain only for a small fraction of cell cycle and hence, the replicating strand will contain a mosaic of dark and light regions. The more is the time of exposure to BrdU, the more dark regions representing the BrdU incorporated regions are expected to be observed. Below are representative images of metaphase spreads showing replication bands (Fig. 2).

The amount of BrdU incorporation (Fig. 3) and average number replicon clusters (Fig. 4) were only marginally different among the treatment groups where BrdU treatment was given during the last one hour (late replicating chromosome) of metaphase progression. This indicates TSA treatment did not drastically influence the replication of heterochromatin in Indian Muntjac cells.

However, the region of BrdU incorporation (length of replication bands in a cluster) were comparatively higher in TSA and radiation plus TSA groups as compared to radiation alone group as observed in Fig. 5(a).

In 2 h and 4 h BrdU treated cells which includes the mid and early replicating bands or the euchromatic region, the percentage of BrdU incorporated regions (Fig. 3) along the length of the chromosome as well as the number of active replicon clusters were significantly higher in all groups compared to radiation alone group, drug plus TSA group being highest (P<0.05) in the 4h BrdU incubation experiment. This implies radiation exposure significantly delayed the replication of euchromatic region of chromosome containing relatively open DNA topology owing to its active nature. Open DNA topology also is more susceptible to radiation induced DNA damage. Radiation plus TSA induced faster replication in those parts of the chromosome comparable to that of control cells indicating efficient removal of the DNA damages which impede DNA replication progression.

5. DISCUSSION
Soon after the Hiroshima and Nagasaki nuclear bombing, the world became serious about the hazards of ionizing radiation exposure and the search for radiation countermeasure agents became one of the top priorities in research and development field. Biological radiation countermeasure includes radio protectors where the agent is administered before exposure to radiation and needs to be present in the
body before exposure to impart its benefits, mitigators are agents those are administered during or shortly after radiation exposure and before the appearance of any symptom while therapeutics are mainly symptom based and are administered only after the symptoms appear. Radiation mitigators are of specific importance since their application in the events of radiation accidents to attain the masses is highly important. In the recent past we have demonstrated the ability of HDAC inhibitors to mitigate radiation injuries at various system, organ and organism level. Trichostatin A is one among them that has been shown to increase survival of lethally irradiated mice and restore normalcy in reproductive system as well\(^\text{13}\).

In this study we have demonstrated the ability of trichostatin A to modulate replication in Indian muntjac cells. Cell proliferation has been shown to be highly affected by radiation exposure which includes delayed cell cycle progression and even cell inactivation\(^\text{14}\). Radiation exposure induces DNA damage and damaged DNA is normally not replicated without faithful repair of the damage which may add to the delay in cell cycle progression. Exposure to ionizing radiation has been shown to enhance the HDAC activity in various cells and tissues\(^\text{13,15}\). Enhanced HDAC activity will lead to chromatin compaction and will impede replication as well besides other downstream DNA metabolism processes. Treatment with TSA as has been done in this study or possibly any other HDACi, will facilitate decompaction of DNA and hence allow smooth progression of replication. In the present study, we compared the pattern of replication of irradiated and TSA treated cells to investigate if TSA can overcome radiation induced delay in replication which indirectly would mean faithful repair completion of damaged DNA. As anticipated, TSA treated irradiated group showed firing of more number of replication origins (replicons) as well as increased incorporation of BrdU in chromosome indicating faster and efficient replication. Indirectly, TSA treated irradiated group cells has normal DNA topology with successful elimination of radiation induced DNA damages which can support normal replication process.

### 6. CONCLUSION

This study demonstrates TSA can enhance repair and replication of DNA and hence can act as a mitigator of radiation injury. However, this study has certain limitations which need to be taken care before giving any concluding remark. One such limitation of the study is the sample size (25 metaphases per group) besides only one chromosome from the karyotype has been considered. The method used is quite old and newer methods are now available including fluorescence dyes to substitute BrdU incorporation method and highly efficient imaging systems and software for better analysis. A well-planned experiment in the same direction considering those limitations will certainly help to prove TSA as a radiomitigator.

### CONFLICT OF INTEREST

Authors declare no conflict of interest.

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### REFERENCES


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CONTRIBUTORS

Dr Mrinalini Tiwari did her graduation in biotechnology from DAVV, Indore and PhD in the Toxicology from Jamia Hamdard University, New Delhi. Currently she is working at NIRM, Delhi in the field of stem cell research. In the current study she was involved in the experimental work, acquisition and analysis of data.

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