STUDY ON THE ROLE OF ENDOGENOUS GLUTATHIONE IN CELLULAR DAMAGE INDUCED BY RADIATION OF DIFFERENT QUALITIES

ABSTRACT

BY

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Literature Review:

During the past few decades, radiation research has developed into specialised sub-disciplines, from basic physics and chemistry to tumor biology and experimental radiation therapy. Although the radiobiological effects have been extensively investigated for X-ray and γ-ray, little work has been directed towards heavy ion beam. With the exploration of the outer space, the research of high Linear Energy Transfer (LET) radiation has attracted more and more attention. Since heavy ions were first applied to cure cancer at Lawrence Berkeley Laboratory, U.S, promising results have been reported when compared with the conventional radiotherapy for soft tissue sarcoma, bone sarcoma and prostate cancer. Now scientist in many institutes such as Gesellschaft fur Schwerionforschung (GSI) in Germany, heavy ion medical accelerator at Chiba (HIMAC) in Japan, and heavy ion research facility in Lanzhou (HIRFL) in China have designed accelerators to deliver ion beams for treatment and started basic research on cancer therapy with heavy ions such as lithium, carbon, neon, oxygen and argon. Modern cancer radiobiology has identified a number of innovations towards improvement of radiation treatment. Besides this, present day radiation safety research is focused on risk assessment, e.g. studies on influence of low dose exposure of radiation of different qualities.

Random energy deposition by ionising radiation induces a wide variety of DNA lesions which includes, single strand break (SSB), double strand break (DSB), oxidised bases and apurinic-apyrimidinic sites. Ionizing radiation induces damage in DNA by direct ionisation and through

generation of hydroxyl radicals that attack DNA and induce some or all of the above lesions (indirect interaction). DSBs are generally considered the most biologically damaging lesion produced by ionising radiation and if left unrepaired, then it can lead to permanent cell cycle arrest, induction of apoptosis, or mitotic cell death caused by loss of genomic material.

DNA damage caused by low or high LET ionizing radiation can lead to untoward effects in cells if unrepaired or misrepaired. With specific regard to carcinogenesis, genomic mutation caused by ionising radiation are widely thought to arise from DNA damage that is subsequently converted into a mutation as a result of processing by DNA repair mechanisms or that is converted into a heritable mutation when DNA undergoes replication. The ability to repair DNA DSBs generated by ionising radiation is important for survival and for maintaining genomic integrity of the cell.

Higher eukaryotic cells primarily repair DSB by one of the two genetically separable pathways, nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ operates at all cell cycle stages and predominating in G_0 phase of the cell cycle. NHEJ repair broken ends with little or no requirement for sequence homology and involves the XRCC4-LIG4 complex and the DNA dependent protein kinase (DNA-PK) holoenzyme, consisting of the DNA end-binding heterodimer Ku70/80 and the catalytic subunit DNA-PKcs. Cell lines defective in any of these genes are generally highly ionising radiation sensitive and have marked deficiency in DSB repair.

HR, which is mainly important during S and G_2 phase, utilises extensive homology to faithfully restore the sequence at the break site by processes that involve proteins of the Rad52 epistasis group. Most of the DNA breaks induced in mammalian cells are processed by NHEJ or HR within a few hours of exposure and are either rejoined, leaving normal chromosomes or misrejoined, leading to structural chromosome aberrations.

Endogenous thiols have long been thought to affect the sensitivity of cells towards radiation and chemicals. Glutathione (GSH), a tripeptide containing glutamic acid, cysteine and glycine(γ-glutamyl-cysteinyl-glycin), was one of the first chemical compounds used as a radio-protector and protection of bacterial and mammalian cells against ionizing radiation. GSH is characterized by its reactive thiol group –SH and its γ-glutamyl bond that makes it resistant to normal peptidase activity. Glutathione is essential for:

a) maintenance of the thiols of proteins (and other compounds) and of antioxidants
b) reduction of ribonucleotides to form the deoxyribonucleotide precursor of DNA, and
c) protection against oxidative damage, free radical damage and other types of toxicity.
d) Accelerate the recovery process of the lesions produced by radiation.

Buthionine sulfoximine (BSO) specifically depletes the endogenous GSH by inhibiting the enzyme γ-glutamylcysteine synthetase and increases

cellular radiosensitivity. It is demonstrated that BSO-mediated GSH depletion increased radiation induced chromosome aberration (CA) except exchange aberration. This can be explained because of the reduction in the DNA shielding effect of GSH and failure in rejoining of DNA DSB. It is observed that under the influence of higher GSH level in irradiated cells, the frequency of deletion reduced and frequency of exchanges increased. This depicts the involvement of GSH in DNA DSB joining / misjoining and acts as a cofactor in enzymatic repair process.

Bleomycin (BLM) mimics the effects of low LET radiation. For this reason, BLM-damaged DNA is frequently used as a model for radiation-induced DNA strand breaks during the study of repair or mutagenicity. Combined treatment of BLM and radiation induces higher frequency of CAs, particularly, exchange aberrations and interstitial deletions. Treatment of cells with BSO, the GSH depleting agent, showed drastic reduction in the frequency of exchange aberration and huge increase in the frequency of terminal deletions. This reduction in the effect of BLM in GSH-depleted cells could be explained on the basis of the failure of lesions interaction and also due to lack of reactivation of the oxidised BLM by the reducing agent GSH that is usually present endogenously.

In order to confirm the role of GSH in such DNA lesions interaction the present investigation has taken three different approaches :

1. Allow the interaction of lesions induced by X-rays and BLM at 4°C in presence of GSH and compare it with the similar treatment at 37°C.
2. Allow the interaction of lesions induced by X-rays and BLM in

presence of agent that selectively blocks DNA repair pathway.

3. Allow the interaction of lesions induced by X-rays and BLM in DNA-repair deficient cell lines.

Till date, synergistic effect of BLM and high LET radiation on chromosome aberration is not known. Because of the difference in the molecular nature of the damage induced by low and high LET radiation, it is interesting to study and compare the pattern of interaction of the DNA lesion induced by BLM and heavy ion.

Therefore, the objectives of this study is:

1. To evaluate the pattern of induction of delay in cell proliferation and DNA damage after low and high LET radiation exposure and the influence of GSH on it.
2. To establish the role of GSH on interaction of DNA lesions induced by high and low LET radiation.
3. Is GSH a radio-protector or radio-modifier?

We have utilised the BIO beam line of Inter University Accelerator Centre (IUAC), New Delhi. Two ion species Carbon (^{12}C) and (^{7}Li) beam is utilised for our experiment. The details of the beam are shown in the table:

Ion Species	Energy (MeV)	LET (KeV/μm)	Fluence (particles/cm^2)	Dose Equivalent (Gy)
Carbon Ion (^{12}C)	85	287	2.3×10^6 6.9×10^6	1.06 3.17
Lithium Ion (^{7}Li)	50	60	1.1×10^7 3.2×10^7	1.06 3.07

X-radiation is utilised from Faxitron Cabinet X-ray systems (Model No. 43855D, 110KVp, 3mA, Beryllium window thickness 0.76mm; Faxitron X-Ray Corp, Wheeling, IL, USA), installed in Department of Zoology, North Eastern Hill University.

In order to compare the data obtained for heavy ions and X- rays, the fluences Φ of particles can be transformed into corresponding doses using the formula:

$$\text{Dose (Gray)} = 1.6 \times 10^{-9} \times \left(\frac{dE}{dX}\right)\left(\frac{KeV}{\mu m}\right) \times F\left(\frac{P}{cm^2}\right) \times \frac{1}{\rho}\left(\frac{cm^3}{g}\right)$$

Where, dE/ dX= LET

$\rho = 1 g/cm^{-3}$ is the density of the stopping material (water)

F= the particle fluence

Role of GSH in radioprotection in high and low LET irradiated cells:

In order to study the role of GSH in radioprotection against chromosome damage, CHO cells are treated with 2mM GSH for 3 hours before irradiation. In some set of the experiment, GSH depleting chemical DL-

Buthionine-(S,R)-Sulfoximine (BSO) is added for 5 hours at a concentration of 0.2mM before irradiation. Metaphase cells were collected at 14, 28 and 42 hours of BrdU addition after radiation exposure. This time interval corresponds to the 3-generation time of control cells. The chromosome aberration (CA) was scored from first cycle metaphases.

From the study, we found that GSH has no role in radioprotection against ^{12}C and ^{7}Li beam. This can be explained, because damage from high LET radiation is primarily due to direct interaction, and because the relative yields of free radicals and reactive oxygen species decreases with increasing LET, protection against high LET radiation by GSH is more difficult to achieve. Thus, depending on the differences in the interaction of heavy ion with the cell system, GSH may not be able to exert its role as radioprotector as efficiently as it does in case of low dose of X-radiation. Exogenous addition of BSO sensitized the cells to radiation. BSO-mediated GSH depletion could reduce the shielding effect and enhance DSB induction by the free radicals produced by radiolysis due to radiation. On irradiation of CHO cells with charged particles and low LET radiation, the spectrum of aberration is dominated by deletion. Though good frequency of chromosome and chromatid-type exchange and chromatid break is also observed.

Radiation induces exchange aberrations which is thought to arise as a consequence of illegitimate reunion (misrejoining) of free ends from different DNA DSBs. The GSH pre-treated cells when exposed to ^{12}C or ^{7}Li beam, the frequency of exchange aberrations though increased but is not as high as observed in X-irradiated cells with GSH pre-treatment.

Thus, GSH may be involved or an important component of the enzymatic machinery that is needed for DSB joining of the lesions induced by X-radiation. GSH is not as efficient in DNA DSB joining of the lesions induced by ^{12}C and ^{7}Li beam and this could be attributed due to induction of more complex and clustered lesions which is being more difficult to repair. It was observed that high-LET particles induced damage was more localized and less efficiently rejoined than after X- irradiation. Indeed, the number of residual DNA breaks increased with particles LET and the delivered dose.

Analysis of the influence of GSH on cell cycle kinetics irradiated with low and high LET radiation in CHO cells has been done simultaneously with the aberration scoring. In this study, it was observed that the induction of delay in cell cycle was more in ^{12}C than ^{7}Li beam. The delay in cell cycle is related to the aberration burden of the cells. The present study is emphasized on to study the influence of GSH on high and low LET radiation induced delay in cell cycle. Both addition of GSH and BSO did not show any influence on the cell cycle kinetic pattern of the irradiated cells. Usually both GSH and BSO reduced the delay induced by low LET radiation, but in the present study it failed to show any influence on such delay. However, most surprisingly it is noted that GSH pretreatment could not reduce the delay induced by X-rays in CHO cells.

Influence of GSH in interaction of DNA DSB lesion induced by Bleomycin and high/low LET radiation:

We have made an attempt to study and compare the role of GSH on DNA DSB interaction induced by BLM in combination with X-radiation, Carbon ion beam (^{12}C LET 287 KeV/μm) or Lithium ion beam (^{7}Li LET 60 KeV/μm) in CHO cells. CHO cells are treated with 2mM of GSH for 3 hrs before irradiation. After one hr of GSH treatment, BLM is given to the cells at a concentration of 10μg/ml. The cells are incubated for 2 hrs after BLM treatment, followed by exposure to high LET radiation. The medium is decanted off from the petriplates and covered with a polypropylene sheet of 6μm thickness before irradiation. After irradiation fresh medium supplemented with serum is added to the plates and incubated for 1hr. Culture is set in presence of 6μg/ml of BrdU. Cells are harvested at 14, 28 and 42 hrs after BrdU addition. Same experimental protocol was used for low LET radiation experiments. However, the cells were harvested only at 14 and 28 hrs after BrdU addition.

Involvement of GSH in DNA DSB rejoining can be studied with analysis of increase in exchange aberration formation, which results due to illegitimate reunion of (misrejoinig) of free ends involving different DNA DSB. In case of GSH pre-treatment to BLM + high/low LET radiation exposed cells, the frequency of exchanges and deletions was increased. From our observation we can see that the elevation of the frequency of exchanges on GSH pre-treatment is higher in BLM + X-radiation treated cells than that of BLM + high LET- radiation exposed cells. The frequency of deletion did not decrease with GSH pre-treatment in BLM + high LET-

radiation exposed cells. This may be because of the complex and clustered damage produced by high LET radiation. While isolated damages are repaired efficiently, clustered DNA lesions have been suggested to be more difficult to repair, and in general are considered as DNA damages that are repair-resistant or non-repairable with a high mutagenic potential and, therefore, considered as highly significant endpoints. Thus, GSH may be unable participate in DNA DSB lesions joining/misjoining in a efficient manner which is induced by BLM + high LET radiation.

Involvement of GSH in DNA DSB joining / misrejoining pathway:

Rejoining of DNA DSB induced in DNA by ionising radiation and other physical or chemical agents can be achieved either by Homologous recombination (HR) or Non Homologous End Joining (NHEJ). NHEJ pathway is regarded as the dominant mechanism for DSB repair in vertebrates, especially in G_0 and G_1 phases of the cell cycle, although HR is also of importance particularly during S- and G_2 phases.

We chose Human Peripheral Blood Lymphocytes (HPBL) as our experimental model. It is acknowledged by the earlier workers that NHEJ is the most active repair pathway involved in the G_0 human lymphocytes. Increase in DNA DSB interaction is observed on treating the cells with BLM and radiation in HPBL in presence of exogenous GSH at RT as well as 4°C.

If the DNA DSB interaction takes place through NHEJ pathway, then increase in exchanges in presence of exogenous GSH, depicts the role of GSH in NHEJ repair pathway. In order to justify the statement that GSH

has a role to play in NHEJ pathway, we made an attempt to inhibit the crucial component of NHEJ pathway i.e. DNA-PKc by Vanillin (3-methoxy-4-hydroxybenzaldehyde). Addition of vanillin to the lymphocyte culture treated with BLM and radiation, reduced the exchanges, although not significantly.

Recently, Iliakis *et al.*, have proposed the existence of two types of NHEJ pathways: D-NHEJ (DNA-PK dependent) and a B-NHEJ (back up non-homologous end joining). While D-NHEJ is presumably involved in the fast DSB repair component with low level of DSB misrejoining, B-NHEJ is involved in the slow DSB repair component with high level of DSB misrejoinig. Therefore, it seems that vanillin, an inhibitor of DNA-PK, interferes the D-NHEJ pathway and blocks DNA misjoining at low level. Exogenously addition of GSH to the above treatment, increased the frequency of exchanges marginally. Thus, a slight increase in the exchange frequency did not provide any strong evidence of involvement of GSH in B-NHEJ pathway.

Another attempt was made to study the role of GSH on exchange aberration formation in the cells that are mutant in NHEJ repair pathway. We carried out our experiment in NHEJ deficient V33 cell line. The experiment is compared with AA8 cell line i.e. the parental line of CHO. High frequency of chromatid break and chromatid-type exchanges was induced by radiation in V33 cells. The present high frequency of chromatid type aberrations following irradiation in V33 cells could be due to accumulation of unrepaired DSB which could enter into S phase without G_1 arrest since repair deficient cells have inefficient G_1 checkpoint. Hence,

a combined deficiency of DNA DSB repair and cell cycle regulation can account for the increased exchange type aberrations in NHEJ deficient cell lines.

A high frequency of chromosome exchange is observed in irradiated V33 cells. This exchange aberration could be formed by B-NHEJ pathway which is involved in slow DSB repair component with high level of DSB misrejoining. The present V33 cells, having DNA-PK mutant, are unable to join DNA DSB by D-NHEJ pathway and therefore observed chromosome-type exchanges mainly formed by B-NHEJ pathway. Poor influence of BSO on chromosome-type exchanges was observed in V33 cells indicating the less involvement of GSH in B-NHEJ pathway. As a whole it seems that the presence of GSH before radiation increased the frequency of chromosome type exchanges in AA8 and chromatid type than chromosome type exchanges in V33 cells. Whereas in the presence of BSO during irradiation, the frequency of chromosome type exchanges was decreased in AA8 cells but not in V33 cells where the frequency of chromatid type exchanges was reduced marginally. The present data indicate the involvement of GSH in DNA DSB joining irrespective of NHEJ or HR pathway.

GSH as modulator of DNA repair:

Endogenous thiols, especially the tripeptide reduced glutathione (GSH), are known to play an important role in cellular defence against radiation. However, there are evidences suggest that GSH may not be an efficient protector of DNA. The present study will determine whether modulation

of endogenous GSH level protects or potentiates the amount of chromosomal damage induced by ionizing radiation. Human blood lymphocytes were isolated and then treated with either GSH (for 1 hr) or buthionine sulfoximine (BSO; GSH depleting agent for 5 hrs) before X-irradiation. DNA damage was analyzed by scoring chromosome aberrations (CA) and by comet assay. The level of endogenous GSH was measured in lymphocytes treated with GSH, BSO or X-rays. A roughly 8 and 21% increase in endogenous GSH level was observed after 1 and 3 hrs treatment with exogenous GSH, and this reduced the frequency of all types of CA and aberrant metaphases induced by 1 and 2 Gy of X-rays and also decreased the tail moment as determined by a comet assay, suggesting radiation protection. Such uniform protection by GSH pretreatment was not visible while cells were exposed to 3Gy or above. Interestingly, in GSH-depleted lymphocytes, the frequency of radiation induced CA was increased in a non-uniform manner. Therefore, an increase in the level of endogenous GSH in lymphocytes unable to reduce chromosomal damage induced by 3Gy or above, whereas decrease in the level of GSH enhanced the frequency of CA at all radiation doses in a non-uniform manner. It seems that GSH did not act as a radioprotector against DNA damage induced by higher dose X-rays rather it acts as a modulator of DNA repair activity.

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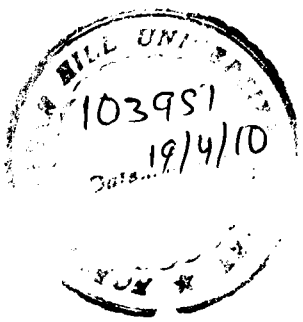

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DECLARATION

I, Geetanjali Pujari, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other university/ institute.

This is being submitted to the North Eastern Hill University for the degree of doctor of Philosophy in Zoology.

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Dedicated to my

Loving Younger brother

Late. Bikash Pujari

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Contents

Abbreviations

AGT	**Average Generation Time**
ATM	**Ataxia telangiectasia mutated**
B-NHEJ	**Backup- Non Homologous End Joining**
BLM	**Bleomycin**
BrdU	**5'-bromodeoxyuridine**
BSO	**D,L-Buthionine Sulfoximine**
^{12}C	**Carbon ion beam**
CA	**Chromosome Aberration**
CHO	**Chinese Hamster Ovary**
Chtd. Bk.	**Chromatid Break**
Del	**Deletion**
DNA	**Deoxyribonucleic acid**
D-NHEJ	**DNA-PK-dependent Non Homologous End Joining**
DNA-PKc	**DNA-dependent Protein Kinase catalytic subunit**
DSB	**Double Strand Break**
DMEM	**Dulbecco's Modified Eagles Medium**
Exch.	**Exchange**
FBS	**Foetal Bovine Serum**
FPG	**Fluorescence Plus Giemsa Technique**
GSH	**Reduced Glutathione**
GSSG	**Glutathione Disulfide**

Gy	**Gray**
HPBL	**Human Peripheral Blood Lymphocytes**
HR	**Homologous Recombination**
hrs	**Hours**
^{7}Li	**Lithium Ion Beam**
LET	**Linear Energy Transfer**
M1	**First Metaphase Cycle**
M2	**Second Metaphase Cycle**
M3	**Third Metaphase Cycle**
mM	**Millimolar**
µM	**Micromolar**
NHEJ	**Non Homologous End Joining**
OER	**Oxygen Enhancement Ratio**
PBS	**Phosphate Buffer Saline**
PHA	**Phytohaemagglutinin**
PK	**Protein Kinase**
RBE	**Relative Biological Effectiveness**
RI	**Replicative Index**
RT	**Room Temperature**
RPMI	**Rosewell Park Memorial Institute**
SCGE	**Single Cell Gel Electrophoresis**
SSB	**Single Strand Break**
SSC	**Saline sodium Citrate**
Van	**Vanillin**

GENERAL INTRODUCTION

Literature Review:

Radiation has always been present in the environment. However, mankind was not directly aware of its existence until the end of the 19th century, when a flurry of scientific discoveries was made. In 1895, Wilhelm Roentgen discovered X-rays. In 1896, Henri Becquerel discovered the spontaneous emission of radiation from uranium, a phenomenon he called 'radioactivity'. And in 1898, Marie Curie discovered radium, which is luminescent as well as having radioactive isotope (Lide 2000).

Beyond the revolution they caused in basic physics, these discoveries were put to immediate practical use. The first diagnostic x-ray was produced in January 1896, only a few months after Roentgen made his discovery. Today, the field of diagnostic radiology with its various modalities affects the great majority of people in the developed world. It is to be mentioned that the radiobiological research started in the context of medical radiology, it gradually progressed to more fundamental questions concerning the effects of different types of radiation on all types of biological systems. Consequently, radiobiological research came to involve investigators from various fields, not only biologists and clinicians, but also physicists and chemists.

In the early 20th century, ionizing radiation was used in the hope of eradicating tumors. With modern technology and scientific knowledge regarding dosimetry, the field of radiation therapy is being highly

evolved. At present, considerable efforts are being made in the field of radiobiology, to elucidate the mechanisms governing DNA damage and repair. Such knowledge could provide the means to modify radiation response in both healthy and malignant tissues, thereby improving the therapeutic effects still further.

It was also realized that ionizing radiation could have adverse health effects. An association between skin cancer and radiation among radiologists were found as early as 1902, and as much has been learned since then about the risks of radiation, mainly from the epidemiological studies on atomic bomb survivors and radiation workers. Present day radiation safety research is focused on risk assessment, e.g. studies on influence of low dose exposure of radiation of different qualities.

A long-standing paradigm in radiation biology has been that many effects induced by ionizing radiation, including its carcinogenic effects and ability to kill cancer cells, are the result of DNA damage arising from the action of ionizing radiation in cell nuclei, especially interactions of ionizing radiation and its products with nuclear DNA (Goodhead 1994; Iliakis 1991). Consistent with this view, ionizing radiation can undoubtedly damage DNA by directly ionizing DNA itself and also by indirect processes in which DNA reacts with numerous radiolytic reactive products, e.g., OH•, H•, O_2 and H_2O_2, that are generated in aqueous fluid surrounding DNA. Whether through direct or indirect process of damage, both pathways appear to make a significant contribution to DNA damage *in vivo* (Chapman *et al.* 1975).

Ionizing radiation induces numerous types of DNA damage including single and double strand breaks (SSB and DSB respectively), base damage and DNA-protein crosslinks. It is widely accepted that DNA DSBs are the critical lesions in the pathways leading from the initial energy deposition by radiation to radiobiological damage at sub-cellular and cellular levels, including gene mutations, chromosome aberration (CA), neoplastic transformation and clonogenic inactivation. The spatial correlation of DSB, both in terms of geometrical distance and in terms of 'genomic distance' (i.e. in base pairs), is thought to influence the DSB reparability.

Differences in the damage induction, especially in the yields of complex DSB and other types of clustered lesions, have been shown to correlate with the differences in the response to radiation of diverse qualities. Ionizing radiation can be grouped into two main categories: sparsely and densely ionizing radiation. The qualitative property that distinguishes the two is the spatial distribution of energy transfer in the surrounding matter. The initial events, eventually leading to radiation induced biological effects, are the ionization and excitation of atoms and molecules of the irradiated matter. X-ray and gamma photons deposit their energy via secondary electrons, set in motion by the incident photons at all depths in the target matter, and are therefore sparsely ionizing. In contrast, the energy deposition of charged particles such as α-particles and accelerated ions, is restricted to a limited volume adjacent to the primary particle track, and a more dense ionization pattern occurs.

The molecular nature of DNA damage and the production of DSB are expected to be determined by the spatial distribution of the ionization events, which itself depends on the physical properties of energy deposition and the chemical environment of the DNA. The amount of induction of DSB is dependent on **Linear Energy Transfer (LET)**.

For use in radiobiology and radiation protection the physical quantity that is useful for defining quality of radiation is LET. LET focuses attention on the linear rate of energy absorption by the absorbing medium as the charged particle traverses the medium. In 1962 the International Commission on Radiological Units (ICRU) defined LET as-

'LET of charged particles in a medium is the quotient *dE/dl,* where, *dE* is the average energy locally imparted to the medium by a charged particle of specified energy in traversing a distance of *dl'*.

Different types of radiation have different levels of LET. X- rays, γ-rays and electrons are known as low LET radiation. Neutrons, heavy ions and pions are classified as high LET radiation.

Radiobiology of heavy ions has recently attracted increasing interest in particular for its applications in two important fields. The first application is in radiation therapy of cancer and its importance in radioprotection.

Heavy charged particle is used in radiotherapy – is due to its two major advantages-

1. The particle beams exhibit a superior dose depth distribution and have a smaller lateral scattering than any other conventional

radiotherapeutic beam like photons or electrons or even neutrons. It is characteristic feature of all ion beams that the energy deposition by heavy charged particles increases with increasing penetration depth and is maximal shortly before the particles are stopped.

2. Heavy ions exhibit an elevated relative biological effectiveness (RBE) in the region of increased energy deposition, which diminishes differences in the radioresponse between fast and slowly proliferating cells. In addition, with high RBE, the repair capacity of the cells is selectively reduced.

The second application is in space radiation biology: in fact, cosmic radiation has a heavy ion component, and the understanding of its radiobiological effect is crucial for risk evaluation in space (Durante 1996).

Tumor therapy by high LET radiation can be performed either internally ie, endoradiotherapy, which includes irradiation with nuclides located directly in the tumor tissue, or, by external beam of accelerated particles such as Helium, Lithium, Carbon, Oxygen etc. Endo-radiotherapy using radioactive nucleides hold promise for the treatment of spread cancer diseases, while external irradiation with high LET beam is more suitable for deep-seated, voluminous tumors. Hypoxic cells are less radioresistant to high LET radiation than to low LET radiation. Poorly oxygenated cells are present in many tumors. There is strong evidence that hypoxic cells may reduce the probability of local tumour eradication by conventional radiation therapy (Peter 2001). In order to cure hypoxic as well as relatively radiation resistant

tumours (chordomas and chondrosarcomas of the skull base, prostate, non-small cell lung cancer) and very resistant tumours such as glioblastomas, the idea is to utilize the high LET and the high RBE of the Bragg peak of the ion beam for conformal dose delivery to the tumour tissue. This is possible with the light ion beam of Helium, Lithium, Beryllium and Boron, and even with Carbon ions, where the entrance part of the depth dose distribution is still of a relatively low LET. If the Bragg peak from such beams can be placed solely in the target volume, a high therapeutic effect on the tumour might be achieved, without serious impact on normal tissues.

The first clinical use of light ion (helium) beams was undertaken as early as in 1957 by the San Francisco Medical Centre at the Lawrence Berkeley Laboratory in California, USA. In 1977, radiotherapy with heavier ion beams (neon) was also introduced at Berkeley. Carbon, silicon and argon ion beams were also used. Since 1957, more than 3,500 patients have been treated worldwide (Turesson *et al.* 2003).

There has been a long debate over the relative biological effectiveness (RBE) of ionizing radiation of differing qualities in inducing genetic damage at equivalent low exposure levels. With radiations of high LET, the ionizations in the irradiated cell are clustered as dense tracks. In case of low LET radiation are distributed sparsely as small clusters within the cells.

In very broad terms, with low LET radiation the frequency of induced genetic damage is related to dose and dose rate. At high rate of exposure, chromosome damage increases with increasing dose roughly

in proportion to the square of the dose giving a curvilinear dose/effect response. If the radiation is given at low dose rate, the response curve may approximate to linearity between dose and effect, so that damage induced by low LET radiations may be repaired and their repair is time dependent. In contrast, with high LET radiation, little repair seems to be possible, the aberration frequency increases linearly with dose and decreasing dose rate does not reduce the effects. High LET radiations thus are far more effective than low LET radiation at low level of exposure, and RBE decreases with increasing levels of damage.

One of the difficult challenges facing radiation biology is understanding the important molecular damage induced by ionizing radiation in cellular DNA and its relationship to the biological effect. Cells exposed to radiation during G_0/G_1 phase of the cell cycle causes CA through chromosome breakage and large scale rearrangement of the pieces. Such aberrations are informative about initial radiation damage, about DNA repair/misrepair pathways and about cell nucleus ultrastructure. In addition, they are important to the main areas of applied radiobiology- biodosimetry, cell killing during radiotherapy and carcinogenesis risk estimation.

High linear energy transfer (LET) ions have been shown to induce DNA strand breaks, as well as chromosome breakage and rearrangements of high complexity involving several breaks. Most of these lesions are more frequent and, above all, more complex when induced by particles as opposed to sparsely ionizing radiations (Ritter *et al.* 1992 ; Testard *et al.* 1997).

Most DNA breaks induced in human cells by ionizing radiation are processed within a few hours of exposure and are either rejoined, leaving normal chromosomes, or misrejoined, leading to structural chromosome aberrations (Obe *et al.* 2002). Whether caused by low or high LET ionizing radiation, any form of DNA damage can lead to untoward effects in cells if unrepaired or misrepaired. With specific regard to carcinogenesis, genomic mutation caused by ionising radiation are widely thought to arise from DNA damage that is subsequently converted into a mutation as a result of processing by DNA repair mechanisms or that is converted into a heritable mutation when DNA undergoes replication. The ability to repair DNA DSBs generated by ionising radiation is important for survival and for maintaining genomic integrity of the cell.

Higher eukaryotic cells primarily repair DSB by one of two genetically separable pathways, nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ is the predominant mechanism in mammalian cells. The NHEJ pathway is active throughout the cell cycle in all vertebrate tissues, and it is a major pathway for repair of DSB during G_0, G_1 and early S phase. NHEJ involves the DNA end-binding heterodimer Ku70/Ku80, the catalytic subunit of the DNA-PK(DNA PKcs), the XRCC4 gene product and DNA ligase IV (Jeggo 1998; Karran 2000; Smith and Jackson 1999). Cell lines with mutations in any of these genes are radiation sensitive and show marked deficiencies in DSB repair (Allalunis-Turner *et al.*

1995; Biedermann *et al.* 1991; Chang *et al.* 1993), and defect in DNA PKcs inactivates V(D)J recombination.

HR, also plays a crucial role in DSB repair in vertebrate cells (Brenneman *et al.* 2000; Pierce *et al.* 1999). HR functions during late S and G_2 of dividing cells to repair DSB. HR utililizes extensive homology to faithfully restore the sequence at the break site by processes that involve proteins of the Rad 52 epistasis group (Haber 2000; Thacker 1999; Thompson and Schild 2001). In human cells, the main steps in HR are thought to be mediated by the single strand binding protein RPA; the human homologs of *Saccharomyces cerevisiae* Rad51, Rad52 and Rad54 (Baumann and West 1998); and the Rad51 paralogs XRCC2, XRCC3, Rad51B, Rad51C,and Rad51D (Thacker 1999; Thompson and Schild 1999).

Stress on the study of potential application of chemicals with radioprotective property has become necessary because of the emerging nuclear advancement. It has also been considered possible that radiation therapy for cancer patients could be improved by the use of radioprotectors to protect normal tissue. Organism survival in the presence of low-dose, low dose rate levels of background ionizing radiation suggests the occurrence of physiological adaptive mechanisms, supported by nutrients, which protect against excessive radiation damage. Early investigators attempted to use radioprotectors to help elucidate the mechanisms of interaction of radiation and molecules of biological importance. Gerschman *et al.* (1954) hypothesized that both radiation injury and oxygen poisoning occur

through the formation of reactive oxygen species (ROS). They demonstrated that sulfhydryl agents such as cysteine, glutathione, β-mercaptoethylamine (cysteamine), and other antioxidants, shown to protect mice against the lethal effects of radiation, could also increase survival of mice exposed to high oxygen tensions.

Thiols dominated the field of radiation protection from the very beginning. To elucidate the mechanism(s) of action of these chemical radioprotectors, following hypothesis have been suggested by various authors,

Sulphydryl compounds may:

1. act as scavenger of free radicals;
2. stabilize the radiosensitive molecules of the cell and thereby increase their resistance to radiation;
3. accelerate the recovery process of the lesions produced by radiation;
4. donate hydrogen atoms to the molecules damaged by free radicals and bring them back to their biologically active form;
5. create a biochemical shock in the cell which, by slowing down the metabolism, brings the cell into a state of greater radioresistance, or which brings about, by some unknown mechanism, a condition favourable to the repair of the radiation induced DNA lesions.
6. Protects cells against oxidative damage.
7. Existence as an important component of a system using pyridine nucleotides to provide a reducing atmosphere essential for the integrity of cell membranes.

8. Key role in amino acid transport and multiple metabolic pathways such as synthesis of proteins, nucleic acids and leukotrienes.
9. Regulation of enzyme activation and immune response.
10. Acting as a reservoir of cysteine. GSH is also proposed to be involved in homeostasis and detoxification of metal ion in biological system.

Glutathione (GSH) is a water-soluble tripeptide composed of the amino acids glutamine, cysteine, and glycine. The thiol group is a potent reducing agent, rendering GSH the most abundant intracellular small molecule thiol, reaching millimolar concentration in some tissues. As an important antioxidant, GSH plays a role in the detoxification of a variety of electrophilic compounds and peroxides via catalysis by glutathione-S-transferases (GST) and glutathione peroxidases (GPx). The importance of GSH is evident by the widespread utility in plants, mammals, fungi and some prokaryotic organisms (Anderson 1998).

The metabolism of GSH has been studied and demonstrated by late Alton Meister and his colleagues. Glutathione status is homeostatically controlled, being continuously self adjusting with respect to the balance between GSH synthesis (by GSH synthetase enzyme), its recycling from GSSG (by GSH reductase), and its utilization (by peroxidases, transferases, transhydrogenases and transpeptidases).

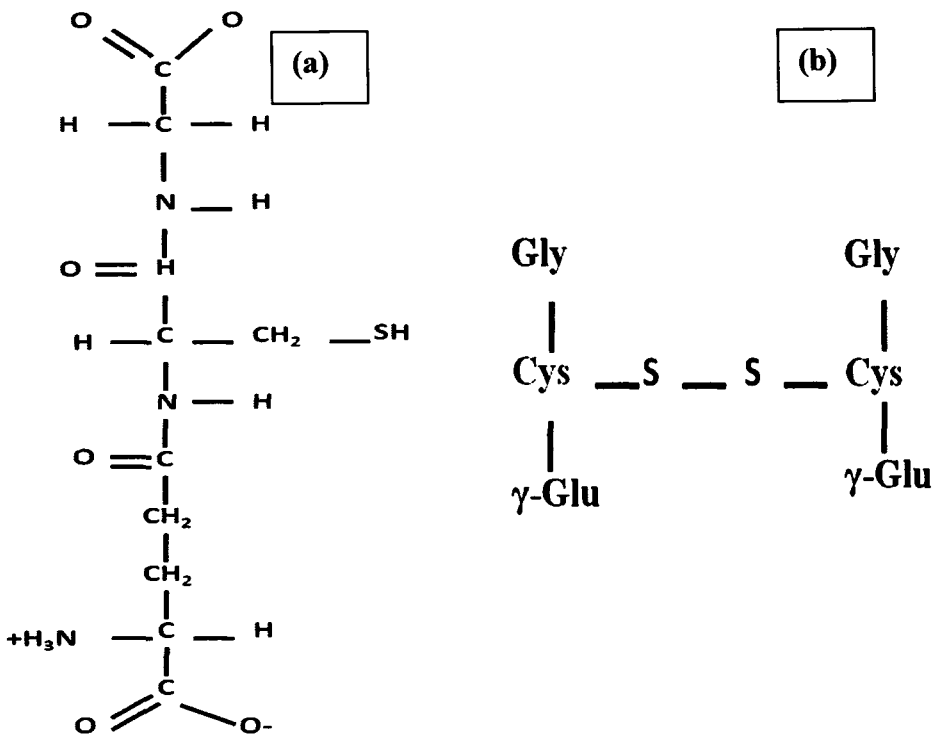


Fig (a) Structure of oxidized glutathione (GSSG) and

(b) reduced glutathione (GSH)

The intracellular GSH concentration, typically 0.5-10mM reflects a dynamic balance between the rate of GSH synthesis and the combined rate of GSH consumption within the cell and its loss through efflux. Glutathione synthesis occurs within the cells in two closely linked, enzymatically controlled reactions that utilize ATP and draw on nonessential amino acids as substrates. First, cysteine and glutamate are combined (by the enzyme γ-glutamyl cysteinyl synthetase. The γ-glutamyl cysteinyl synthetase is rate limiting for GSH synthesis and regulation of γ-glutamyl cysteinyl synthetase expression and activity is

critical for GSH homeostasis. The buildup of GSH acts to feedback-inhibit this enzyme, thereby to ensure homeostatic control over GSH synthesis.

The second GSH synthesis reaction combines gamma-glutamylcysteine with glycine to generate GSH catalyzed by GSH synthetase. Excessive accumulation of gamma-glutamylcysteine in the absence of its conversion to GSH can lead to its conversion to 5-oxoproline by the enzyme gamma-glutamyl cyclotransferase. Buildup of 5-oxoproline can have adverse consequences due to metabolic acidosis.

The GSH pool is drawn on for 3 major applications: (a) as cofactor for the GSG-S-transferases in the detoxicative pathways; (b) as substrate for the gamma-glutamyl transpeptidases, enzymes which are located on the outer cell surface and which transfer the glutamine moiety from GSH to other amino acids for subsequent uptake into the cell; and (c) for direct free-radical scavenging and as an antioxidant enzyme cofactor. The GSH transferases are a large group of isozymes that conjugate GSH with fat soluble substances as the major feature of liver detoxification.

Glutathione is an essential cofactor for antioxidant enzymes, namely the GSH peroxidases (both Se dependent and non-Se-dependent forms exist) and the more recently described phospholipid hydroperoxide GSH peroxidases. The GSH peroxidases serve to detoxify peroxides (hydrogen peroxide, other peroxides) in the water-phase, by reacting them with GSH; the latter enzymes use GSH to detoxify peroxides generated in the cell membranes and other lipophilic cell phases. This is

one instance of the water-soluble GSH providing electrons to help reduce oxidized biomolecules located away from the water phase.

Enzymes collectively known as GSH transhydrogenases use GSH as a cofactor to reconvert dehydroascorbate to ascorbate, ribo-nucleotides to deoxyribonucleotides, and for a variety of -S-S- <--> -SH inter-conversions.

After GSH has been oxidized to GSSG, the recycling of GSSG to GSH is accomplished mainly by the enzyme glutathione reductase. This enzyme uses as its source of electrons the coenzyme NADPH (nicotinamide adenine dinucleotide phosphate, reduced). Therefore NADPH, coming mainly from the pentose phosphate shunt, is the predominant source of GSH reducing power.

In mammalian tissues, GSH has a rapid metabolic turnover; for example it has a half life of 2-4 hrs in the liver (Douglas and Mortensen 1956) and 50-90 hrs in erythrocytes (Edler and Mortensen 1956). In most cases about 5% of GSH is present in oxidized form (GSSG) (Kosower and Kosower 1974). The balance between reduced and oxidized forms is maintained mainly by the action of two enzymatic systems involving glutathione peroxidase and glutathione reductase, but protein bound GSH also probably plays an important role.

GSH was one of the first chemical compounds used as a radio-protector (Latarjet and Ephrati 1948), and protection of bacterial and mammalian cells against ionizing radiation by this and sulphydryl compounds is well known (Maisin 1966). Much of research have been carried out which have proved, the tripeptide GSH play an important role in the

protection of cells against the damaging effect of ionizing radiation of low LET radiation (Chatterjee and Jacob Raman 1986; Chattopadhyay and Chatterjee 1999) and chemicals (Chatterjee *et al.* 1989; Chattopadhyay *et al.* 1997; Dev-Giri and Chatterjee 1998; Syng-ai *et al.* 2002). Evidence has accumulated on the importance of glutathione (GSH) as an inherent cellular radioprotector (Revesz *et al.* 1963; Ohara and Terasima 1969). Several reports in the literature suggest a direct correlation between glutathione (GSH) levels in cells and their radiosensitivities. Reduction of the intracellular GSH levels, by drugs that bind or oxidize it, results in enhanced radiosensitivity and an increase in the radiosensitizing effects of hypoxic cells sensitizers (Bump *et al.* 1982; Biaglow *et al.* 1983; Meister and Anderson 1983). A possibility of controlling glutathione (GSH) levels can be specifically is provided by buthionine sulphoximine (BSO). By inhibiting gamma-glutamylcysteine synthetase activity, this substance prevents GSH synthesis at concentrations that have no, or little, side effects and effectively depletes the GSH contents of cells (Griffith and Meister 1979; Dethmers and Meister 1981). A considerable enhancement of the effectiveness of some radiosensitizers has been demonstrated on such cells (Biaglow *et al.* 1983a; Hodgkiss and Middleton 1983), suggesting possible practical applications. The rate and extent of GSH depletion, and the subsequent pattern of GSH regeneration after a single dose of BSO varies in different tissues depending on the rates of GSH metabolism and pharmacokinetics of BSO. Mice treated with BSO exhibit a rapid decline in GSH in kidney, liver, pancreas and muscle

and, after prolonged treatment, also showed a reduced concentration of GSH in other tissues (Griffith and Meister 1979).

Over the past few years, advantage has been taken of the GSH-deficient cell strains in extensive studies to clarify the role of GSH in the radiation response of the cells, and especially in characterizing the radiobiological oxygen effect. Studies conducted with GSH^- cells on the *postirradiation rejoining of radiation induced SSB revealed a repair* function of endogenous GSH not heretofore recognized (Edgren *et al.* 1981). When GSH^- cells were exposed to radiation under hypoxic conditions, practically all the induced SSB were gradually rejoined within about 1hr of aerobic incubation, independently of the dose of radiation. Similar observations were made with a great number of different GSH^+ cells. In contrast, when irradiations were performed under aerobic conditions, GSH- cells failed to repair a considerable part of the induced SSB during an identical incubation period. Thus about 30% of the SSB remained unrejoined in the GSH- cells, whereas in the GSH+ cells rejoining was again practically complete.

Earlier findings have shown that under the influence of higher GSH level in irradiated cells, the frequency of deletions was reduced and the frequency of exchange aberrations was increased (Chattopadhyay *et al.* 1999). This observation is important since the role of GSH has been clearly demonstrated in DNA synthesis under certain conditions (Holmgreen 1979) and as a cofactor in enzymatic repair processes in the cells (Xue *et al.* 1988). Most animal studies of radioprotectors have employed gamma- or X-ray sources and, to a lesser extent, neutrons or

other high-LET sources. Several studies have shown that chemical agent which cause a radiation sensitivity of mammalian cells exposed to low LET radiation are less effective in changing sensitivity with high LET radiation (Barendsen *et al* 1966; Todd 1967). The biological effects of heavy charged particles have been studied extensively but limited data exist in literature regarding the role of GSH in cytogenetic damage induced in cells irradiated with high LET radiation.

Therefore, the objectives of the thesis is:

1. To evaluate the pattern of induction of delay in cell proliferation and DNA damage after low and high LET radiation exposure and influence of GSH on it.
2. To establish the role of GSH on interaction of DNA lesions induced by high and low LET radiation.
3. Is GSH a radio-protector or radio-modifier?

CHAPTER: 1

Induction of CA and delay in cell proliferation in high and low LET irradiated cells and influence of GSH on it.

Literature Review:

Studies aimed at understanding the mechanisms involved in cellular effects of heavy ion irradiation have been stimulated by the application of accelerated particles in the radiation therapy of tumors, which relies both on advantageous physical and radiobiological properties of the ion beam.

One of the most important physical characteristics of particle beams in relation to their biological effects is the in homogenous microscopic pattern of energy deposition compared to radiation of low linear energy transfer. Charged particle tracks show a high density of ionization events along the center of the particle path, and a decay of local dose deposition with increasing radial distance from the trajectory. This leads spatially localized energy deposition within the cellular targets, being the basis for high LET effects following particle irradiation.

DNA double strand breaks (DSB) are considered important with evidence suggesting that both their initial level (Prise *et al.* 1987) or residual (unrepaired) level (Frankenberg *et al.* 1981) may be related to cell lethality. Many studies have measured the relative biological effectiveness (RBE) for DSB induction for various types of radiation. It was found that the RBE values remaining around 1.0 for all types of radiation studied, including heavy ions (Weber and Flentje 1993; Heilmann *et al.* 1995). These findings have been explained on the basis of the increased complexity of lesions produced by high LET radiations due to the formation of multiply damaged sites (Ward 1995).

These are formed by the highly localized deposition of energy which are produced as densely ionizing tracks interact with the DNA.

Experimental data are accumulating which suggest that the DNA DSB may be the primary lesion leading to Chromosome aberration (Bryant 1984; Bender *et al.* 1974). Chromosome aberrations (CA) are one of the most reliable and sensitive biomarkers for estimating radiation exposure. Various studies have shown that high LET radiations are far more effective per unit dose in producing complex aberration than are X- or γ- ray. Chromosome rearrangements induced by sparsely ionizing radiations are well known and cytogenetic analyses of irradiated human lymphocytes have been widely applied to biological dosimetry (Lloyd *et al.* 1992).

Much less is known about CA induced by high LET particles. Such particles induce DNA strand breaks, as well as chromosome breakage and rearrangement of high complexity (Ritter *et al.* 1992). High LET radiation tracks produce highly localized clustered damage within the DNA and also spatially separated sites of damage along the path of the radiation track (Goodhead 1991; Goodhead *et al.* 1993; Rydberg 1996). With the introduction of Fluorescence in situ hybridization (FISH) (Pinkel *et al.* 1986), where whole chromosomes are 'painted' a high proportion of complex chromosome aberration is observed after exposure to both high-LET radiation in normal human fibroblast (Griffin *et al.*1995), peripheral blood lymphocytes (Testard *et al.* 1997) and Chinese hamster splenocytes (Grigorova *et al.* 1998) and also low

LET radiation at doses greater than 3Gy (Brown and Kovacs 1993; Tucker *et al.* 1993; Simpson and Savage 1996).

The repair of DNA damage is a critical step that could lead from the initial damage, to broken or rearranged chromosomes, cell death or cancer. In order to prevent this, the cells have evolved in various ways to restore the genome, and early evidence of the occurrence of repair of X-ray induced damage in mammalian cells was provided by Elkind and Sutton in 1960. It is well known that high LET irradiation can cause damage that is more difficult to repair, and that this largely explains the high RBE values for severe outcome. The explanation of the restricted repair capacity is believed to be a greater complexity of induced DSBs, due to clustered damage (Goodhead 1994). Indeed slower repair and a larger proportion of residual damage following high LET exposure have frequently been reported (Ritter *et al.* 1977) and non-repairable DSBs and unrejoined chromatin fragments are closely correlated to cell killing. To identify the repair dynamics involved in high LET radiation-induced DNA damage, phospho-H2AX (γH2AX) foci formation was analyzed after cellular exposure to iron ions (Fe-ions, 500 MeV 200 KeV/μm). It was observed that Fe-ion induced foci remained for longer times than X-radiation induced foci. These findings suggest that Fe-ion induced damage is repaired more slowly than X-radiation induced damage, possibly because Fe-ion induced damage or lesions are more complex or extensive (Takahashi *et al.* 2008).

The increased complexity may lead to misrejoining of breaks, ie. two lesions are rejoined together by the cellular repair mechanism. Several end-points that are believed to require misrejoining events e.g, chromosome exchanges (Durante *et al.* 1995) and interstitial deletions (Thacker 1992) are induced at increased frequencies by increasing LET values. A possible explanation may be found in the clustering of breaks seen for high LET radiation. The numerous breaks within a cluster, or in the immediate vicinity of high LET particle track, are more likely to be misrejoined, suggesting the involvement of higher order chromatin structure in the formation of CA derived from induced DSBs.

Studies on the comparative effects of high- and low- LET radiation on cultured cells must be done not only to elucidate the mechanism of action of ionizing radiation on living cells but also to establish the radiobiological basis for the high- LET radiotherapy of cancer. In case of colony-forming ability as a measure of cell damage, the effects of high- but not low- LET radiation are characterized by the reduction or absence of an initial shoulder in the cell survival curve (Deering and Rice 1962; Todd 1967; Skarsgard *et al.* 1967), diminished or no repair of sublethal radiation damage (Skarsgard *et al.* 1967), a lowered value of OER (Rice 1962, Barendsen *et al.* 1966), and a smaller variation of radiosensitivity during the cell cycle (Hall *et al.* 1972; Raju *et al.* 1975).

In addition to the central role of the repair capacity for clustered DNA DSBs in relation to the mechanism of radiation action, regulation of cell cycle progression in response to DNA damage is a subject of great

concern. Depending on the irradiation dose and especially after exposure to charged particles, extreme cell cycle delays have been reported (Scholz *et al.* 1994). The coordination of DNA repair and cell cycle arrest pathway is considered to be of prime importance for the suppression of late radiation effects like genomic instability and carcinogenesis.

Radiation doses, which can be used in the radiotherapeutic treatment of cancer, can be limited by the tolerance of normal tissues to doses that are insufficient to kill radiation-resistant hypoxic cells in tumor. An agent that could protect normal tissues from radiation damage while having less or little effect on tumor could produce a therapeutic gain. Experimental and clinical studies of radioprotective and radiosensitizing drugs are conducted to improve the cure rate of tumors. It is preferable that the protective agents selectively protect normal aerobic tissues, while the sensitizing agents selectively sensitize hypoxic tumor cells (Yuhas and Storer 1969).

Cells contains a complex inter-related antioxidant defence system consisting of non protein thiols (Glutathione, Cysteine), protein thiols, SOD, catalase, NADPH, Vitamin C and E, and various enzyme systems; these serve to protect against free radicals generated during normal oxidative metabolism or radiation exposure. The presence of exogenous thiol compound at the time of irradiation has been shown to protect enzymes and cells *in vitro* as well as animals *in vivo* against radiation-induced lethality.

We have concentrated our efforts on establishing the radioprotective action of the intracellular thiols species, Glutathione (GSH), which constitutes 90% of the total non-protein thiol pool in most cells. Glutathione, is synthesized within the cells, is a component of a pathway that uses NADPH to provide cells with their reducing mileu. This is essential for (a) maintenance of the thiols of proteins (and other compounds) and of antioxidants (eg. Ascorbate, alpha-tocopherol), (b) reduction of ribonucleotides to form the deoxyribonu- cleotide precursor of DNA, and (c) protection against oxidative damage, free radical damage and other type of toxicity.

It has been proposed that GSH within the cell nucleus and in particular when close to DNA is important in determining cellular radiosensitivity (Edgren 1987, Prise *et al.* 1992). In order to study and understand the role of GSH, there is a need to manipulate the level of exogenous GSH concentration. Buthionine sulphoximine (BSO) is a reversible inhibitor of γ-glutamyl cysteine synthetase, the key enzyme in GSH biosynthesis (Griffith and Meister 1979). Administration of BSO leads to decreased GSH levels in virtually all tissues including developing embryos (Hales and Brown 1991). Although a decrease in the level of cellular glutathione is not lethal, however, it has been shown to enhance the cytotoxicity of a variety of agents, including ionising radiation and heavy metals. Extensive work has been carried out on the role of GSH as radioprotectant in mammalian cell system. In Chinese Hamster Ovary cells in vitro, BSO treatment (resulting in 80% GSH depletion) has no effect on aerobic radiosensitivity, although

there is an increase in hypoxic radiosensitivity (Clark *et al.* 1983). This suggests that BSO competes with oxygen for the reactive species generated, resulting in reduced radiation damage. However, the same reaction in the absence of oxygen enhances the anoxic pathway of radiation damage. It is observed that depletion of GSH by BSO reduces the frequency of chromosome aberration produced by radiomimetic chemical bleomycin in human lymphocytes (Chattopadhyay *et al.* 1997) and increases the frequency of chromosome aberration induced by arecoline (Deb and Chatterjee 1998) and mitomycin C (Dev-Giri and Chatterjee 1998) in mouse bone marrow cells. It has been shown that there was a significant reduction in the frequency of micronuclei induced by 1Gy γ-rays after BSO treatment in polychromatic erythrocytes of mouse bone marrow cells although the GSH level after 2hrs BSO (20mg/Kg) treatment were unaltered (Sarma *et al.* 1996). BSO has been used to evaluate the effect of GSH depletion on radiosensitization of mouse bone marrow cells *in vivo* and human peripheral blood lymphocytes *in vitro*. Work carried out by Chattopadhyay *et al.* (1999) showed that presence of BSO increased cellular radiosensitivity in mouse bone marrow cells and blood lymphocytes with respect to chromosome aberration. It was also observed that the exchange type of aberrations reduced in BSO-pretreated cells in spite of significant enhancement of chromatid and deletion-type of aberrations. It is hypothesized that the increase in frequency of radiation induced CA apart from exchange aberrations in

BSO-treated cells could be due to reduction of DNA shielding effect, failure in rejoining of DNA DSB free ends, and apoptosis.

Work has been performed to study the influence of endogenous GSH level on radiation-induced delay through the cell cycle. It is observed that addition of exogenous GSH removed the radiation induced delay more convincingly in early first-division cells than late division cells. GSH pre-treatment reduced the radiation–induced CA significantly in late arising metaphases irradiated G_0 stage but failed to reduce delay in the course of the cell cycle. It was observed that the degree of protection of CA by GSH-pretreatment was lower in G_1 irradiated cells than in G_0 irradiated cells. It is also observed that BSO-treatment enhanced the frequency of CA almost uniformly at all stages of the cell cycle, nevertheless the extent of radiation-induced delay was not enhanced. The presence of GSH reduced the level of both p53 and p21 proteins. Such reduced level of p53 and p21 could also be the additional factor for GSH-mediated reduction in radiation induced delay in the cell cycle, besides protection of cell membrane and cytoplasmic proteins. On the other hand, irradiation to BSO-treated cells increased the frequency of CA, showed higher level of p53 and p21 and maintained delay in the cell cycle induced by irradiation (Ray and Chatterjee 2007).

Fragmentary report exists in literature that could explain the role of GSH as radioprotector against high LET radiation. As studies have shown that chemical agents which cause a pronounced change in the radiation sensitivity of mammalian cells exposed to low-LET radiation

are less effective in changing sensitivity with high LET radiations. These studies include OER measurement made over a range of LET values (Barendsen *et al.* 1966; Todd 1967) and measurements of the effectiveness of the radioprotective agent cysteamine, also for a range of LET values (Barendsen and Walter 1964). Experiment performed to measure the radiation protection of cysteamine for high-LET radiations in experimental modal V79 showed that the extent of protection decreased with increasing LET value.

Work has been carried out to study the effect of high linear energy transfer (LET) radiation on DNA *in vitro*, both in protective and non-protective environments. Two hydroxyl radical scavengers, tris(hydroxymethyl) aminomethane and 2-mercaptoethanol, were compared for their ability to protect SV40 DNA from radiation damage over a wide LET range. In general, a decrease in single-strand breaks (SSBs) relative to double-strand breaks (DSBs) was observed as LET increased. This effect was more pronounced when a radioprotector was present. Comparison of the relative biological efficiency (RBE) of radiation damage as LET increased showed a peak of DSB production in the mid-LET range. An explanation for this increase in DSB production efficiency has been proposed based on the particle track structure of high-LET radiation (Stanton et al 1992).

The extent of DNA damage and effect on cell cycle proliferation induced by low LET radiation with respect to GSH status of the cell has been explored much extensively. There is no information available on the role of GSH on high LET radiation induced CA and cell cycle

delay in mammalian cell system. Therefore, it will be interesting to compare the influence of GSH on the effect of low and high LET radiation induced DNA damage and delay in cell proliferation. To examine the mechanistic basis for the LET-specific cytogenetic changes we determined the frequency and type of CA induced by ^{7}Li ion, ^{12}C ion and X-radiation.

Materials and Methods

Experimental model:

Our study was performed in Chinese Hamster Ovary (CHO) Cell line.

Chinese Hamster Ovary (CHO) Cell Line Culture:

Chemicals for cell culture:

- Dulbecco's Modified Eagles Medium (DMEM), Penicillin-Streptomycin, Trypsin-EDTA, JRH Biosciences, USA.
- Foetal Calf Serum (FCS), Biological Industries, Israel.
- Glutathione Reduced, Sigma, USA, used at a concentration of 2 mM.
- DL- Buthionine-(S,R)-Sulfoximine (BSO), Sigma, USA, used at a concentration of 200μM.
- 5-bromo-2-deoxyuridine (BrdU), Sigma, USA; BrdU powder is dissolved in autoclaved Millipore water to prepare a working solution of 100μg/ml.
- Colcemid, Gibco USA.
- Bis-benzimide (Hoechst 33258), Sigma, USA, used at a concentration of 50μg/ml in double distilled water.

- Giemsa Stain Solution, BDH Chemicals Ltd, UK.

All other chemicals used are of analytical grade.

CHO Cell line is ordered from National Centre for Cell Science (NCCS) Pune, India. The cells are routinely cultured in DMEM supplemented with 10% Foetal Calf Serum and 1% antibiotic Penicillin and Streptomycin. The cells are incubated in 5% CO_2. Inverted phase contrast microscope is used for visualizing the cells. Culture is examined daily to observe the morphology, color of medium and density of the cells. Cells are irradiated as a monolayer culture.

Irradiation

Low - LET Radiation:

Throughout this work we have used Faxitron Cabinet X-ray systems (Model No. 43855D, 110KVp, 3mA, Beryllium window thickness 0.76mm; Faxitron X-Ray Corp, Wheeling, IL, USA), for X-irradiation.

High LET ions:

The Pelletron at the Inter University Accelerator Centre (IUAC), New Delhi, provided the accelerated ions. Two different ions ^{12}C and ^{7}Li were extracted from the ion source and accelerated to energies of 85 and 50 MeV respectively. Beam properties are listed in the Table.

Table 1.1: Beam properties

Ion Species	Energy (MeV)	LET (KeV/μm)	Fluence (particles/cm^2)	Dose Equivalent (Gy)
Carbon Ion (^{12}C)	85	287	2.3×10^6	1.06
			6.9×10^6	3.17
Lithium Ion (^{7}Li)	50	60	1.1×10^7	1.06
			3.2×10^7	3.07

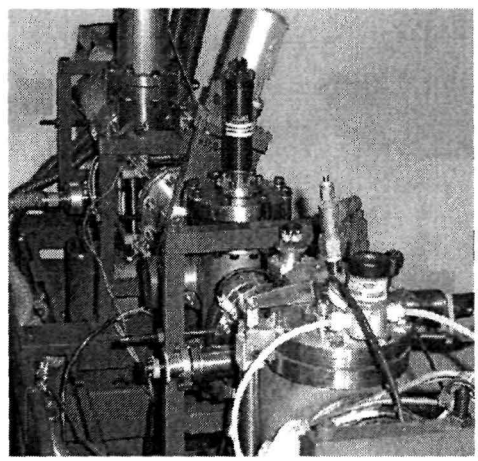


Fig 1.1: Part of Vault Area

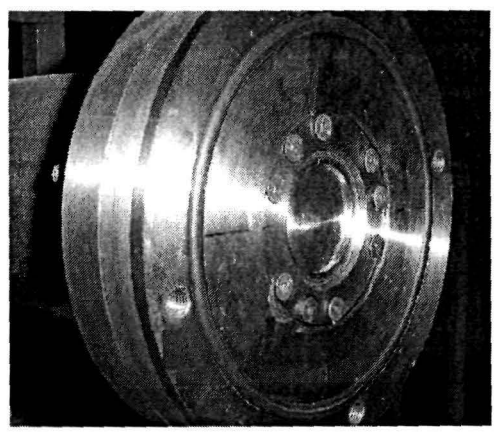


Fig 1.2: Exit window of the beam line

The ion beams were directed, from the accelerator to the BIO beam line through a high vacuum beam transport system. The primary heavy ion beam from the pelletron is diffused using a gold foil and low flux beam is obtained at the exit window made of 6.25mg/cm^2 thick aluminium foil. A Silicon surface barrier (SSB) detector is placed at the position where the sample is irradiated and the beam energy is measured. The positive current signal from the diffuser foil was integrated using a current integrator. The ratio of current signal to that of flux measured using SSB detector at the same position is measured by multiple trials and the calibration factor is obtained. The current signal is fed to a preset controller to terminate the beam after irradiation using a Faraday cup.

In order to compare the data obtained for heavy ions and X- rays, the fluences Φ of particles can be transformed into corresponding doses using the formula:

$$\textbf{Dose (Gray)} = 1.6 \times 10^{-9} \times \left[\frac{dE}{dX}\right] \left[\frac{KeV}{\mu m}\right] \times F \left[\frac{P}{cm^2}\right] \times \frac{1}{\rho} \left[\frac{cm^3}{g}\right]$$

Where, dE/ dX= LET

ρ = 1g/cm^{-3} is the density of the stopping material (water)

F= the particle fluence

Target Preparation:

Cells are seeded at a density of 0.8×10^6 in 35mm diameter cell culture petriplates, 15 hrs prior to radiation exposure so that the cells reached 80% confluent state. Ten hrs after cells were seeded, BSO at a concentration of 0.2mM was added for 5 hrs before irradiation. In case of reduced GSH, it was added 12 hrs after cells were seeded at a concentration of 2mM for 3 hrs before radiation. At the time of irradiation, the medium is decanted off from the petriplates. A 6μm thick polypropylene film is used to cover the plates. The cells were irradiated under sterile condition at atmospheric pressure and were exposed to the ions through the polypropylene film. Soon after irradiation fresh medium supplemented with 10% FCS is added to the cells. BrdU is added to the medium at a concentration of 6μg/ml. The cultures are incubated at 37°C, 5% CO_2 and 95% humidity. The cells are harvested at 14, 28 and 42 hours after BrdU addition.

Metaphase Chromosome Preparation and differential staining

Cells are given 2 hrs of colcemid treatment (0.01μg/ml) before harvesting to accumulate mitotic metaphase cells. In order to differentiate between metaphases in the first, second or third post irradiation cycle, the fluorescence-plus-Giemsa (FPG) staining technique according to Perry and Wolff (1974) was applied. Chromosome preparations were made according to standard techniques, i.e. cells were trypsinised, treated for 15 min with

hypotonic solution (0.075M KCl) and fixed in methanol: acetic acid (3:1). After three washes in fixative, the cell suspension was dropped in chilled wet slides.

After 24 hrs of slide preparation, the cells were stained for 15min with Hoechst 33258 at a concentraton of 50μg/ml in dark. The slides were then rinsed in distilled water and mounted in 2 X SSC (Saline Sodium Citrate, pH 6.8) and kept in sunlight for about 30-40 mins depending upon the intensity of light. The slides are then rinsed in distilled water and stained in 3% Giemsa for 5-6 mins, followed by rinsing in distilled water. The slides are dried and mounted in DPX.

By FPG technique, BrdU, which is a thymine analogue, present on the newly synthesized strand, binds to Hoechst 33258 in the presence of UV. Therefore, when Giemsa staining is done, one arm of the chromosome appears darkly stained and the other arm is lightly stained. This helps in distinguishing between the first, second and subsequent cell division cycles.

Scoring and statistical analysis:

Slides were coded at random. The aberration studied were Chromosome exchanges, Chromatid exchanges, Deletion and Chromatid breaks. All aberrations were scored from first cycle metaphase since the IAEA guidelines suggest that reliable data can be obtained if fixation is made in the maximal part of the first mitotic wave (Bianchi *et al.* 1982). The number of second, third and subsequent metaphases were also noted for

the calculation of the Average Generation Time (AGT) in order to determine the cell proliferation kinetics.

AGT is the ratio of BrdU duration in hours and the Replicative Index (RI) of the cells.

$$\mathbf{AGT=} \frac{\textbf{BrdU duration (hrs)}}{\textbf{Replicative Index (RI)}}$$

Where, RI= (1×M1+2×M2+3×M3)/ Total number of cells scored.

The statistical significance of the difference between the control and the treated groups were determined. Aberrant metaphases were tested using the 2×2 Contingency χ^2-test. The different types of aberrations studied were compared using the simple χ^2 test.

Fig 1.3: 1st Cycle Metaphase

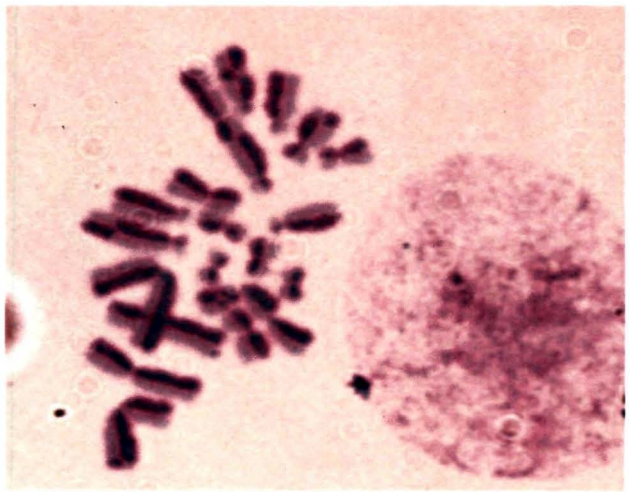


Fig 1.4: 2nd Cycle Metaphase

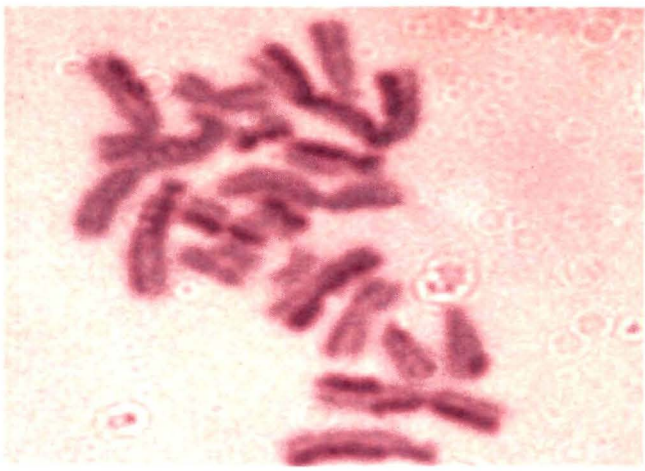


Fig 1.5: 3rd Cycle Metaphase

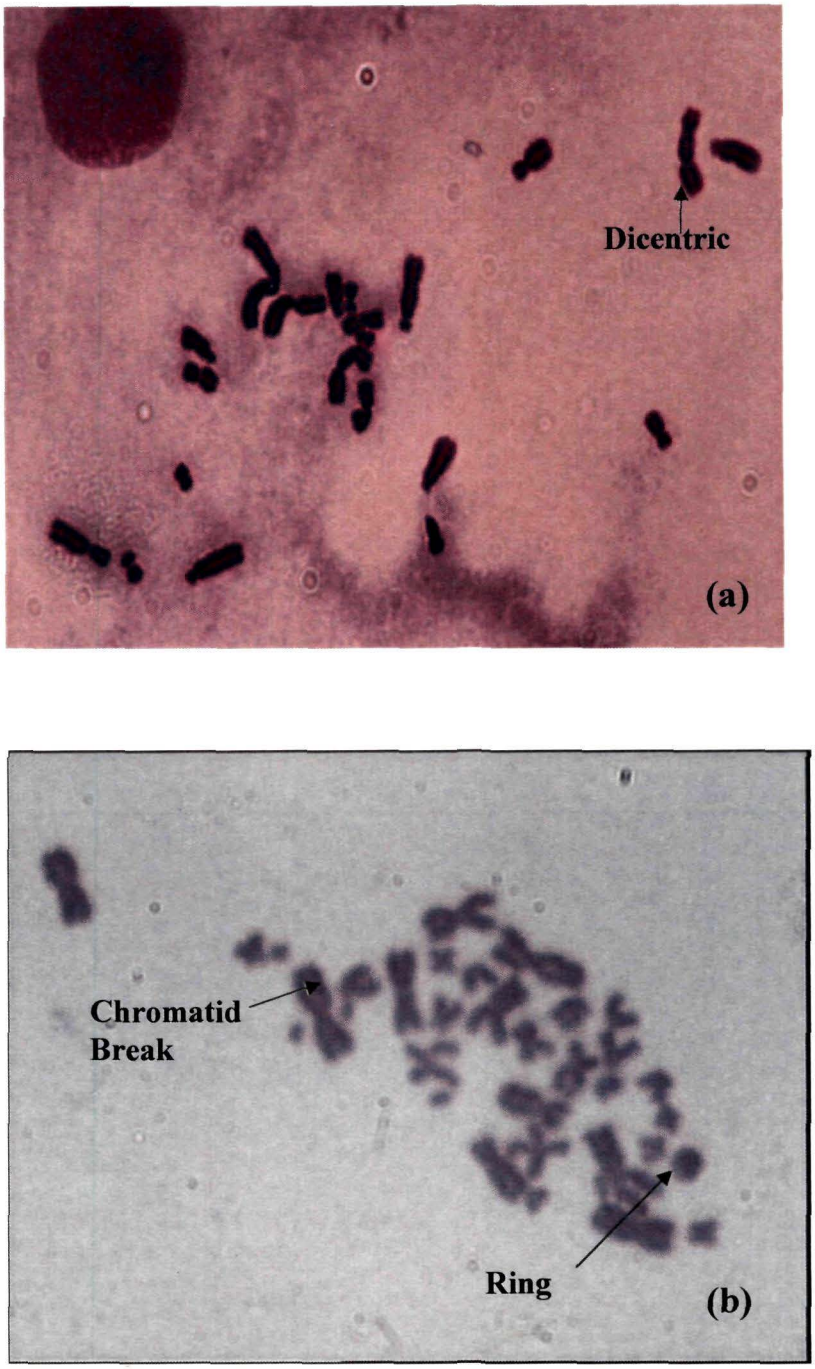


Fig 1.6: Aberration in CHO cell line irradiated with heavy ion beam (a) Dicentric (b) Ring and Chromatid break.

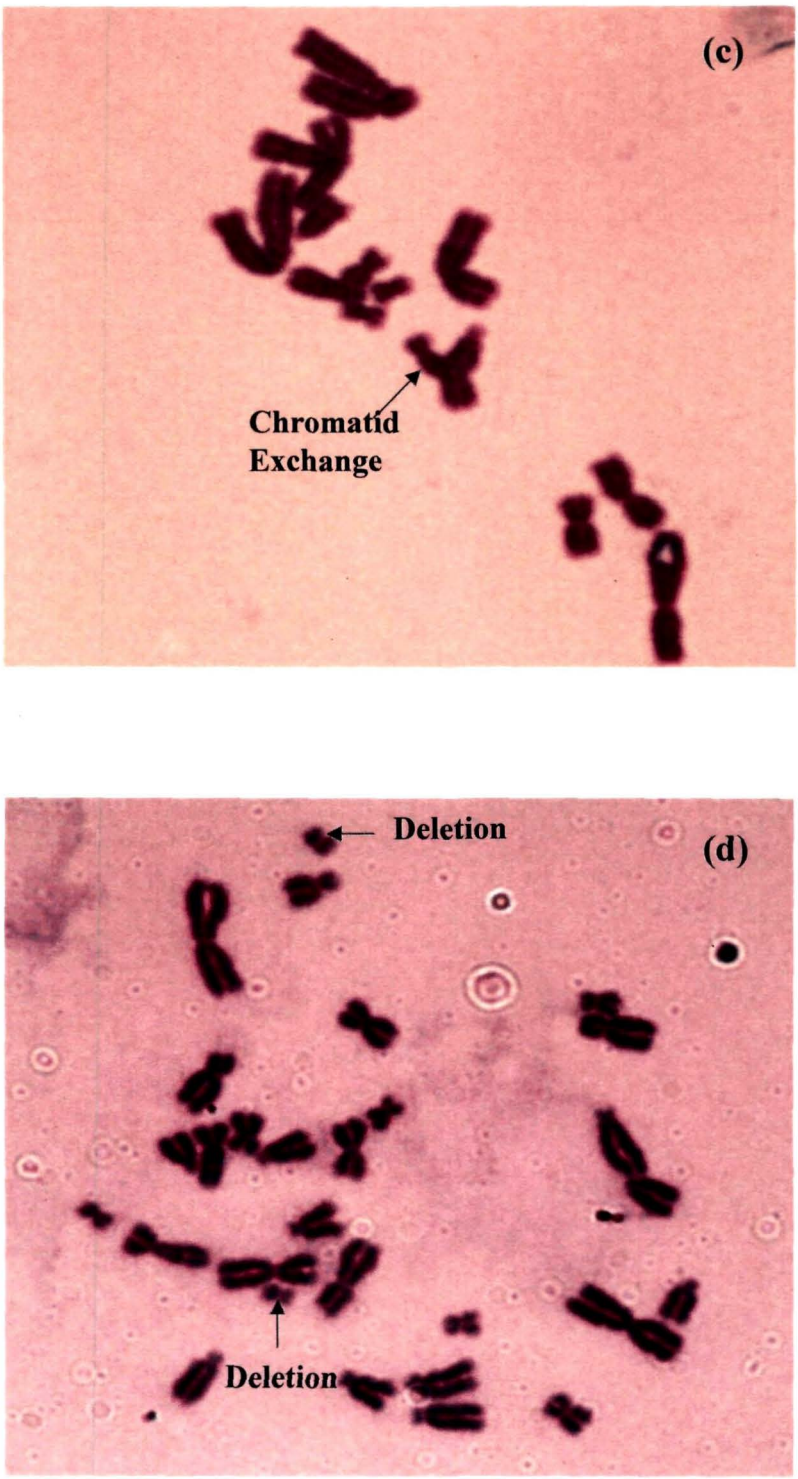


Fig 1.7: Aberration in CHO cell line irradiated with heavy ion beam (c) Chromatid exchange and (d) Deletions

Result:

1. CA of ^{12}C, ^{7}Li and X-irradiated CHO cells with respect to GSH status:

Table 1.2: CA in CHO cells irradiated with ^{12}C beam (LET 287 KeV/μm) with respect to GSH status.

Exptal. Condition	Aberrant Metaphase %	Total Metaphase	Chrom. Exch. %	Chtd. Exch. %	Del. %	Chtd Bk. %
14 Hours						
Untreated	7	109	0	0	7	2
1.06 Gy	61	120	19	8	42	7
GSH+1.06 Gy	66	203	30	14	39	5
BSO+1.06 Gy	67	102	10	7	76@	8
3.17 Gy	76	111	32	10	52	2
GSH+3.17 Gy	77	151	40	15	44	6
BSO+3.17 Gy	75	110	29	6	82@	20
Untreated	5	111	0	0	5	4
1.06 Gy	56	117	10	8	46	4
GSH+1.06 Gy	66	154	20	12	50	1
BSO+1.06 Gy	63	84	5	0	76@	5
3.17 Gy	71	87	15	8	66	3
GSH+3.17 Gy	75	89	26	10	76	4
BSO+3.17 Gy	71	107	8	9	101@	4
28 Hours						
1.06 Gy	74	81	25	12	56	6
GSH+1.06 Gy	76	46	36	13	48	4
BSO+1.06 Gy	78	46	17	9	148$	4
3.17 Gy	100	50	34	16	86	10
GSH+3.17 Gy	97	38	39	18	89	13
BSO+3.17 Gy	100	32	28	16	169@	9
1.06 Gy	70	54	17	4	85	2
GSH+1.06 Gy	77	62	26	2	71	3
BSO+1.06 Gy	77	44	11	0	113$	7
3.17 Gy	91	128	24	10	147	16
GSH+3.17 Gy	100	51	38	15	166	18
BSO+3.17 Gy	87	54	21	7	176@	11

@ Significant at $P<0.05$, # Significant at $P<0.01$, $ Significant at $P<0.001$ simple $\chi 2$ test (compared to respective control).

Chrom. Exch.= Chromosome Exchange; Chtd. Exch.= Chromatid Exchange; Del.= Deletion; Chtd. Bk.= Chromatid Break.

Table 1.3: Pooled data of CA in CHO cells irradiated with ^{12}C beam (LET 287 KeV/μm) with respect to GSH status.

Fixation Time (hrs)	Exptal Condn.	Abberant Metaphase %±SEM	Total Cell	Chrom. Exch. %±SEM	Chtd. Exch% ±SEM	Del. % ±SEM	Chtd. Bk%± SEM
14	Untreated	6±1	220	0	0	6±1	3±1
	1.06 Gy	59±3	237	15±5	8±0	44±2	6±2
	GSH+1.06 Gy	66±0	357	25±5@	13±1@	45±6	3±2
	BSO+1.06 Gy	65±2	186	8±3	4±4	76±0#	7±2
	3.17 Gy	74±3	198	24±9	9±1	59±7	3±1
	GSH+3.17 Gy	76±1	240	33±7	13±3	60±16	5±1
	BSO+3.17 Gy	73±2	217	19±11	8±2	92±10@	12±8
28	1.06 Gy	72±2	135	21±4	8±4	71±15	4±2
	GSH+1.06 Gy	77±1	108	31±5	8±5	60±12	4±1
	BSO+1.06 Gy	78±1	90	14±3	5±5	131±12#	6±2
	3.17 Gy	94±3	178	29±5	13±3	117±30	13±3
	GSH+3.17 Gy	99±2	89	38±1	17±2	128±39	16±3
	BSO+3.17 Gy	94±7	86	25±1	12±5	173±4@	10±4

@ Significant at P<0.05, # Significant at P<0.01, $ Significant at P<0.001 simple $\chi 2$ test (compared to respective control).

Chrom. Exch.= Chromosome Exchange; Chtd. Exch.= Chromatid Exchange; Del.= Deletion; Chtd. Bk.= Chromatid Break.

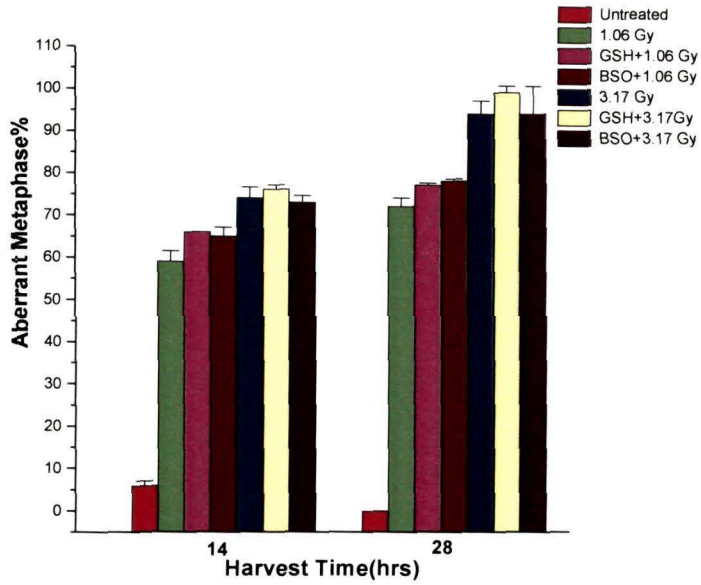


Fig 1.8: Aberrant metaphase percentage in CHO cells irradiated with ^{12}C beam of LET 287 KeV/μm with respect to GSH status.

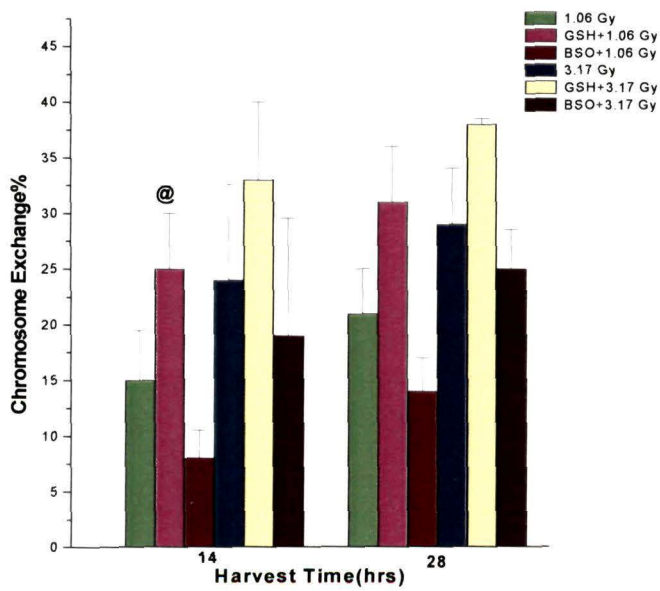


Fig 1.9: Chromosome exchange percentage in CHO cells irradiated with ^{12}C beam of LET 287 KeV/μm with respect to GSH status.

Table 1.4: CA in CHO cells irradiated with ^{7}Li beam (LET 60 KeV/μm) with respect to GSH status.

Exptal. Condition	*Aberrant Metaphase %*	*Total Metaphase*	*Chrom. Exch. %*	*Chtd. Exch. %*	*Del %*	*Chtd. Bk. %*
14 Hours						
Untreated	7	120	0	0	7	3
1.06 Gy	51	109	14	2	61	15
GSH+1.06 Gy	62	92	24	3	21	4
BSO+1.06 Gy	55	112	9	9	80#	4
3.07 Gy	67	121	24	3	47	12
GSH+3.07 Gy	74	117	29	16#	53	4
BSO+3.07 Gy	71	113	18#	12	79@	4
Untreated	3	109	0	0	1	2
1.06 Gy	44	103	5	4	35	3
GSH+1.06 Gy	50	129	18	7@	36	2
BSO+1.06 Gy	50	103	8	3	52@	5
3.07 Gy	63	120	14	9	59	8
GSH+3.07 Gy	66	89	28	13	63	7
BSO+3.07 Gy	68	100	14	7	84@	10
28 Hours						
1.06 Gy	61	21	19	0	71	5
GSH+1.06 Gy	77	26	27	0	62	8
BSO+1.06 Gy	70	33	17	7	97@	2
3.07 Gy	94	51	27	14	120	8
GSH+3.07 Gy	97	36	33	8	129	0
BSO+3.07 Gy	91	43	23	2	165@	0
1.06 Gy	65	25	16	0	64	8
GSH+1.06 Gy	74	31	23@	0	65	6
BSO+1.06 Gy	76	21	5	0	95@	10
3.07 Gy	88	32	25	9	72	16
GSH+3.07 Gy	94	31	32	19	84	6
BSO+3.07 Gy	84	25	20	8	100@	8

@ Significant at $P<0.05$, # Significant at $P<0.01$, $ Significant at $P<0.001$ simple $\chi 2$ test (compared to respective control).

Chrom. Exch.= Chromosome Exchange; Chtd. Exch.= Chromatid Exchange; Del.= Deletion; Chtd. Bk.= Chromatid Break.

Table 1.5: Pooled data of CA in CHO cells irradiated with ^{7}Li beam (LET60 KeV/μm) with respect to GSH status.

Fixation Time (hrs)	Exptal Condn.	Abberant Metaphase %±SEM	Total Cell	Chrom. Exch. %±SEM	Chtd. Exch% ±SEM	Del. % ±SEM	Chtd. Bk% ±SEM
14	Untreated	5±2	229	0	0	4±3	3±1
	1.06 Gy	48±4	212	10±5	3±1	36±1	9±6
	GSH+1.06 Gy	56±6	221	21±3@	5±2	29±8	3±1
	BSO+1.06 Gy	53±3	215	9±1	6±3	66±14#	5±1
	3.07 Gy	65±2	241	19±5	6±3	53±6	10±2
	GSH+3.07 Gy	70±4	206	29±1	15±2	58±5	6±2
	BSO+3.07 Gy	70±2	213	16±2	10±3	82±3@	7±3
28	1.06 Gy	63±2	46	13±3	0	68±4	7±2
	GSH+1.06 Gy	76±2	57	25±2	0	64±2	7±1
	BSO+1.06 Gy	73±3	54	11±6	7±0	96±1@	6±4
	3.07 Gy	91±3	83	26±1	3±1	96±24	12±4
	GSH+3.07 Gy	96±2	67	33±1	14±6	107±21	3±3
	BSO+3.07 Gy	88±4	68	22±2	5±3	133±31@	8±8

@ Significant at P<0.05, # Significant at P<0.01, $ Significant at P<0.001 simple $\chi 2$ test (compared to respective control).

Chrom. Exch.= Chromosome Exchange; Chtd. Exch.= Chromatid Exchange; Del.= Deletion; Chtd. Bk.= Chromatid Break.

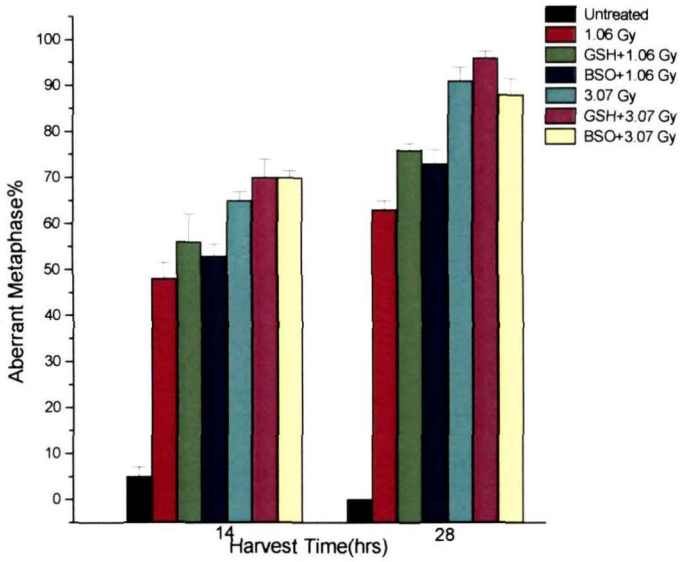


Fig 1.10: Aberrant metaphase percentage in CHO cells irradiated with ^{7}Li beam of LET 60 KeV/μm with respect to GSH status.

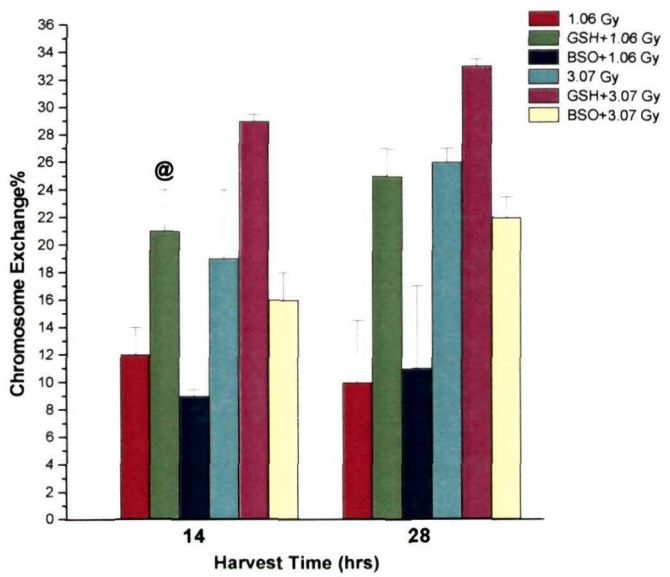


Fig 1.11: Chromosome exchange percentage in CHO cells irradiated with ^{7}Li beam of LET 60 KeV/μm with respect to GSH status.

Table 1.6: CA in CHO cells irradiated with X-radiation with respect to GSH status.

Fixation Time	Exptal Condn.	Abberant Metaphase	Total Cell	Chrom. Exch.%	Chtd. Exch%	Del. %	Chtd.Bk %
14 Hrs	Untreated	4	108	0	0	0	4
	1Gy	17	93	4	0	19	12
	GSH+1Gy	17	117	12	3	7	8
	BSO+1Gy	26	87	3	0	34@	14
	3Gy	48	83	25	2	36	13
	GSH+3Gy	51	86	40@	12	23	14
	BSO+3Gy	58	103	17	0	42	15
	6Gy	74	81	38	6	69	3
	GSH+6Gy	81	84	45	8	55	2
	BSO+6Gy	89	79	32	0	80@	13
28Hrs	Untreated	0	10	0	0	0	0
	1Gy	25	72	4	0	29	13
	GSH+1Gy	22	86	8	1	21	9
	BSO+1Gy	31	96	2	0	50@	1
	3Gy	64	166	36	4	42	1
	GSH+3Gy	67	196	49@	6	31	5
	BSO+3Gy	71	84	24	5	60	4
	6Gy	86	108	43	6	74	17
	GSH+6Gy	92	87	51	9	69	6
	BSO+6Gy	89	98	34	4	79	21

@ Significant at $P<0.05$, # Significant at $P<0.01$, $ Significant at $P<0.001$ simple $\chi 2$ test (compared to respective control).

Chrom. Exch.= Chromosome Exchange; Chtd. Exch.= Chromatid Exchange; Del.= Deletion; Chtd. Bk.= Chromatid Break.

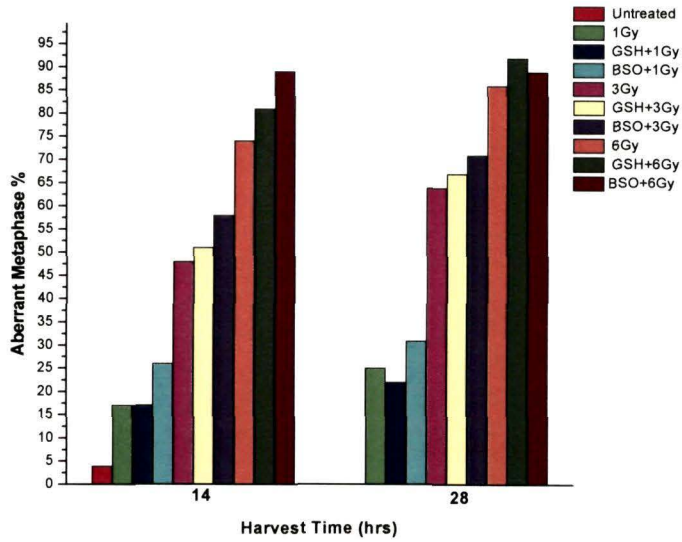


Fig 1.12: Aberrant metaphase percentage in CHO cells irradiated with X-radiation with respect to GSH status.

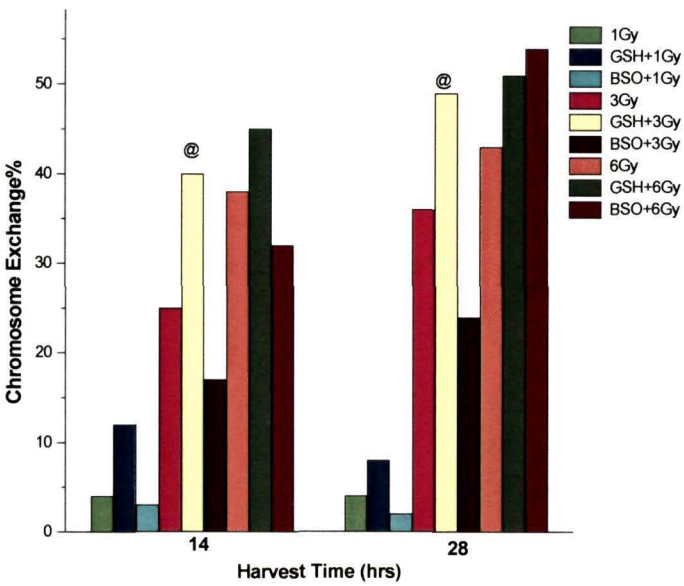


Fig 1.13: Chromosome exchange percentage in CHO cells irradiated with X-radiation with respect to GSH status.

CA induction by high LET and X-radiation

Chromosomal damage induced in CHO cells by ^{12}C, ^{7}Li and X-radiation, was examined at 14, 28 and 42 hrs of fixation time. Following differential staining the analysed cells were identified to be either at first, second or third post-irradiation mitosis. Since the number of cells in first metaphase is relatively lesser in 42 hrs harvested cells, the aberrant metaphase data is ignored at 42 hrs. We have considered the 42 hrs-harvested cells for the analysis of cell cycle kinetics only.

On comparison we could observe that carbon beam (LET 287 KeV/μm) is more effective in inducing CA than lithium beam (LET 60 KeV/μm). CA induced by X-radiation is comparatively lesser than Lithium ion irradiated cells. CA can be studied from the Table 1.3, 1.5 and 1.6, at 14 hrs, the aberrant metaphase percentage of 1.06 Gy of ^{12}C beam exposed CHO cells is 59±3; the induction of CA percentage by 1.06 Gy ^{7}Li beam is 48±4 and only 17% for X-radiation at dose of 1Gy. On increasing the dose to 3.17 Gy of ^{12}C, the frequency of aberrant metaphase was increased to 74±3. Dose of 3.07 Gy of ^{7}Li beam induced 65±2% of aberrant metaphase and 1 Gy of X-radiation induced 48% aberrant metaphase.

There is a steep increase in the frequency of aberrations with the increase in cell fixation time from 14 to 28 hrs in cells irradiated with any of the three radiation types. The increment in aberration percentage with increase in harvest time is more pronounced on heavy ion irradiated cells than in cells exposed to X-radiation.

In order to study the role of Glutathione (GSH) in radioprotection against chromosome damage, we treated the cells with 2mM of GSH for 3 hrs before irradiation. In some set of the experiment, GSH depleting chemical DL- Buthionine-(S,R)-Sulfoximine (BSO) is added for 5 hrs at a concentration of 200μM before irradiation.

Role of GSH as radioprotector against CA in CHO cells exposed to ^{12}C and ^{7}Li beam:

From the data we could observe that exogenous addition of GSH has no role to play in radioprotection against DNA damage in ^{12}C and ^{7}Li beam irradiated cells. The aberrant metaphase percentage increased from 59±3 to 66±0 on exogenous GSH addition prior to exposure of cells to 1.06 Gy of ^{12}C beam irradiation. Increase in aberrant metaphase percentage from 48±4 to 56±6 in pre-treatment of GSH in 1.06 Gy of ^{7}Li irradiated cells was also observed. Similar trend was also observed on GSH addition to cells at higher dose (~ 3Gy) of heavy ion irradiated cells. On fixing the cells at 28 hrs, no protection was observed by exogenous GSH addition to the cells prior to heavy ion irradiation.

Role of GSH as radioprotector against CA in CHO cells exposed to X-radiation:

We could observe that at 14 hrs fixation, exogenous GSH did not show any protection in the frequency of aberrations induced by X-rays to CHO cells. On increasing the harvest period to 28 hrs, we could

observe marginal protection by exogenous GSH addition in 1Gy of X-irradiated cells.

At 3 and 6 Gy, exogenous supply of GSH failed to show protection against CA. The aberration frequency increased from 64% to 67% on GSH addition in 3Gy X-irradiated cells harvested at 28 hrs. The aberrant metaphase increased from 86% to 92% on exogenous GSH addition in 6 Gy X-irradiated cells at the same fixation time.

Effect of BSO on radiosensitization of CHO cells exposed to heavy ion or X-radiation:

Depletion of GSH level by BSO increased the aberration frequency in both heavy ion and X-irradiated cells harvested at 14 hrs. The frequency of aberrations was increased from 59±3 to 65±2% on BSO addition in 1.06 Gy ^{12}C irradiated cells at 14 hrs. At the same dose of ^{7}Li irradiated BSO-treated cells the aberration frequency was increased to 53±3% from 48±4%. Likewise, on exogenous BSO addition to 1Gy X-irradiated cells the aberration percentage increased from 17 to 26% harvested at 14 hrs. Depletion of GSH by BSO has negligible effect on the aberration percentage in both heavy ion and X-irradiated cells at 28 hrs.

Spectrum of CA types and influence of GSH on ^{12}C, ^{7}Li and X-irradiated CHO cells :

The spectrum of aberration is dominated by deletion. A good number of chromosome exchange, chromatid exchange and chromatid break is

also observed. We could observe a high frequency of chromatid type aberration in ^{12}C beam irradiated cells. ^{7}Li ion beam irradiated cells also showing chromatid-type exchange. In CHO cells exposed to X-radiation, the frequency of chromatid type aberration is comparatively lesser than ^{12}C beam exposed cells.

On addition of exogenous GSH, the frequency of exchanges was increased in heavy ion irradiated cells and for deletion the frequency did not alter in a significant manner. However, in X-irradiated CHO cells, the percentage of exchanges was increased and the frequency of deletion was decreased on exogenous supply of GSH to CHO cells compared to the positive control. BSO has been used to evaluate the effect of GSH depletion on radiosensitization of CHO cells. A marked increase in the induction of deletions was observed in BSO treated CHO cells exposed to heavy ion or X-radiation. The frequency of exchanges was decreased in all the cases after BSO-treatment.

2. Cell Cycle Kinetics of irradiated CHO cells with respect to GSH status:

The data are in the tabular form as follows :

Table 1.7: Cell cycle kinetics of CHO cells irradiated with ^{12}C beam (LET 287 KeV/μm) with respect to GSH status.

Experimental Condn.	*M1%*	*M2%*	*M3%*	*TM*	*AGT (hrs)*
14 Hours					
Untreated	100	0	0	121	14.00
1.06 Gy	100	0	0	171	14.00
GSH+1.06 Gy	100	0	0	227	14.00
BSO+1.06 Gy	100	0	0	106	14.00
3.17 Gy	100	0	0	136	14.00
GSH+3.17 Gy	100	0	0	204	14.00
BSO+3.17 Gy	100	0	0	129	14.00
Untreated	96	4	0	163	13.46
1.06 Gy	100	0	0	129	14.00
GSH+1.06 Gy	100	0	0	188	14.00
BSO+1.06 Gy	100	0	0	95	14.00
3.17 Gy	100	0	0	110	14.00
GSH+3.17 Gy	100	0	0	105	14.00
BSO+3.17 Gy	100	0	0	131	14.00
28 Hours					
Untreated	0	98	2	184	13.86
1.06 Gy	23	77	0	394	15.55
GSH+1.06 Gy	26	73	1	246	16.00
BSO+1.06 Gy	34**	66	0	182	16.66
3.17 Gy	40	58	2	174	17.39
GSH+3.17 Gy	56*	44	0	85	19.58
BSO+3.17 Gy	58**	42	0	77	19.72
Untreated	0	95	5	120	13.79
1.06 Gy	19	81	0	370	15.47
GSH+1.06 Gy	22	78	0	390	15.73
BSO+1.06 Gy	17	83	0	304	15.91
3.17 Gy	51	49	0	257	18.79
GSH+3.17 Gy	54	46	0	140	19.17
BSO+3.17 Gy	40	60	0	164	17.52
42Hours					
Untreated	0	5	95	221	14.23
1.06 Gy	11	76	13	151	20.79
GSH+1.06 Gy	8	56	36	145	18.34
BSO+1.06 Gy	3	59	38	133	17.87
3.17 Gy	27	46	27	200	24.70
GSH+3.17 Gy	16	53	31	88	19.53
BSO+3.17 Gy	14	42	44	135	18.34
Untreated	0	2	98	215	14.09
1.06 Gy	7	50	43	137	15.90
GSH+1.06 Gy	10	42	48	205	17.64
BSO+1.06 Gy	6	57	37	323	17.72
3.17 Gy	11	52	37	134	18.50
GSH+3.17 Gy	12	51	38	259	18.58
BSO+3.17 Gy	8	56	36	186	18.42

Table 1.8: Cell cycle kinetics of CHO Cells irradiated with ^{7}Li beam (LET 60 KeV/μm) with respect to GSH status.

Exptal. Condn.	*M1%*	*M2%*	*M3%*	*TM*	*AGT(hrs)*
14 Hours					
Untreated	100	0	0	127	14.00
1.06 Gy	100	0	0	117	14.00
GSH+1.06 Gy	100	0	0	119	14.00
BSO+1.06 Gy	100	0	0	116	14.00
3.07 Gy	100	0	0	136	14.00
GSH+3.07 Gy	100	0	0	125	14.00
BSO+3.07 Gy	100	0	0	115	14.00
Untreated	87	13	0	145	12.61
1.06 Gy	97	3	0	126	13.59
GSH+1.06 Gy	98	2	0	144	13.72
BSO+1.06 Gy	100	0	0	107	14.00
3.07 Gy	100	0	0	127	14.00
GSH+3.07 Gy	100	0	0	114	14.00
BSO+3.07 Gy	100	0	0	109	14.00
28 Hours					
Untreated	0	100	0	105	14.00
1.06 Gy	15	85	0	175	15.14
GSH+1.06 Gy	22	78	0	195	15.73
BSO+1.06 Gy	21	79	0	187	15.64
3.07 Gy	39	61	0	148	17.39
GSH+3.07 Gy	46	54	0	148	18.18
BSO+3.07 Gy	48	52	0	164	17.19
Untreated	0	97	3	166	13.72
1.06 Gy	12	88	0	226	14.89
GSH+1.06 Gy	16	84	0	207	15.22
BSO+1.06 Gy	9	91	1	240	14.58
3.07 Gy	31	69	0	134	16.47
GSH+3.07 Gy	30	70	0	160	16.47
BSO+3.07 Gy	31	69	0	134	16.56
42Hours					
Untreated	0	4	96	101	14.19
1.06 Gy	3	42	55	113	16.67
GSH+1.06 Gy	1	44	55	296	16.47
BSO+1.06 Gy	2	39	59	266	16.28
3.07 Gy	7	44	49	139	17.36
GSH+3.07 Gy	7	62	31	152	18.60
BSO+3.07 Gy	5	35	60	188	16.47
Untreated	0	1	99	135	14.05
1.06 Gy	0	38	62	145	16.03
GSH+1.06 Gy	0	67	33	120	18.02
BSO+1.06 Gy	0	46	54	127	16.54
3.07 Gy	8	57	35	105	18.42
GSH+3.07 Gy	8	53	39	103	20.09
BSO+3.07 Gy	9	53	38	113	18.34

Table 1.9: Cell cycle kinetics of CHO Cells irradiated with X-rays in presence of exogenous BSO or GSH.

Experimental Condition	*M1%*	*M2%*	*M3%*	*TM*	*AGT(hrs)*
14 Hrs.					
Untreated	97	3	0	119	13.59
1Gy	100	0	0	107	14.00
GSH+1Gy	100	0	0	126	14.00
BSO+1Gy	100	0	0	96	14.00
3Gy	100	0	0	91	14.00
GSH+3Gy	100	0	0	93	14.00
BSO+3Gy	100	0	0	120	14.00
6Gy	100	0	0	101	14.00
GSH+6Gy	100	0	0	97	14.00
BSO+6Gy	100	0	0	87	14.00
28 Hrs.					
Untreated	5	94	1	301	14.21
1Gy	17	76	7	434	14.81
GSH+1Gy	20	73	7	390	14.97
BSO+1Gy	26	72	2	394	15.91
3Gy	31	69	0	565	16.56
GSH+3Gy	38*	62	0	552	17.17
BSO+3Gy	34	66	0	287	16.86
6Gy	45	55	0	274	18.06
GSH+6Gy	50	50	0	269	18.66
BSO+6Gy	48	52	0	329	18.42

* Significant at $P<0.052X2$ $\chi 2$ Contingency test (compared to respective control).

M1= First Cycle Metaphase; M2= Second Cycle Metaphase; M3=Third Cycle Metaphase; TM= Total Metaphase; AGT= Average Generation Time.

Cell Cycle Kinetics of CHO cells irradiated with ^{12}C beam, ^{7}li beam and X-radiation with respect to GSH status:

Delay in proliferation of cells was measured in terms of increase in the frequency of first-cycle metaphase following treatment in comparison to that of untreated controls. With the increase in dose, delay in cell cycle is observed in all the three types of radiation exposed cells. ^{12}C beam of higher LET inducing more cell cycle delay than ^{7}Li beam and X-rays.

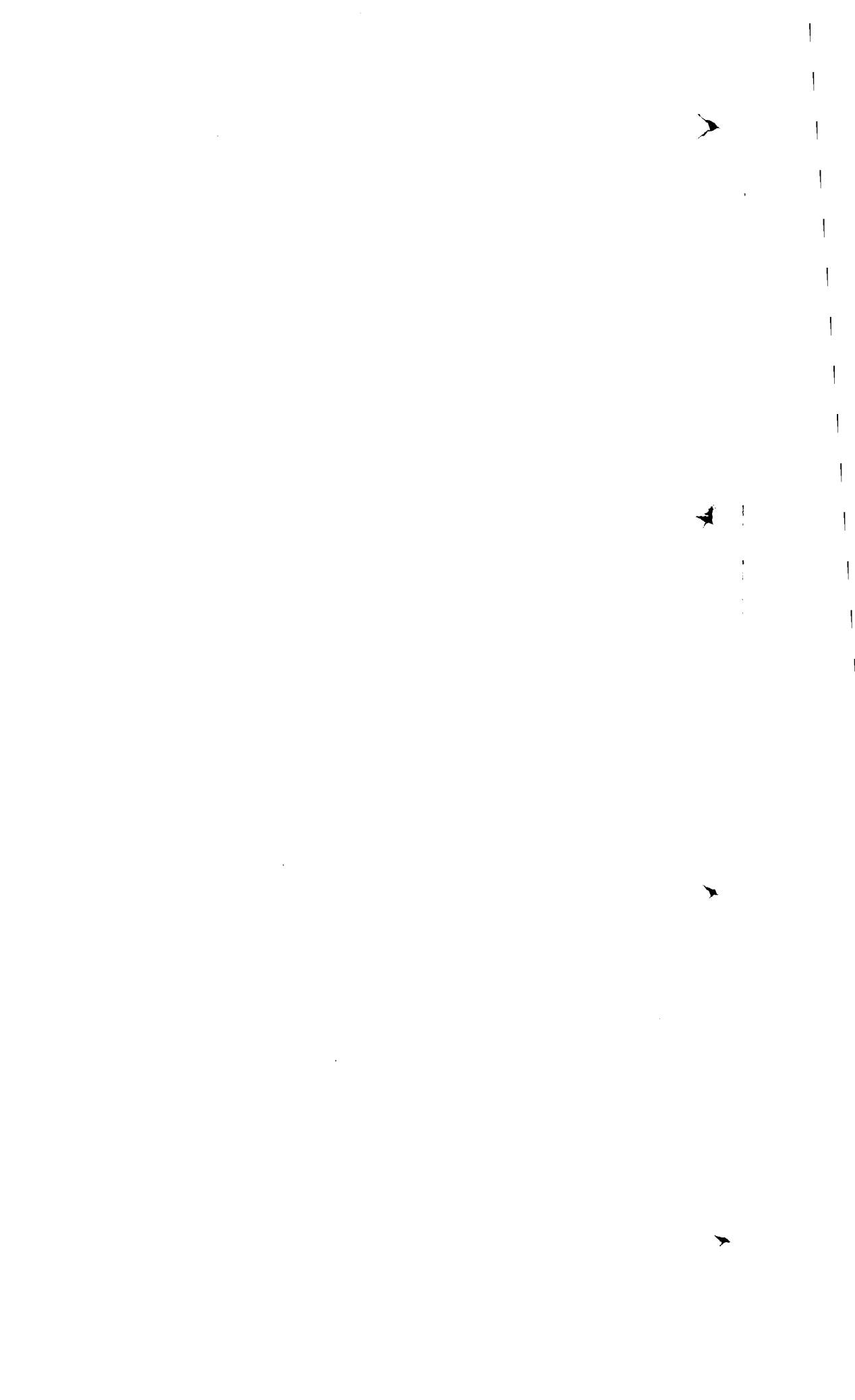
At 14 hrs all the cells are in M1 phase. On harvesting the cells at 28 hrs, the frequency of M1% was increased with increase in dose. The case is true on irradiation of CHO cells with any of the three radiation types. The degree of delay induction is reduced in the cells fixed at later hours.

The objective of the present analysis is to evaluate the influence of GSH level on radiation induced delay in the cell cycle. We could observe that exogenous supply of GSH is unable to show any protection to ^{12}C, ^{7}Li ion beam irradiated cells. BSO addition also has negligible effect on cell cycle kinetics in heavy ion irradiated cells. Conflicting observation in the present experiment is that GSH is not showing protection in cell cycle kinetics of X-irradiated CHO cells even at low dose.

Discussion:

To illustrate the effect of heavy ion beam and X-radiation on CA and cell cycle kinetics with respect to GSH status of the cell, the experiment was performed in CHO cell line. Cells were irradiated with X-radiation and heavy ion ^{12}C and ^{7}Li beam with LET 287 KeV/μm and 60 KeV/μm, respectively. Metaphase cells were collected at 14, 28 and 42 hrs of BrdU addition after radiation exposure. This time interval corresponds to the 3-generation time of control cells. To confine the CA analysis to genuine first post-irradiation metaphase the Fluorescence Plus Giemsa staining technique (FPG) is applied.

From the result, it is observed that the frequency of aberration increases with increase in LET of radiation. Carbon beam with the higher LET is more effective in inducing chromosome damage than lithium beam. X-radiation induces comparatively lesser percentage of aberrations than heavy ion beam. As LET increases, there is an increased probability of larger energy event depositions in volumes of the DNA and an increased probability of more than one lesion being formed close together within a few base-pairs. It is thought that an average of 2-6 ionizations within a 1-4 nm volume of the DNA are the most likely events required for the formation of a DNA DSB (Brenner and Ward 1992). Due to clustering of ionisation by high LET particles, the DSBs induced are believed to be more severe and less repairable. Recently it was shown that high LET heavy ion irradiation induces a lower number of initial chromosome breaks with minimal repair when compared with low LET irradiation. These results at the chromosome



level substantiate and extend the notion that high LET radiation produces complex-type DNA double strand breaks (DSBs) (Sekine *et al.* 2008)

The aberration frequency increases significantly with the increase in dose and also with the increase in sampling time. Heavy ion exposed cells show higher frequency of CA with the increasing harvest time than in X-irradiated cells. In extended time-course studies a drastic increase in the number of aberrations with sampling time has been observed after particle irradiation, while after the exposure to sparsely ionizing radiation a less pronounced effect has been found (Nasonova *et al.* 2001). This difference in the time-course of chromosomal damage is particularly important for the determination of accurate RBE values. It can be explained as the exposure of cells to particles results in an inhomogeneous energy deposition per cell. The inhomogeneity is determined by different number of particle hits per cell nucleus and by the non-uniform dose distribution inside particle track. Cells with low number of particle traversals and correspondingly low chromosomal damage enter mitosis earlier than cells with a high number of its correspondingly more chromosome damage. In contrast, when X-rays are applied, the energy is fairly uniformly deposited leading to a more homogenous distribution of aberrations and delay within the exposed cell population (Ritter *et al.* 1996).

The spectrum of aberration is dominated by deletions. Besides deletions, chromosome and chromatid-type exchanges are also observed. The analysis of the aberration types in CHO cells show that

high LET ^{12}C and ^{7}Li radiation beam is inducing very high percentage of deletions. This probably reflects an increase in complexity or clustering of breaks with high LET radiation. The high frequency of exchange aberrations was observed in ^{12}C and ^{7}Li ion exposed cells. It has been suggested that as LET increases, the DSBs are found close together within the cell making the interaction to cause chromosomal exchanges more probable (Brenner 1990).

An interesting observation seen is that, ^{12}C and ^{7}Li beam is inducing a higher percentage of chromatid type of aberration than X-irradiated cells, though the cells are being irradiated at G1 phase. Primarily DSBs would lead to chromosome-type aberrations. The elevated number of chromatid type aberration in heavy ion irradiated cells can be explained by the Bender *et al.* (1974) as well as Chadwick and Leenhouts (1978), that, DNA single strand break could be involved in the formation of chromatid type of aberrations. If such DNA lesions remain unrepaired throughout G1 then they can be converted into DSB via the process of replication and thus leading to chromatid type aberration in subsequent mitosis.

Radiation induced mitotic delay is a well-known phenomenon that occurs in a variety of cell types. The application of FPG technique enable us to differentiate the cells in first, second and subsequent cycle. Counting the number of cells in M1, M2 and M3 phase gives the idea of cell cycle proliferation of CHO cells exposed to high and low LET radiation. Cell cycle delay is LET-dependent, high LET ^{12}C beam (LET 287 KeV/μm) induced more delay than ^{7}Li beam (60 KeV/μm).

Following high LET exposure (11 MeV u(-1) C ions (LET = 153.5 keV /μm) normal fibroblasts suffer a transient delay into S-phase and into mitosis as well as a prolonged, probably permanent cell cycle arrest in the initial G_0/G_1-phase. Cells that reach the first mitosis at early times carried less aberrations than those collected at later times indicating a relationship between cell cycle delay and the number of aberrations (Tenhumberg *et al.* 2007). Similar increase in delay in cell cycle was observed in a study (Testard *et al.* 1997) where such delay can be linked with oxygen particles with different LET and also to the fluence. The cell cycle was slowed with increasing fluences and LET values and they proposed that such delay must be linked to the cell death and G_2 block previously seen in cells irradiated with high-LET particles (Lucke-Huhle *et al.* 1984). It was surprising that the cell cycle was not more delayed for fluences $>10^7$ particles/cm^2 when the LET was the highest. This could be due to high mortality, induced by too many impacts in a nucleus.

There exists a close relationship between strand break rejoining and division delay. The time constant for rejoining process is larger after heavy ion irradiation because heavy ion induces more complex DNA lesion due to very high local energy deposition inside the particle track. Assuming a correlation between DSBs rejoining and cell cycle delay, these complex damages would lead to more pronounced cell cycle delay (Ritter *et al.* 1977).

Role of GSH as radioprotector in CHO cells exposed to high and low LET radiation:

The primary objective of the present study is the analysis of the influence of endogenous GSH level on heavy ion and low LET radiation induced CA and cell cycle delay. GSH is one of the most important factors playing an important role for the cellular sensitivity towards radiation.

Ionizing radiation produces widespread oxidative damage to DNA by both direct and indirect mechanisms. Direct action produces disruption of chemical bonds in the molecular structure of DNA while the indirect effects results from highly reactive free radicals such as OH•, H• and e_{aq}^- produced during the radiolysis of water, and their subsequent interaction with cellular DNA. About two third of the biological damage by low LET X- radiation is due to indirect action. ^{7}Li and ^{12}C beam belongs to densely ionizing radiation, which is more effective in causing biological damage because more of its energy is released in clusters of ionizing events, giving rise to more severe local damage. Direct action is the dominant process in the interaction of high-LET particles with biological materials. Indirect action can be modified by chemical sensitizers or radioprotectors.

From our observation, pretreatment of GSH prior to low dose of X-rays, seemed to protect the cells. It is reported that protective effects of GSH against low LET radiations are due to its scavenging ability for free radicals. GSH play an important role in the protection of cells from reactive oxygen species. As well as acting as the cofactor for the

glutathione peroxidase, where it provides two electrons for the reduction of hydrogen peroxide to water, it can also act as a radical-scavenging antioxidant. From our observation, it is seen that GSH showed protective effects against low LET radiation in a dose dependent manner. On increasing the dose of X-radiation, GSH is unable to act as radioprotector. The protective effect from any radioprotectant can be detected around 10^{-12} sec after irradiation. At that time, the radioprotector begin to repair chemical damage molecules and also react with chemical intermediates that indirectly damage these molecules. Protection of GSH depends on the ability to reduce the intracellular concentration of free radicals and the reactive oxygen species that are produced within the first millisecond after irradiation.

From this study, we found that GSH has no role in radioprotection against ^{12}C and ^{7}Li beam. This can be explained, because damage by high LET radiation is primarily due to direct interaction, and since the relative yields of water radiolytic products and reactive oxygen species decreases with increasing LET, protection against high LET radiation by GSH is more difficult to achieve. Thus, depending on the differences in the interaction of heavy ion with the cell system, GSH may not be able to exert its role as radioprotector as efficiently as it does in case of low dose of X-radiation.

Depletion of intracellular GSH by BSO sensitized the cells exposed to low and high LET radiation with respect to CA. It is presumed that a portion of cellular GSH binds on DNA or DNA-bound proteins and

provides a shielding effect against radiation. Thus, BSO-mediated GSH depletion could reduce the shielding effect and enhance DSB induction which is primarily due to direct action of high LET radiation.

Increase in exchange aberration and decrease in the frequency of deletion is observed on GSH pretreatment in low LET X-irradiated cells compared to respective control. Exchange aberration is thought to arise as a consequence of illegitimate reunion (misrejoining) of free ends from different DNA DSB (Cornforth and Bedford 1993). Our observation of an increase in exchange aberrations and decrease in deletions could be due to enhancement in rejoining (both restitution and illegitimate reunion) of radiation-induced DNA DSB under the influence of increased endogenous GSH. This statement is supported by the observation that the exchange frequency decreased and deletion increased on BSO pretreatment to the cells irradiated with X-radiation. However, GSH pre-treated cells when exposed to ^{12}C or ^{7}Li beam, the exchange frequency though increased but is not as high as it was observed in X-irradiated cells. Unlike GSH+X-radiation treated cells, decrease in deletion frequency is not observed with the increase in exchanges in GSH+ heavy ion exposed cells. Thus, GSH may be involved or an important component of the enzymatic machinery that is needed for DSB joining of the lesions induced by X-radiation. GSH is not as efficient in DNA DSB joining of the lesions induced by ^{12}C and ^{7}Li beam and this could be attributed due to induction of more complex and clustered lesions which is being more difficult to repair. It was observed that high-LET particles induced damage was more localized

and less efficiently rejoined than after X- or γ-ray irradiation. Indeed, the number of residual DNA breaks increased with particles LET and the delivered dose (Jenner *et al.* 1993, Heilmann *et al.* 1995).

Analysis of the influence of GSH on cell cycle kinetics irradiated with low and high LET radiation in CHO cells has been done simultaneously with the aberration scoring. In this study, it was observed that the induction of delay in cell cycle was more in ^{12}C than ^{7}Li beam. The delay in cell cycle is related to the aberration burden of the cells (Ritter *et al.* 1994, 1996). It is known that cell cycle delays are LET and dose-dependent (Blakeley *et al.* 1985), and it was indicated that disruption in cell cycle progression will differ significantly for high- and low-LET exposures (Ritter *et al.* 1996, 2000). High LET radiation produces energy deposition focused along a particle track. Some cells will be heavily damaged and severe delay could occur in these cells, while cells hit by delta-rays alone will suffer modest damage, and other cells with no hits will progress normally through the cell cycle (Cucinotta *et al.* 1998). This is in contrast to low LET radiation exposures, such as γ-rays, where a more even distribution of damage could induce a uniform distribution of cell-cycle delays. The influence of cell cycle delay is of particular importance in the biodosimetry analyses of astronauts, since exposure to the extremely complex mixed radiation field in space could result complicated mitotic delay patterns when their lymphocytes are cultured directly after flight.

The present study is emphasized on to study the influence of GSH on high and low LET radiation induced delay in cell cycle. Both addition of GSH and BSO did not show any influence on the cell cycle kinetic pattern of the irradiated cells. Usually both GSH and BSO reduced delay induced by low LET radiation, but in the present study it failed to show any influence on such delay. However, most surprisingly it is noted that GSH pretreatment could not reduce delay induced by X-rays in CHO cells. Such treatment always showed reduction in delay in human peripheral lymphocytes and also in mouse bone marrow cells (Ray and Chatterjee 2006, 2007). More studies are needed to see that such effect is unique for certain cell line or not.

CHAPTER: 2

Role of GSH in the interaction of DNA lesions induced by High/Low LET radiation and Bleomycin

Literature Review

Large number of studies suggest that DNA double strand break (DSB) induced by ionizing radiation is the critical lesions which if unrepaired or misrepaired can cause chromosome aberrations (CAs), cell death as well as mutations and cell transformation (Frankenberg-Schwager 1990; Iliakis 1991). Radiation induces exchange aberrations which is thought to arise as a consequence of illegitimate reunion (misrejoining) of free ends from different DNA dsbs (Cornforth and Bedford 1993). Such misrejoining may be expected to depend on the number and proximity of the breaks. Various studies have shown that high LET radiations are far more effective per unit dose in producing complex aberration than are X- or γ- ray. Chromosome rearrangements induced by sparsely ionizing radiations are well known and cytogenetic analyses of irradiated human lymphocytes have been widely applied to biological dosimetry (Lloyd *et al.* 1992).

Much less is known about CA induced by high LET particles. Such particles induce DNA strand breaks, as well as chromosome breakage and rearrangement of high complexity (Ritter et al 1992). High LET radiation tracks produce highly localized clustered damage within the DNA and also spatially separated sites of damage along the path of the radiation track (Goodhead 1991; Goodhead *et al.* 1993; Rydberg 1996). With the introduction of Fluorescence in situ hybridization (FISH) (Pinkel *et al.* 1986), where whole chromosomes are 'painted' a high proportion of complex CA is observed after exposure to both high-LET

radiation in normal human fibroblast (Griffin *et al.*1995), peripheral blood lymphocytes (Testard *et al.* 1997) and Chinese hamster splenocytes (Grigorova *et al.* 1998) and also low LET radiation at doses greater than 3Gy (Brown and Kovacs 1993; Tucker *et al.* 1993, Simpson and Savage 1996).

It was demonstrated by Preston (1982) that if the DNA damage produced by two agents is repaired at very different rates then the probability of producing a synergistic effect on aberration frequency is low. On the other hand, if the damage from both agents is repaired rapidly, then there is a high probability of producing a synergistic or interactive effect. Therefore, the present study considered these possibilities with an aim to investigate the pattern of interaction of DNA DSBs induced by high or low LET radiation with bleomycin (BLM) induced DNA lesions.

Bleomycin (BLM) is an antitumor antibiotic used in the treatment of squamous cell carcinoma, testicular carcinoma and malignant melanoma (Crooke and Umezawa 1978). BLM is isolated from the supernatant of *Streptomyces verticillus* culture (Vig and Lewis 1978). The clinical effectiveness of BLM is based on its cytotoxicity backed up by its interaction with DNA, chelation of metal ions and generation of oxygen free radicals in the presence of molecular oxygen. Studies with purified DNA suggest that the cytotoxic effects of BLM may be due to the disruption of the physical integrity of DNA (Sausville *et al.* 1978a,b). Bleomycin-induced genetic damage to DNA in mammalian cells includes single- and double- strand breaks, release of free base

residues, inhibition of DNA synthesis, and the production of chromosomal aberrations (Vig and Lewis 1978).

Under physiological conditions, polynuceotide degradation by BLM requires dioxygen and an appropriate metal ion as cofactor; Fe^{2+} can certainly support this process and Cu^{+} may also suffice. One of the most remarkable facets of DNA cleavage involve the pyrimidine nucleotides in 5'-GC-3' and 5'-GT-3' sequences; a subset of these are cleaved with particularly high efficiency. The molecular basis for the choice of 5'-Gpyr-3' sequences as preferred cleavage sites is poorly understood, although it may be related to the fact that the minor groove of DNA is relatively wide and shallow at such sites. Before causing a break, BLM binds to one DNA strand and exchanges Cu^{2+} for Fe^{2+} in the presence of oxygen. While still bound to the DNA, BLM can be reactivated in the presence of reducing agents. In this way it can make another break in close vicinity to the first break (Chatterjee *et al.* 1989).

Bleomycin (BLM) mimics the effects of low LET radiation. BLM has been known to produce DSB possessing 5'-P and 3'-Phosphoglycolate (3'-PG) termini that are blunt. Low LET radiation also produces approx. 50% of the breaks that have 3'-PG termini while the rest has 3'-P ends. For this reason, BLM-damaged DNA is frequently used as a model for radiation-induced DNA strand breaks during the study of repair or mutagenicity (Dar and Jorgensen 1995).

Combined treatment of BLM and radiation induces higher frequency of CAs, particularly, exchange aberrations and interstitial deletions.

Treatment of cells with BSO, the GSH depleting agent, showed drastic reduction in the frequency of exchange aberration and huge increase in the frequency of terminal deletions. This reduction in the effect of BLM in GSH-depleted cells could be explained on the basis of the failure of lesions interaction and also due to lack of reactivation of the oxidised BLM by the reducing agent GSH that is usually present endogenously (Dutta *et al.* 2005).

It is known that low LET and high LET radiations act differently on DNA because of the differing degrees of spatial clustering of ionisations and DSBs. In contrast to sparsely ionising radiation, high LET radiation leads to the induction of more complex and highly localized DNA damage along particle tracks. Thus, high LET radiation induced DNA damage is a clustered lesion consisting of multiple strand breaks, base alterations action produced by the direct interaction between DNA and the charged particle, which deposits its energy densely along its path. Both the localisation and complexity of high LET radiation induced DSB may influence the cellular capacity to repair such damage. The DNA damage induced by charged particles is associated with slower rejoining of DSBs (Lobrich *et al* .1998).

Till date, synergistic effect of BLM and high LET radiation on chromosome aberration is not known. Because of the difference in the molecular nature of the damage induced by low and high LET radiation, it is interesting to study and compare the pattern of interaction of the DNA lesion induced by BLM and heavy ion. By earlier work, GSH has been reported to be involved in the repair and mis-repair of the DNA

DSB lesion in mammalian cells exposed to combined treatment of BLM and low LET radiation. Therefore, this work considered these possibilities with an aim to investigate the role of GSH in DNA DSB-rejoining by treating the cells with BLM and heavy ion ^{12}C and ^{7}Li beam and X-rays.

Materials and Method:

Experimental Model:

Chinese Hamster Ovary (CHO) Cell line.

Chemicals:

- Dulbecco's Modified Eagles Medium (DMEM), Penicillin Streptomycin, Trypsin-EDTA, Colcemid, Gibco, USA.
- Foetal Calf Serum (FCS), Biological Industries, Israel.
- Glutathione Reduced, Sigma Chemical Company, USA, used at a concentration of 2 mM.
- DL- Buthionine-(S,R)-Sulfoximine (BSO), Sigma Chemical Company, USA, used at a concentration of 200μM.
- Bleocip (Bleomycin Injection), Cipla, India, used at a concentration of 10μg/ml.
- 5-bromo-2-deoxyuridine (BrdU), Sigma Chemical Company, USA; for the experiment the BrdU powder is dissolved in autoclaved Millipore water to prepare a working solution of 100μg/ml.

- Bis-benzimide (Hoechst 33258), Sigma USA, This is used at a concentration of 50μg/ml in double distilled water.
- Giemsa Stain Solution, BDH Chemicals Ltd., UK.

High LET ions:

The Pelletron at the Inter University Accelerator Centre (IUAC), New Delhi, provided the accelerated ions. Two different ions ^{12}C and ^{7}Li were extracted from the ion source and accelerated to energies of 85 and 50 MeV respectively. Beam properties are listed in the Table.

Table 2.1: Beam properties

Ion Species	**Energy (MeV)**	**LET (KeV/μm)**	**Fluence (particles/cm^2)**	**Dose Equivalent (Gy)**
Carbon Ion (^{12}C)	85	287	2.3×10^6	1.06
			6.9×10^6	3.17
Lithium Ion (^{7}Li)	50	60	1.1×10^7	1.06
			3.2×10^7	3.07

X- irradiation

We have used Faxitron Cabinet X-ray systems (Model No. 43855D, 110KVp, 3mA, Berylium window thickness 0.76mm; Faxitron X-Ray Corp, Wheeling, IL, USA), for X-irradiation.

Cells are seeded at a density of 0.8×10^6 in 35mm petriplates, 15 hrs prior to radiation exposure so that the cells reached at 80% confluent state. In case of reduced GSH, it was added 12 hrs after cells were seeded at a concentration of 2mM for 3 hrs. After one hr of GSH treatment, BLM is given to the cells at a concentration of 10μg/ml. The cells are incubated for 2 hrs after BLM treatment, followed by high/low LET radiation. The medium is decanted off from the petriplates and covered with a polypropylene sheet of 6μm thickness before irradiation. After irradiation fresh medium supplemented with serum is added to the plates and incubated for 1hr. Culture is set in presence of 6μg/ml of BrdU. Cells are harvested at 14, 28 and 42 hrs after BrdU addition (cells were fixed at 14 and 28 hrs only, in case of X-radiation experiments). We are not considering the 42 hrs harvest cells for CA studies, due to the relatively lesser number of cells in M1 cycle.

Preparation of metaphases and differential staining

The preparation of the metaphases and differential staining were obtained by the same procedure as mentioned in chapter 1.

Scoring and statistical analysis:

Slides are coded randomly and are studied for chromosome exchanges, chromatid exchanges, deletions and chromatid breaks. The statistical significance of the difference between the control and the treated groups were determined. Aberrant metaphases were tested using the 2x2 χ^2 Contingency test. The different types of aberrations studied were compared using the simple χ^2 test.

Result:

Table 2.2: CA induced in CHO cells exposed to combined treatment of BLM and ^{12}C beam (LET 287 KeV/μm) with respect to GSH status.

Experimental Condition	*Aberrant metaphase %*	*Total Metaphase*	*Chrom. Exch. %*	*Chtd. Exch. %*	*Del. %*	*Chtd Bk. %*
14 Hours						
Untreated	7	109	0	0	7	2
BLM(10μg/ml)	33	120	7	5	22	1
1.06 Gy	61	120	19	8	42@	7
BLM+1.06 Gy	73*	126	34@	7	52@	21
GSH+BLM+1.06 Gy	76	127	39	10	47	8
3.17 Gy	76	111	32	10	52	2
BLM+3.17 Gy	83	103	49@	17	63@	10
GSH+BLM+3.17 Gy	89	87	54	23	75@	11
Untreated	5	111	0	0	5	4
BLM(10μg/ml)	39	154	13	0	14	16
1.06 Gy	56	117	10	8	46#	4
BLM+1.06 Gy	74*	84	25#	14@	51	0
GSH+BLM+1.06 Gy	75	96	31	6	42	5
3.17 Gy	71	87	15	8	66	3
BLM+3.17 Gy	81	80	29@	15	67	4
GSH+BLM+3.17 Gy	84	77	35	14	78@	3
28 Hours						
1.06 Gy	74	81	25	12	56	6
BLM+1.06 Gy	89*	44	39@	14	68@	0
GSH+BLM+1.06 Gy	93	68	44	16	74	7
3.17 Gy	100	50	34	16	86	10
BLM+3.17 Gy	97	38	50@	21	105@	13
GSH+BLM+3.17 Gy	100	54	54	15	117@	9
1.06 Gy	70	54	17	4	85	2
BLM+1.06 Gy	84*	70	29@	15@	100@	14
GSH+BLM+1.06 Gy	87	63	34	12	110@	18
3.17 Gy	91	128	24	10	147	16
BLM+3.17 Gy	94	116	31	10	200#	9
GSH+BLM+3.17 Gy	97	61	39	10	213@	16

* Significant at P<0.05,**Significant at P<0.012×2 χ2Contingency test; @ Significant at P<0.05,

Significant at P<0.01, $ Significant at P<0.001 simple χ2 test (compared to respective control).

Chrom. Exch.= Chromosome Exchange; Chtd. Exch.= Chromatid Exchange; Del.= Deletion; Chtd. Bk.= Chromatid Break.

Table 2.3: Pooled data of CA in CHO exposed to combined treatment of BLM and ^{12}C beam (LET 287 KeV/μm) with respect to GSH status.

Fixation Time	*Exptal Condn.*	*Abberant Metaphase% ±SEM*	*TM*	*Chrom. Exch.% ±SEM*	*Chtd. Exch% ±SEM*	*Del. % ±SEM*	*Chtd. Bk% ±SEM*
14	Untreated	6±1	220	0	0	6±1	3±1
	BLM(10μg/ml)	36±3	274	10±3	3±3	18±4	9±8
	1.06Gy	59±3	237	15±5	8±0	44±2	6±2
	BLM+1.06Gy	74±1**	210	30±5$^{\$}$	11±4$^{\#}$	51±1	11±11
	GSH+BLM+ 1.06Gy	76±1	223	35±4$^{\#}$	8±2	48±3	7±2
	3.17Gy	74±3	198	24±9	9±1	59±7	3±1
	BLM+3.17Gy	82±1	183	39±10$^{@}$	16±1	65±2	7±3
	GSH+BLM+3.17Gy	87±3	164	46±10	19±5	77±2	7±4
28	1.06Gy	72±2	135	21±4	8±4	71±15	4±2
	BLM+1.06Gy	87±3**	114	34±5$^{@}$	15±1$^{@}$	84±16$^{@}$	7±7
	GSH+BLM+ 1.06Gy	90±3	131	39±5	14±2	92±18	13±6
	3.17Gy	94±3	178	29±5	13±3	117±31	13±3
	BLM+3.17Gy	96±2	154	41±10$^{@}$	16±6	153±47$^{@}$	11±2
	GSH+BLM+3.17Gy	99±2	115	47±8$^{\$}$	13±3	165±48	13±4

** Significant at P<0.01 2×2 $\chi 2$ Contingency test; @ Significant at P<0.05, # Significant at P<0.01, $ Significant at P<0.001 simple $\chi 2$ test (compared to respective control).

TM= Total Metaphase; Chrom. Exch.= Chromosome Exchange; Chtd. Exch.= Chromatid Exchange; Del.= Deletion; Chtd. Bk.= Chromatid Break.

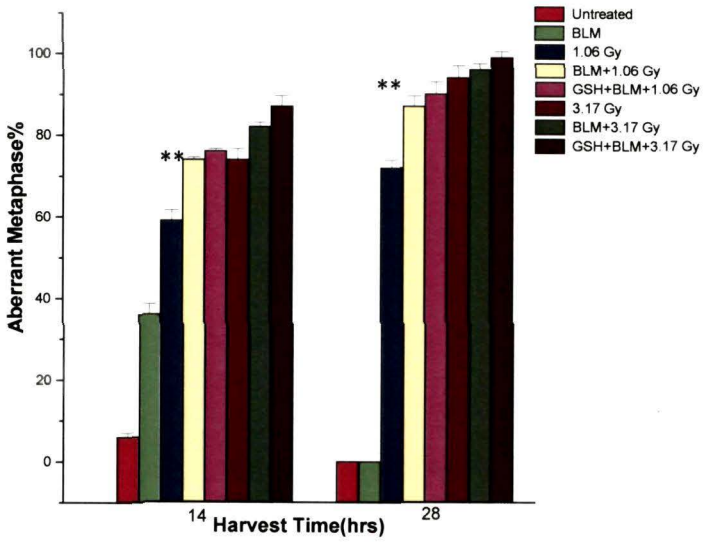


Fig 2.1: Aberrant metaphase percentage in CHO cells exposed to combined treatment of BLM and ^{12}C beam (LET 287 KeV/μm) with respect to GSH status.

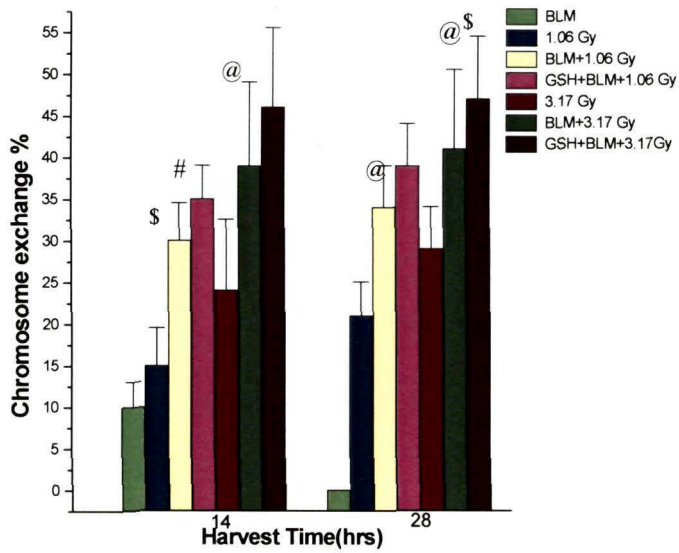


Fig 2.2: Chromosome exchange aberration induced in CHO cells exposed to combined treatment of BLM and ^{12}C beam (LET 287 KeV/μm) with respect to GSH status.

Table 2.4 CA in CHO cells exposed to combined treatment of BLM and ^{7}Li beam (LET 60 KeV/μm) with respect to GSH status.

Experimental Condition	*Aberrant Metaphase %*	*Total Metaphase*	*Chrom. Exch. %*	*Chtd. Exch. %*	*Del. %*	*Chtd. Bk. %*
14 Hours						
Untreated	7	120	0	0	7	3
BLM(10μg/ml)	28	111	7	0	21	0
1.06 Gy	51	109	14	2	37	15
BLM+1.06 Gy	68*	108	29#	3	43	10
GSH+BLM+1.06 Gy	78	83	36	10	52	6
3.07Gy	67	121	24	3	47	12
BLM+3.07 Gy	77	104	34@	8	54	13
GSH+BLM+3.07 Gy	81	99	40	14	63	6
Untreated	3	109	0	0	1	2
BLM(10μg/ml)	40	88	14	7	19	6
1.06 Gy	44	103	5	4	35	3
BLM+1.06 Gy	61*	79	20@	9	48@	2
GSH+BLM+1.06 Gy	67	81	28	14	52	2
3.07Gy	63	120	14	9	59	8
BLM+3.07 Gy	72	79	32$	16	67	9
GSH+BLM+3.07 Gy	78	77	36	17	75	6
28 Hours						
1.06 Gy	61	21	19	0	71	5
BLM+1.06 Gy	89*	28	32@	4	82	7
GSH+BLM+1.06 Gy	85	53	42	2	75	8
3.07Gy	94	51	27	14	120	8
BLM+3.07 Gy	95	39	38	21	144@	26
GSH+BLM+3.07 Gy	85	53	42	2	162@	19
1.06 Gy	65	25	16	0	64	8
BLM+1.06 Gy	77*	31	29$	3	74	13
GSH+BLM+1.06 Gy	81	43	37$	2	81	12
3.07Gy	88	32	25	9	72	16
BLM+3.07 Gy	92	36	39@	14@	83	14
GSH+BLM+3.07 Gy	92	51	43@	22@	94	10

* Significant at P<0.0 2×2 $\chi 2$ Contingency test; @ Significant at P<0.05, # Significant at P<0.01, $ Significant at P<0.001 simple $\chi 2$ test (compared to respective control).

Chrom. Exch.= Chromosome Exchange; Chtd. Exch.= Chromatid Exchange; Del.= Deletion; Chtd. Bk.= Chromatid Break.

Table 2.5: Pooled data of CA in CHO cells exposed to combined treatment of BLM and ^{7}Li beam (LET 60 KeV/μm) with respect to GSH status.

Fixation Time	*Exptal Condn.*	*Abb Metaphase %±SEM*	*TM*	*Chrom. Exch.% ±SEM*	*Chtd. Exch% ±SEM*	*Del. % ±SEM*	*Chtd. Bk%± SEM*
14	Untreated	5±2	229	0	0	4±3	3±1
	BLM(10μg/ml)	35±5	199	11±4	6±0	20±1	3±3
	1.06 Gy	48±4	212	10±5	3±1	36±1	9±6
	BLM+1.06 Gy	65±4**	187	25±5$^{\$}$	6±3	46±3	6±4
	GSH+BLM+1.06 Gy	73±6	164	32±4	12±2$^{@}$	52±0	4±2
	3.07Gy	65±2	241	19±5	6±3	53±6	10±2
	BLM+3.07 Gy	75±3	183	33±2$^{\$}$	12±4$^{@}$	61±8	11±2
	GSH+BLM+3.07 Gy	80±2	176	38±2	16±1	69±6$^{@}$	6±0
28	1.06 Gy	63±2	46	13±3	0	68±4	7±2
	BLM+1.06 Gy	83±6*	59	31±2$^{@}$	2±1	78±4	10±3
	GSH+BLM+1.06 Gy	83±2	96	40±3	2±0	78±3	10±2
	3.07Gy	91±3	83	26±1	3±1	96±24	12±4
	BLM+3.07 Gy	94±2	75	38±3$^{@}$	18±4	114±31$^{@}$	20±6
	GSH+BLM+3.07 Gy	89±3	104	43±1	12±10	128±34	15±5

* Significant at P<0.05, ** Significant at P<0.01 2×2 $\chi 2$ Contingency test; @ Significant at P<0.05, # Significant at P<0.01, $ Significant at P<0.001 simple $\chi 2$ test (compared to respective control).

TM= Total Metaphase; Chrom. Exch.= Chromosome Exchange; Chtd. Exch.= Chromatid Exchange; Del.= Deletion; Chtd. Bk.= Chromatid Break.

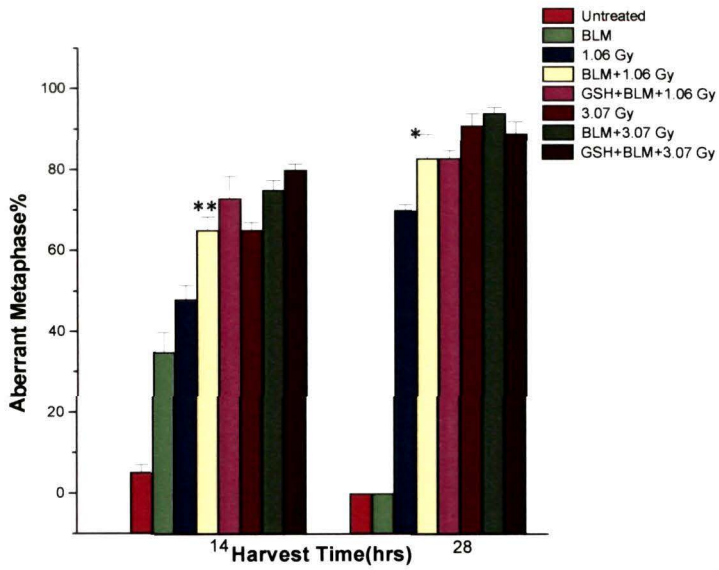


Fig 2.3:Aberrant metaphase percentage in CHO cells exposed to combined treatment of BLM and ^{7}Li beam (LET 60 KeV/μm) with respect to GSH status.

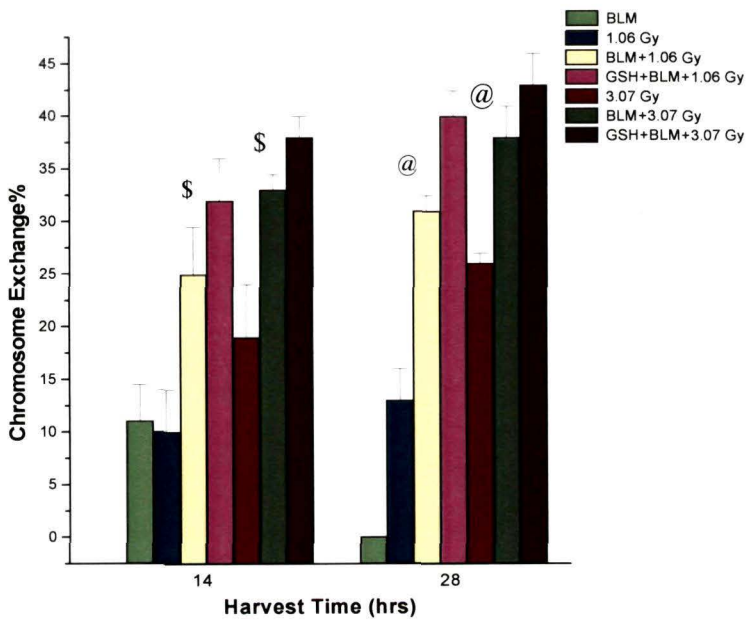


Fig 2.4: Chromosome exchange aberration in CHO cells exposed to combined treatment of BLM and ^{7}Li beam (LET 60 KeV/μm) with respect to GSH status.

Table 2.6: CA in CHO cells exposed to combined treatment of BLM and X-rays with respect to GSH status.

Experimental Condition	*Aberrant Metaphase %*	*TM*	*Chrom. Exch. %*	*Chtd. Exch. %*	*Del. %*	*Chtd. Bk. %*
14 Hrs.						
Untreated	3±0	208	0	0	0	3±0
BLM(10μg/ml)	42±2	210	20±1	3±0	25±3	5±2
1Gy	19±2	206	6±1	0	22±3	5±2
BLM+1Gy	50±2**	184	37±4#	3±2	57±5#	7±3
GSH+BLM+1Gy	50±3	198	58±3@	0	37±5@	12±2
3Gy	49±2	212	26±3	2±1	38±3	5±1
BLM+3Gy	63±2*	192	49±4@	8±2	56±5@	12±3
GSH+BLM+3Gy	70±3	180	71±3#	2±1	50±5	12±1
28 Hrs.						
1Gy	25±2	188	6±1	0	29±3	10±3
BLM+1Gy	56±2**	164	39±4$	3±2	68±8#	10±2
GSH+BLM+1Gy	58±4	118	60±5#	4±1	57±5	15±3
3Gy	64±2	212	29±3	2±1	42±8	12±2
BLM+3Gy	76±5	124	54±4#	10±2	88±12#	14±1
GSH+BLM+3Gy	82±2	124	62±3	14±6	122±28#	14±2

** Significant at $P<0.01$ 2×2 $\chi 2$ Contingency test. @ Significant at $P<0.05$, # Significant at $P<0.01$, $ Significant at $P<0.001$ simple $\chi 2$ test.

TM= Total Metaphase; Chrom. Exch.= Chromosome Exchange; Chtd. Exch.= Chromatid Exchange; Del.= Deletion; Chtd. Bk.= Chromatid Break.

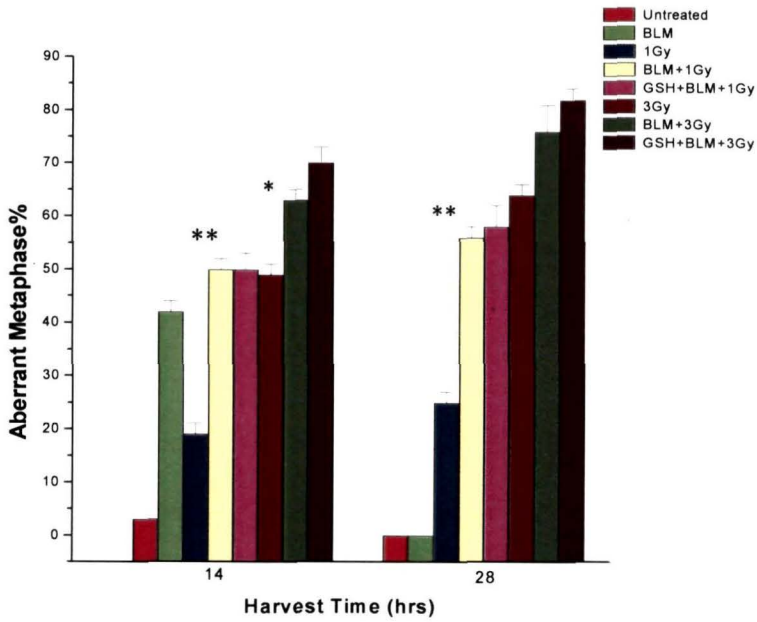


Fig 2.5: Aberrant metaphase percentage in CHO cells exposed to BLM and X-radiation with respect to GSH status.

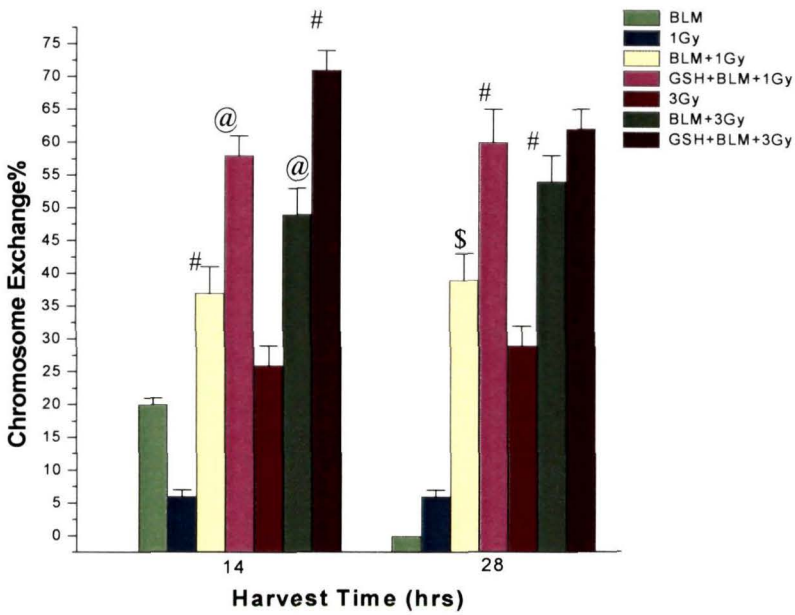


Fig 2.6: Chromosome exchange aberration in CHO cells exposed to combined treatment of BLM and X-radiation with respect to GSH status.

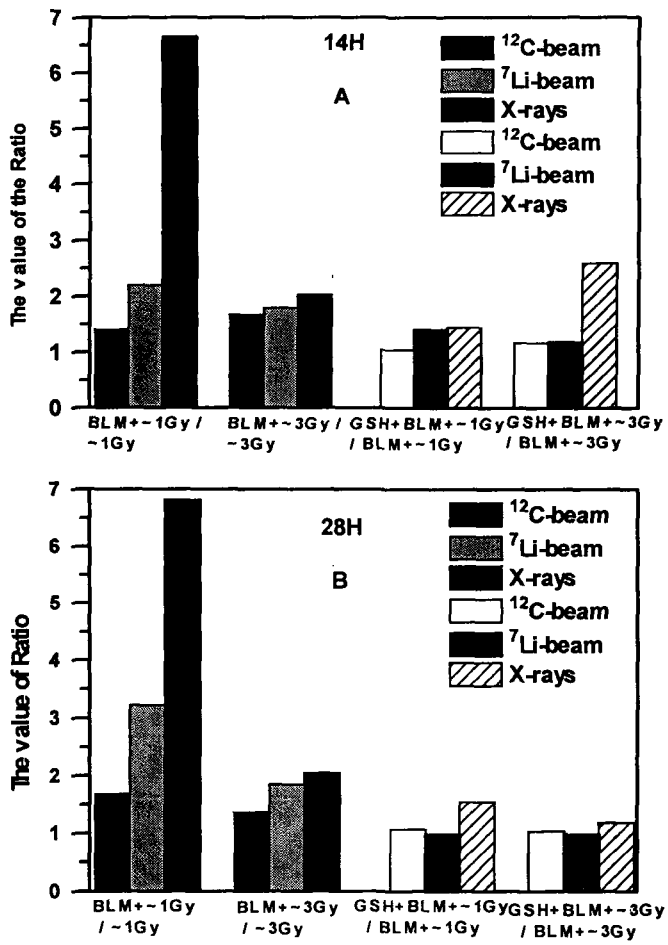


Fig 2·7: The value of the ratio between BLM+Radiation : Radiation and GSH+BLM+Radiation : BLM+Radiation for exchange aberrations in CHO cells fixed at 14H (A) and 28H (B).

Effect of BLM and high/low LET radiation alone and in combination on the induction of CAs in CHO cells with respect to GSH status:

Induction of CAs by ^{12}C (LET 287 KeV/μm), ^{7}Li (LET 60 KeV/μm), X-radiation and Bleomycin (BLM) alone and in combination in CHO cells were studied and the data is represented in tabular format in 2.3, 2.5 and 2.6; and in Fig: 2.1, 2.3 and 2.5.

From the data we can observe that in all the three radiation types, the aberration percentage tend to increase when BLM and radiation is given to the CHO cells in combination. At 14 hrs harvest, 1.06 and 3.17 Gy of Carbon beam irradiated cells show an increase in the frequency of aberrant metaphases from 59 and 74% to 74 and 82%, respectively while combined with BLM-treatment. On increasing the fixation time to 28 hrs, the pattern of increment was similar, however, the frequency of aberrant metaphases was higher than 14hrs fixation time.

Cells irradiated with 1.06 and 3.07 Gy of lithium beam showed the frequency of aberrant metaphases was 48 and 65%, respectively at 14 hrs fixation time. This frequency was increased to 65 and 75% while combined with BLM treatment. At 28 hrs, the pattern of increment was similar, however, the frequency of aberrant metaphases was higher than 14hrs fixation time (Table 2.4 and 2.5).

Similarly, increase in the frequency of aberration is observed after combined treatment of BLM and X-radiation in CHO cells. The frequency of aberrant metaphases was increased from 19 and 49% induced by 1Gy and 3Gy X-rays to 50 and 63% while combined with

BLM-treatment harvested at 14 hrs. Likewise increase in aberrant metaphase percent was also observed on BLM treatment to X-irradiated cells harvested at 28 hrs.

Deletion and exchanges are the most frequent type of aberrations seen in the cells exposed to combined treatment of BLM and low/high LET radiation. On comparing the trend of aberrations, it was observed that the frequency of exchanges and deletions was increased significantly in combined treatment of BLM and radiation with respect to positive control. BLM in combination with X-radiation result in increase in the frequency of exchange. It is worth mentioning that the treatment of cells with either Carbon or Lithium beam in combination with BLM, the aberrant cells showed exchanges of chromatid-type besides usual chromosome-type. The overall induction of chromatid exchanges by X-rays was very low. From the above data it shows clearly that the increment in the frequency of exchanges is significantly higher in BLM and X-radiation treated cells than in cells exposed to combined treatment with BLM and heavy ion (Table 2.3, 2.5 and 2.6). The value of the ratio between BLM+Radiation : Radiation for exchange aberration fixed at 14 hrs and 28 hrs is represented at Fig. 2.7 (a) and (b), which shows clearly the increment of exchange is higher in BLM+X-radiation treated cells than BLM+ high LET radiation exposed cells. Deletion percentage increased when the cells are given combined treatment of BLM and low/high LET radiation.

In this study, GSH was added exogenously to the CHO cells to evaluate the effect of GSH on DNA DSB joining after exposing the

cells to BLM and radiation. Presence of exogenous GSH increased the percentage of exchanges markedly in the BLM and low/high LET radiation treated cells. Interestingly, the degree of increase in the frequency of exchanges is higher in the cells where exogenous GSH is added to BLM and X-radiation exposed cells. The value of ratio between between GSH+BLM+Radiation : BLM+Radiation for exchange aberration in CHO cells fixed at 14 hrs and 28 hrs is represented in Fig. 2.7 (a) and (b). This increase in the frequency of exchanges is also marked by significant decrease in the frequency of deletions in BLM+ X-radiation treated cells.

Table 2.7: M1% of CHO cells exposed to combined treatment of BLM with ^{12}C, ^{7}Li or X-radiation with respect to GSH status.

Fixation Time	*Experimental Condition*	*M1%±SEM*	*Total Metaphase*
14(^{12}C)	Untreated	98±2	284
	BLM(10μg/ml)	100±1	315
	1.06 Gy	100±0	300
	BLM+1.06 Gy	100±0	241
	GSH+BLM+1.06 Gy	100±0	266
	3.17Gy	100±0	246
	BLM+3.17 Gy	100±0	189
	GSH+BLM+3.17 Gy	100±0	187
14(^{7}Li)	Untreated	94±7	272
	BLM(10μg/ml)	98±2	232
	1.06 Gy	99±2	243
	BLM+1.06 Gy	95±5	208
	GSH+BLM+1.06 Gy	100±0	181
	3.07Gy	100±0	263
	BLM+3.07 Gy	100±0	212
	GSH+BLM+3.07 Gy	100±0	219
14(X-rays)	Untreated	94±2	290
	BLM(10μg/ml)	100±0	265
	1Gy	100±0	254
	BLM+1 Gy	100±0	220
	GSH+BLM+1 Gy	100±0	227
	3Gy	100±0	225
	BLM+3 Gy	100±0	219
	GSH+BLM+3 Gy	100±0	222
28(^{12}C)	Untreated	0	304
	BLM(10μg/ml)	1±1	288
	1.06 Gy	21±2***	764
	BLM+1.06 Gy	27±8	640
	GSH+BLM+1.06 Gy	34±9**	482
	3.17Gy	46±6***	431
	BLM+3.17 Gy	58±1*	362
	GSH+BLM+3.17 Gy	59±1	255
28(^{7}Li)	Untreated	0	271
	BLM(10μg/ml)	0	220
	1.06 Gy	14±2	401
	BLM+1.06 Gy	25±10**	366
	GSH+BLM+1.06 Gy	25±2	487
	3.07Gy	35±4***	282
	BLM+3.07 Gy	39±9	322
	GSH+BLM+3.07 Gy	31±8	318
28(X-rays)	Untreated	0	202
	BLM(10μg/ml)	0	250
	1 Gy	13±2	1555
	BLM+1 Gy	17±2	1040
	GSH+BLM+1Gy	15±2	900
	3Gy	30±3	750
	BLM+3.Gy	35±2	397
	GSH+BLM+3 Gy	33±1	450

Fixation Time	*Experimental Condition*	*M1%*±SEM	*Total Metaphase*
42(^{12}C)	Untreated	0	436
	BLM(10μg/ml)	0	295
	1.06Gy	9±2	288
	BLM+1.06Gy	11±5	557
	GSH+BLM+1.06Gy	7±1	352
	3.17Gy	19±9**	334
	BLM+3.17Gy	23±9	359
	GSH+BLM+3.17Gy	26±2	206
42(^{7}Li)	Untreated	0	236
	BLM(10μg/ml)	0	301
	1.06 Gy	2±2	258
	BLM+1.06 Gy	0	287
	GSH+BLM+1.06Gy	1±2	300
	3.07Gy	7±1**	244
	BLM+3.07Gy	12±1	206
	GSH+BLM+3.07Gy	9±2	215

* Significant at P<0.05, ** Significant at P<0.01, *** Significant at P<0.001 2×2 $\chi 2$ Contingency test.

Effect of BLM and high/low LET radiation alone and in combination on cell cycle proliferation in CHO cells with respect to GSH status:

It is observed that on exposure of CHO cells to combined treatment of BLM with ^{12}C or ^{7}Li or X-radiation, result in increase in M1%. Exogenous addition of GSH to the above treatment has negligible difference on the M1%. The table 2.7 shows the data scored at 14, 28 and 42 hrs of fixed cells (For low LET experiments, cells were fixed only at for 14 and 28 hrs). The degree of delay induction was higher in high LET radiation than X-rays. The difference in the delay induction by the BLM and radiation with or without GSH was not significant.

Discussion:

The present study was designed to evaluate the effects of the combined sequential exposures to Bleomycin (BLM) and low doses of X-radiation (low LET radiation) or Carbon ion beam (^{12}C LET 287 KeV/μm) or Lithium ion beam (^{7}Li LET 60 KeV/μm) as high LET radiation in CHO cell lines. It is known that high LET radiations, such as neutrons produce a higher local density of DNA DSB compared to low LET radiation, such as X-rays (Griffin *et al.* 1995; Anderson *et al.* 2000). It has been suggested that the induction of incomplete chromosome exchanges might increase after high LET exposure, because of the more likely probability of incorrect and incomplete rejoining for the more densely packed DNA DSB (Lucas 1998). In order to see the role of endogenous GSH on joining of DNA DSBs, we allow to interact DNA lesions induced by BLM and either X-rays or Carbon-ion or lithium-ion beam in presence and or absence of reduced GSH.

It has also been shown that BLM-induced DNA dsbs are rejoined *in vitro* with an efficiency similar to that measured for radiation-induced DNA dsbs (Cheong and Iliakis 1997). In the present study, DNA lesions induced by BLM (10μg/ml) was interacted with either high or low LET radiation of ~1 and ~3Gy of doses. Earlier there was a study where DNA damage induced by BLM (20μg/ml) and X-rays (2Gy) are stated to be qualitatively and quantitatively similar although higher

frequency of chromatid breaks induced by BLM and exchanges induced by radiation are observed (Chatterjee and Jacob-Raman 1988). Similar kind of induction of CAs by BLM was observed in muntjac lymphocytes where 40μg/ml BLM induced qualitatively and quantitatively similar to 2Gy of X-ray (Chatterjee and Jacob-Raman 1988). In this study, the frequency of aberrant metaphases, exchanges and deletions was increased when BLM was combined with radiation. This increase is more prominent while BLM combined with low LET radiation. Significant increase in the frequency of exchanges and deletions was observed by Preston (1982) in human peripheral blood lymphocytes while BLM 15μg/ml was combined with 1.5Gy X-ray. It was interpreted that since the DNA damage induced by both BLM and X-rays is repaired rapidly, the probability of obtaining a synergistic effect on aberration frequency with a combined treatment is high. It seems that in this synergistic process misrejoining and misrepair of DNA DSBs induced by both BLM and radiation may take place with a high frequency and thus increased the frequency of exchange aberrations. In an another study, investigating the role of GSH in DNA DSB rejoining, the interaction of the lesions induced by BLM and by γ-radiation was studied since the lesions caused by both have similar and apparent rapid rates of repair. It was observed that combined treatment of BLM and radiation induced higher frequency of CAs, in particular the exchange aberrations and interstitial deletions. However, such increased frequency of exchange aberrations was reduced drastically and the frequency of terminal deletions was increased significantly

when combined treatment was given to BSO-pretreated cells (Dutta *et al.* 2005). Non-homologous end joining (NHEJ) is regarded as the dominant mechanism for DSB repair in vertebrates, especially in G_0 and G_1 phases of the cell cycle (Khanna and Jackson 2001). Several lines of evidence suggest that cells with an intact NHEJ pathway can give rise to chromosomal rearrangements in response to induction of high frequencies of dsbs (Richardson and Jasin, 2000; Rothkamm *et al.* 2001). The consistent level of Ku70 protein in all the treated samples was noted and undetectable level of Rad51 protein also observed in the G_0-lymphocytes indicates the involvement of NHEJ pathway in misrejoing of DNA DSBs (Dutta *et al.* 2005). Therefore, in this study it could be that NHEJ pathway involved in such misrejoining of DNA DSBs and increased in the frequency of exchange aberrations.

The interesting observation in this study is the lesser degree of enhancement of CA when BLM induced DNA lesions interacted with high LET radiation induced DNA lesions. The present data indicate that the frequency of CA induced by high LET radiation was higher than X-rays but the DNA lesions induced by X-rays were interacted better with the BLM-induced DNA lesions. The frequency of exchanges was increased by 1.5 to 1.8 fold in combined treatment of BLM and high LET radiation whereas it was increased by 3 fold while BLM treatment combined with X-rays. The differences in chromatid break rejoining and misrejoining after exposure to low- and high-linear energy transfer (LET) radiation was demonstrated by (Durante *et al.* 1998). Cells were irradiated with hydrogen, neon, carbon or iron ions

in the LET range 0.3-140 keV/μm and were incubated at 37°C for various times after exposure. Little difference was observed in the yield of early prematurely condensed chromosome breaks for the different ions. The kinetics of break rejoining was exponential for all ions and had similar time constants, but the residual level of unrejoined breaks after prolonged incubation was higher for high-LET radiation. The kinetics of exchange formation was also similar for the different ions, but the yield of chromosome interchanges measured soon after exposure was higher for high-LET particles, suggesting that a higher fraction of DNA breaks are misrejoined quickly. On the other hand, the rate of formation of complete exchanges was slightly lower for densely ionizing radiation. In another study it was observed that the rejoining of DSB slows down with increasing LET, being probably due to the increasing complexity of DSB. Oxygenation of cells at the time of irradiation affects half-life values, indicating that radiation chemistry plays an important role (Frankenburg-Schwager *et al.* 1994).

It is noted in this study, the huge elevation in the frequency of exchange aberrations induced by combined treatment of BLM and X-rays but not with BLM and high LET radiation, to GSH-pretreated cells. It indicates that the interaction of DNA lesions induced by BLM and X-rays is improved in presence of GSH and such better interaction between DNA lesions produced by BLM and high LET radiation was not observed.

Earlier it was shown that BLM + γ-rays to BSO-treated cells induced higher frequency of aberrant metaphases and deletions than BSO-untreated cells whereas in the later the frequency of exchanges was increased substantially (Dutta *et al.* 2005). In an attempt to clarify the possible role of GSH in biochemical repair processes, the extent of rejoining of radiation induced SSBs was determined upto 1hr after exposure (Edgren *et al.* 1981; Revesz *et al.* 1984) and it was found that the repair system involved in the rejoining of oxically induced SSBs differs from that involved in the rejoining of hypoxically induced SSBs and is clearly dependent upon GSH. It was also shown earlier that GSH/GSH-ester treatment given after irradiating the cells at 4°C reduced the frequency of deletions and increased the frequency of exchange aberrations which could be due to enhancement in rejoining (both restitution and illegitimate reunion) of radiation-induced DNA DSBs under the influence of increased endogenous GSH (Chattopadhyay *et al.* 1999). If the present induced DNA DSBs are the candidates for the NHEJ-repair pathway then increase frequency of restitution and misrejoining in the presence of endogenous GSH indicate the involvement of GSH either directly or indirectly in the joining of such DNA DSBs

It was also demonstrated that the presence of GSH or thiol-radioprotector potentiates the clastogenic action of BLM (Chatterjee *et al.* 1989; Hoffman *et al.* 1993). It was also observed the reduction in the effect of BLM in GSH-depleted cells which could be explained on the basis of the failure of reactivation of the oxidized BLM by reducing

agent GSH which is normally present endogenously. Therefore, in the present study GSH was added 1 hr before BLM-treatment and thus it is likely that number of DNA lesions induced by BLM was increased in the presence of GSH. However, it is clear that such lesions interact in a better way to X-ray induced lesions than high LET radiation induced lesions. Such differences could be attributed due to the differences in the repair kinetics of the lesions induced by BLM and high LET radiation and also due to the qualitative differences in the DNA lesions. It was shown in the yeast cells which were exposed under oxic or anoxic conditions to sparsely (30 MeV electrons) or densely (3.5 MeV α-particles) ionizing radiation that the rejoining of DNA DSB slows down with increasing LET, being probably due to the increasing complexity of DSB. Oxygenation of cells at the time of irradiation affects half-life values, indicating that radiation chemistry plays an important role (Frankenburg-Schwager et al 1994). It is known that the chemistry of the end of DNA strand breaks induced by X-rays and BLM are similar (Henner *et al.* 1982) which is unlikely for BLM and high LET radiation induced strand breaks (Pastwa *et al.* 2003).

The question may arise whether the response shows by the analysis of CAs in different treated samples in this study, does really show the effect of treatment or is it simply due to scoring of different cell populations as a result of cell cycle shift. In fact the cells were fixed at 14, 28, and 42 hrs in this study and was scored 1^{st}, 2^{nd} and subsequent cycles simultaneously with the aberration scoring and the differences in

the frequency of M1 cells between radiation, BLM+radiation and GSH+BLM+radiation were not significant.

The combined use of chemotherapy and radiation therapy in cancer treatment would seem to be a logical and reasonable approach. Local control of tumor has been achieved by high dose radiation therapy combined with systemic chemotherapy in place of surgery, with the hopes well of control of metastatic diseases. Chemotherapeutic drugs appear to enhance the effects of radiation, making the integration of both modalities even more appealing. The experience has been in the most experimental models and in some clinical trials that when drugs and radiation are combined simultaneously, normal tissue toxicity is enhanced (Phillips and Fu 1976; Tubiana 1989). Research continues in this area with an emphasis on gaining better knowledge of the optimal timing of each modality and mechanistically how each agent interacts.

A mode of combination therapy with BLM and irradiation has been evaluated in cancer patients (Edsmyr *et al.* 1985; Tabata *et al.* 2003) and increased levels of GSH are commonly found in the drug-resistant human cancer cells (Nagata *et al.* 2001). Therefore, combined treatment of BLM and low LET radiation to such cancer cells may induce higher level of DNA damage including exchange aberrations which could lead apoptotic cell death. Unstable aberrations such as dicentrics are one of the main causes of cell lethality because of mechanical difficulties in cell division (Nickias *et al.* 1995). It has been shown that the apoptotic process is primed when the dicentric-bearing human peripheral blood lymphocytes attempt to exit from metaphase.

It is possible that unstable aberrations generate changes in the mitotic spindle causing mechanical tension at the kinetochore, activating the mitotic checkpoint and the execution of p53/survivin-dependent apoptosis (Bassi *et al.* 2003). Therefore, the present result may also have some implication in such combination therapy.

CHAPTER: 3

Involvement of GSH in DNA DSB joining and misrejoining pathway

The maintenance of genomic integrity is crucial for the survival and proper functioning of the cell. The important cellular effects of exposure to ionizing radiation arise primarily from the induction of DNA double strand breaks (DSB) as a consequence of the nature of the energy deposition in DNA by ionizations that produce local clustered damage. When ionizing radiation strikes a cell, DSB and other lesions are produced within less than a millisecond. Thereafter, some of the damage is processed more slowly, in enzymatic repair or misrepair reactions, whose outcome often determines the fate of the cells.

Ionizing radiation is very efficient in inducing chromosome aberration (CA) at all stages of the cell cycle both in vivo and in vitro. Two main hypothesis for ionising radiation induced CA formation were put forth by researchers, namely, 'breakage first' by Sax and 'contact first' by Serebovsky 1929 and Revell 1959. While the breakage first hypothesis can account for the origin of CA at all stages of the cell cycle, the contact first hypothesis assumes that most of ionising radiation induced chromosome breaks are efficiently restituted (repaired) while the minor fraction of breaks is either rejoined (misrepaired) or remains open (unrepaired). Appearance of chromosome fragments is due to unrepaired events while the exchange aberrations are due to misrepaired events. Contact first hypothesis assumes that the chromatid exchanges occur in predetermined regions that are in close proximity at the time of irradiation and failure in chromosome exchange formation

will give rise to fragments. Large number of studies suggests that DNA DSB induced by ionizing radiations is the critical lesions which if unrepaired or misrepaired can cause CAs, cell death as well as mutations and cell transformation (Frankenberg-Schwager 1990, Iliakis 1991). Radiation induces exchange aberration which is thought to arise as a consequence of illegitimate reunion (misrejoining) of free ends from different DNA DSBs (Cornforth and Bedford 1993). Such misrejoining may be expected to depend on the number and proximity of the breaks. The majority of models of radiation action hold that the curvilinear dose responses exhibited by eukaryotic cells to sparsely ionizing radiations result from the interaction of pairs of lesions produced in sensitive targets of the cell. Within this conceptual framework, chromosomal exchange aberrations (e.g., interchanges) are believed to occur through the interaction of damaged sites on both chromosomes participating in the exchange. In contrast, the model proposed by Chadwick and Leenhouts (as well as some other models) suggests that such exchanges arise from initial radiation damage to only one chromosome, which then becomes associated with an undamaged chromosome. A particular aspect of this theory is that asymmetrical exchanges, such as dicentrics, may be formed from the rejoining of a broken end of one chromosome to the telomere of another. By using a DNA probe that specifically hybridizes to the telomeric region of human chromosomes, they were able to test this assertion directly. After scanning more than 200 dicentrics produced in normal human fibroblasts by 6 Gy of ^{60}Co γ-rays, virtually none were

found that contained telomeres located between the centromeres of this aberration type. Therefore, since the proposed telomere-break rejoining process, per se, is not necessarily a central element of the Chadwick-Leenhouts model, the theory was modified to exclude this mechanism (Cornforth *et al.* 1989). The kinetics of DNA strand break repair was studied in exponentially-growing CHO cells after X-irradiation with doses of 3, 9, 30, 60 and 90 Gy. DNA strand breaks were measured using the alkaline unwinding technique. For all X-ray doses applied the kinetics of DNA strand break repair consisted of fast, intermediate and slow phases. The latter, which was interpreted as the repair kinetic of DNA double-strand breaks, was best described by an exponential decline. This result indicated that the repair of double-strand breaks was unsaturated for doses up to 90 Gy. The repair kinetics of the breaks of the fast and intermediate phases were found to be dependent on the dose applied (Dikomey *et al.* 1993).

In the previous chapter, we have seen that high LET radiation induced higher frequency of exchange aberrations than low LET radiation. It is also noted that the DNA lesions induced by high LET radiation interact poorly with the lesions induced by BLM. It was shown earlier (Chattopadhyay *et al.* 1999, Dutta *et al.* 2005) and also in these studies that presence of GSH increased the interaction of DNA lesions induced by low LET radiation and BLM much better way than the lesions induced by high LET radiation and BLM. In order to confirm the role of GSH in such lesions interaction we have taken three different approaches :

1. Allow the interaction of lesions induced by X-rays and BLM at 4°C in presence of GSH and compare it with the similar treatment at 37°C.
2. Allow the interaction of lesions induced by X-rays and BLM in presence of agent that selectively blocks DNA repair pathways.
3. Allow the interaction of lesions induced by X-rays and BLM in DNA- repair deficient cell lines.

Cells have two genetically defined pathways for repairing DNA DSBs. The error-prone nonhomolgous end joining (NHEJ) pathway rapidly and promiscuously ligates the end of broken chromosomes while homologous recombination repair (HR) uses homologous template in a sister chromatid or homologous chromosome to perform error-free repair. In quiescent mammalian cells, this process has been reported to take place prevalently by the NHEJ (Jackson 2002) rather than HR pathway, which is mainly important during S or G_2 phases (Johnson and Jaisin 2000).

Homologous Recombination (HR)

The molecular basis and genetic requirements of HR were initially defined by studies in bacteria and yeast but it has become clear that this pathway is well conserved in higher organisms (Cromie *et al.* 2001; Haber 2000). Genetic analysis of *S. cerevisiae* identified a set of genes- RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11 and XRS2-whose products play important roles in HR and whose

defects lead to increased sensitivity to ionizing radiation. Mammalian homologues of essentially all of these factors in the RAD50 group have been described (Wood *et al.* 2001). Although mammalian homologues exist for all of the known *S. cerevisiae* HR factors, the details of HR are likely to be considerably more complex in higher eukaryotes. One indication of this is the existence of several RAD51 paralogues, such as RAD51B,C,D, and other proteins with weaker homology to the catalytic domain of RAD51, such as XRCC2 and XRCC3 (Wood *et al.* 2001). Some of these factors interact directly with RAD51 and their functions appear to be to help the assembly of the RAD51 nucleoprotein filament and/or the selection and interaction with the appropriate recombination substrate.

Non Homologous End Joining (NHEJ):

NHEJ is carried out in large part by DNA-PK and inhibition of DNA-PK activity enhances DNA damage induced by genotoxic agents (Oliveria *et al.* 2002).

DNA PK is a nuclear serine/threonine kinase composed of a catalytic subunit (DNA PKCs) and a DNA binding Ku70/80 subunit. DNA PK is constitutively present at a high level in the nucleus, with the protein level not significantly affected by genotoxic agents, while the activity of DNA-PK is regulated throughout the cell cycle (Lee *et al.* 1997). It has been proposed that Ku recruits DNA-PKcs to DNA, which in turn facilitates DNA-PKcs-DNA interaction, thus including conformation

changes in DNA-PKcs and releasing the catalytic potential of DNA-PK complex (Smith and Jackson 1999).

DNA-PK and NHEJ:

NHEJ begins when Ku binds to both DNA ends of DSBs. Ku would then recruit DNA-PKcs to the site, likely initiating end joining by tethering the two DNA ends together through the formation of a synaptic complex containing two DNA molecules. Electron microscopy showed complexes of two DNA ends brought together by two DNA-PKcs molecules. The DNA associated DNA-PK may recruit other proteins or factors that are involved in NHEJ to form a repair complex. One example is the X-ray cross-complementing protein-DNA ligase IV complex, which helps complete the DNA repair pathway (Leber *et al.* 1998). Another example is the MRN complex, whose nuclease activity may be critical in cleaning up the damaged DNA termini before they can be ligated together.

Another factor that influences the activity of DNA-PK in NHEJ is the autophosphorylation of DNA-PKcs at multiple residues (Thr2609, Ser2612, Thr2638, Thr2647 and possibly, additional residues not currently identified) in response to ionizing radiation *in vivo* and this process occurs in a Ku-dependent manner. Mutation of Thr2609 to Ala leads to radiation sensitivity and impaired DSB rejoining. Thus, these findings establish that Ku-dependent phosphorylation of DNA-PKcs is required for the repair of DSBs by NHEJ.

Other proteins may also interact with DNA-PK to regulate its activity in NHEJ. For example, Ku70/80 can interact with WRN and stimulate WRN exonuclease activity. WRN forms a complex with DNA-PKcs and Ku in solution and WRN can be phosphorylated by DNA-PK (Karmakar *et al.* 2002). However, addition of WRN to a Ku-DNA-PKcs-DNA complex results in the displacement of DNA-PKcs from the DNA, indicating that the tri- protein complex WRN-Ku-DNA-PKcs displacement of DNA-PKcs from the N- and C- terminal regions of WRN, both of which make direct contact with the Ku70/80 heterodimer. These results point to a potential role of WRN in influencing how DNA ends are processed during NHEJ (Li and Comai 2002).

The participation of various DNA DSB repair mechanism in the formation of CA is not yet fully understood. To investigate particularly the role of NHEJ, study is carried out in order to investigate the formation of radiation induced aberrations in a DNA PKCs- proficient cell line M059K and in deficient cell line M059J. On inhibition of DNA PK and ATM activity by wortmannin in M059K, showed that elimination of DNA PK dependent NHEJ can recruit a slow, error-prone repair process, which is DNA-PK independent and favours the increased formation of chromosome aberration. Recently, Iliakis *et al.*, have proposed the existence of two types of NHEJ pathways: D-NHEJ (DNA-PK dependent) and a B-NHEJ (back up non-homologous end joining). While D-NHEJ is presumably involved in the fast DSB repair

component with low level of DSB misrejoining, B-NHEJ is involved in the slow DSB repair component with high level of DSB misrejoinig.

Ionizing radiation is considered to be an S phase independent agent that induces chromosome type aberrations in G_0/G_1 stage and chromatid type aberrations in S/G_2 phases. However, human and rodent cells mutated in some of the gene products such as ATM and Ku80 in NHEJ pathway display both chromosome and chromatid type aberrations after ionising radiation exposure at G_0/G_1 stages (Taylor 1978; Natarajan and Meijers 1979). Work has been carried out in parental cells AA8, NHEJ deficient cells V33, HR deficient cell Irs 1SF, 51-D1 and revertant of V33 mutant, V33-155 cells. It has been suggested that NHEJ is the most critical pathway for DSB repair whose impairment causes CA in all phases of the cell cycle. The extent of CA in HR deficient cell line was considerably less than NHEJ defective cells. The pattern of CAs observed (ratio between chromatid type and chromosome type) following G_1 irradiation provides some interesting insight towards the relative efficiency of the two DSB repair pathways in ionizing radiation induced chromosome damage and some clues for the importance of B-NHEJ in CA formation (Natarajan *et al.* 2008).

In quiescent mammalian cells, the repair pathway is prevalently taken up by NHEJ rather than HR pathway, which is mainly important during S and G_2 phases. Prominent high expression of Ku70 protein in G_0 human lymphocytes treated with BLM or radiation, and undetectable amount of Rad51 protein, indicates the involvement of NHEJ pathway in DNA DSB joining process. Western analysis also showed that the

level of Ku70 protein did not get altered even when the treatment is given to BSO-pretreated cells (Dutta *et al.* 2005). This result indicates the involvement of NHEJ as the predominant repair pathway-taking place in quiescent mammalian cells.

Earlier report of increase in the exchange aberration in irradiated human blood lymphocytes at G_0 stage indicates the involvement of GSH in NHEJ pathway, that is the predominant DNA DSB repair pathway during G_0/G_1 phase of the cell cycle (Dutta *et al.* 2005). For better understanding the role of GSH in DNA DSB joining/misrejoining pathway, we have already mentioned three approaches that had taken in this investigation.

For the purpose to investigate the involvement of GSH in NHEJ pathway, attempt is made to inhibit the activity of DNA PK with Vanillin in human blood lymphocytes treated with Bleomycin and radiation in presence of exogenous GSH. Vanillin (3-methoxy-4-hydroxybenzaldehyde)- a naturally occurring food component that is an inhibitor of NHEJ pathway. Vanillin, a food-flavoring agent, has been given GRAS (generally regarded as safe) status by the Flavor and Extract Manufacturers Association (FEMA) and is recognized as suitable for food use by the Food and Drug Administration (FDA).

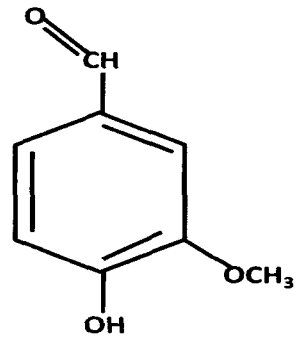


Fig: Structure of Vanillin

Vanillin occurs naturally in the pods of *Vanilla planifolia, Vanilla tahitensis* and *Vanilla pompona* and is also synthesized on a large scale in the food industry for use as a flavouring agent. Vanillin itself exhibits little cytotoxicity, mutagenicity, clastogenicity in model systems-including cultured mammalian cells (Jansson and Zech 1987; Tamai *et al.* 1992). Vanillin selectively blocks DNA repair by the relatively error-prone NHEJ pathway by inhibiting DNA PK. The aldehydic group of vanillin binds preferentially to lysine of DNA PKcs via Schiff Base formation (Chobpattana *et al.* 2000). Thus it modifies the key active site lysine of DNA PK, which is involved in such repair of DNA DSB.

Thus, if vanillin is inhibiting the DNA PK activity, then the chromosome exchanges which result due to misrepair of DNA DSB should decrease in frequency in mammalian cells treated with BLM and X-radiation, which otherwise show a very high percentage of exchanges. So, we designed our experiment where we treated the

HPBLs with vanillin before BLM and X-radiation in order to study the formation of exchange aberration.

In another approach, an attempt was made to assess the role of GSH in NHEJ pathway, by using mammalian cells deficient in NHEJ pathway. The cell lines used in this study are as follows:

Genotype and characteristics of CHO cell line used in this study:

Cell Line	Genotype	Defect	Human homolgues
AA8	Wild-type	-	-
V33	XRCC7$^-$	Deficient in NHEJ	DNA-PK

The chapter will thus deal with the objective to find out the contribution of GSH in DNA DSB repair pathway in mammalian cell system. An interesting insight towards the relative efficiency of GSH in DSB repair pathway can be inferred from the designed experiments to conclude the involvement of GSH in repair pathways.

Experimental Model:

Human Peripheral Blood Lymphocytes (HPBLs)

Chemicals: For HPBL culture:

- RPMI 1640 Medium, Penicillin Streptomycin, Phytohaematoglutinin(PHA), and Colcemid Gibco, USA.
- Foetal Calf Serum (FCS), Biological Industries, Israel.
- Bleomycin, Cipla, India, Used at a concentration of 20μg/ml.

- Vanillin, Sigma Chemical Company, USA, used at concentration of 1.5mM.
- Glutathione Reduced, Sigma Chemical Company, USA, used at concentration of 15 mM.
- DL- Buthionine-(S,R)-Sulfoximine (BSO), Sigma Chemical Company, USA, used at a concentration of 5 mM.
- 5-bromo-2-deoxyuridine (BrdU), Sigma Chemical Company, USA; BrdU powder is dissolved in autoclaved Millipore water to prepare a working solution of 100μg/ml.
- Bis-benzimide (Hoechst 33258), Sigma Chemical Company, USA,This is used at a concentration of 50μg/ml in double distilled water.
- Giemsa Stain Solution, BDH Chemicals Ltd., UK.

All other chemicals used are of analytical grade.

For all HPBL culture, heparinized blood was collected from healthy donors in the age group of 25-30 years and is used immediately after venipuncture.

Protocol of HPBL culture exposed to combined treatment of BLM and radiation in presence of exogenous GSH:

1ml of heparinized blood collected in 25 ml flat-bottomed sterilized beaker

↓

BLM is given at a concentration of 20μg/ml

↓ *Incubated for 2 hrs (4°C and RT)*

Cells irradiated with 2/4 Gy of X-radiation (4°C and RT)

↓

GSH is added immediately after radiation at a concentration of 15mM.

↓ *Incubated for 1 hr (4°C and RT)*

Blood transferred to sterile culture tubes

↓

Culture is set by addition of 0.2 ml of PHA

↓

RPMI 1640 supplemented with 10% FCS is added to the cells.

↓

BrdU at a concentration of 6μg/ml is added to the culture

↓

Culture is harvested at 72 hrs for metaphase chromosome preparation

Protocol of HPBL culture with vanillin treatment in GSH-pretreated cells exposed to combined treatment of BLM and radiation:

Vanillin was dissolved in water by heating at 70°C for 10 mins. Vanillin is added at a concentration of 1.5mM to heparinized peripheral blood samples and the cells are incubated for 3 hrs. Wherever GSH is added exogenously, it is added at a concentration of 15 mM one hour before vanillin treatment. After 3 hrs of vanillin addition, 20 μg/ml BLM is added to the blood and incubated for 2 hrs. Cells are exposed to 4Gy of X-radiation. After irradiation, the blood is incubated for 1 hr and then it

is transferred to sterile culture tubes. The culture is set as mentioned previously and harvested at 72 hrs for chromosome preparation.

Protocol for using V33 and AA8 cell line:

Parental cell AA8, and NHEJ deficient cell line V33, used in the study is a generous gift of Dr. Larry Thompson to Dr. Palitti/Dr. Natarajan's laboratory at the University of Tuscia, Italy.

Cells were cultured in Ham's F-10 medium supplemented with 10% foetal calf serum and antibiotics. The cells were treated with BSO at a concentration of 500μM for about 3 hrs before irradiation. In some of the cases, exogenous GSH was added to the cells 30 mins before X-irradiation. Cells were irradiated with X-radiation at a dose of 0.5, 1, 2, 3, and 4Gy or split dose of 2Gy into 1 +1Gy with a gap of 10 mins or 4Gy into 2+2Gy with a gap of 10 mins. Soon after irradiation, BrdU is added to the cells and harvested at 28 or 33 hrs.

Metaphase chromosome preparation:

The preparation of the metaphases and differential staining were done by the same procedure as mentioned in chapter 1.

Scoring and statistical analysis:

Slides are coded randomly and are studied for chromosome exchanges, deletions and chromatid breaks. The statistical significance of the difference between the control and the treated groups were determined. Aberrant metaphases were tested using the 2x2 χ^2 Contingency test. The different types of aberrations studied were compared using the simple χ^2 test.

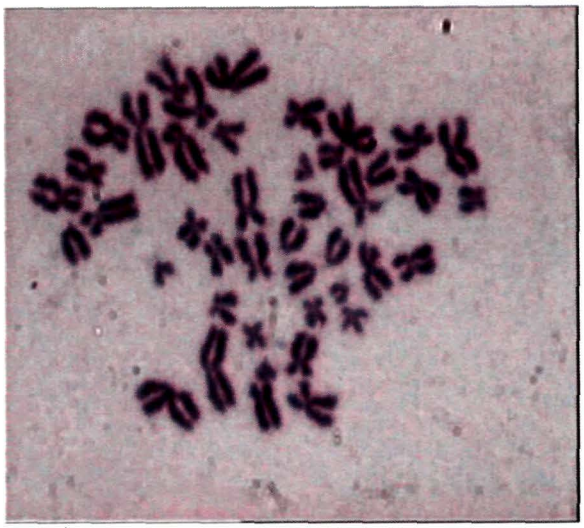


Fig3.1: 1st Cycle Metaphase of Human Blood Lymphocyte

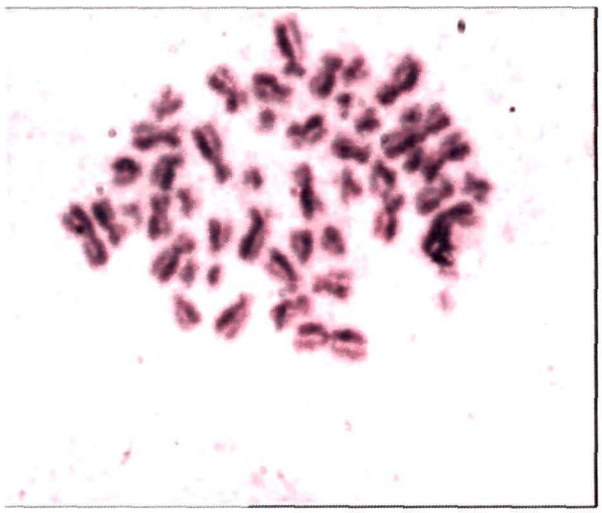


Fig 3.2: 2nd Cycle Metaphase of Human Blood Lymphocyte

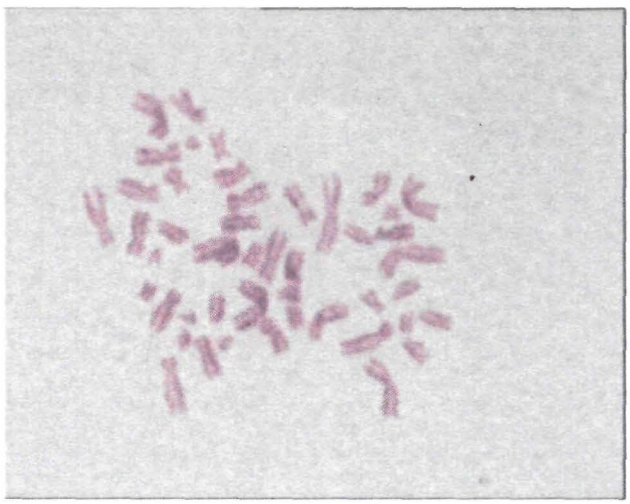


Fig 3.3: 3rd Cycle Metaphase of Human Blood Lymphocyte

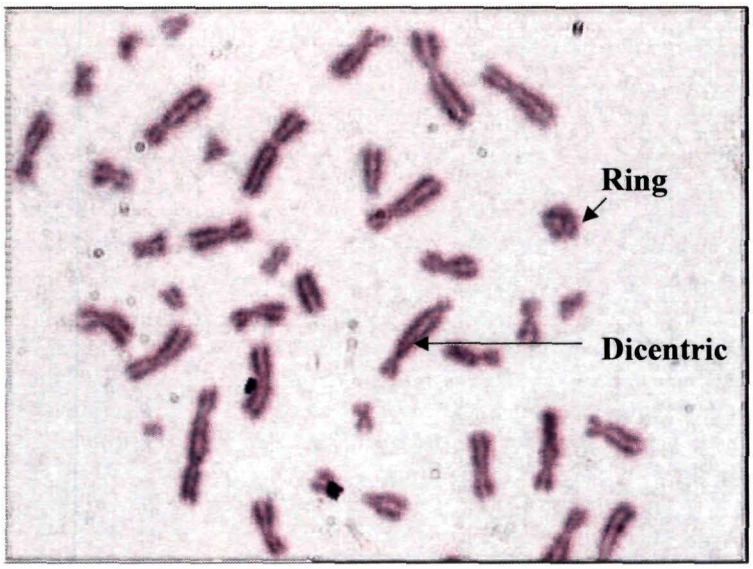


Fig 3.4: Exchange aberration in human blood lymphocytes treated with X-radiation and Bleomycin

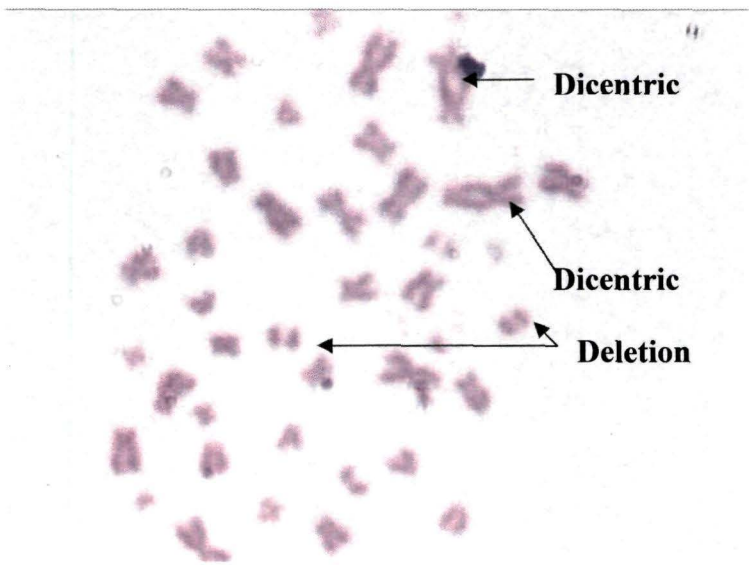


Fig 3.5: Dicentric and Deletion in human blood lymphocytes

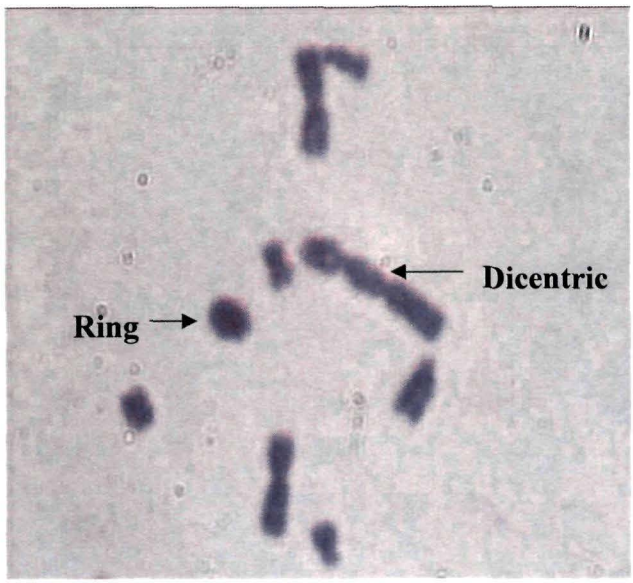


Fig 3.6: Chromosome exchange in AA8 cell line after X-irradiation

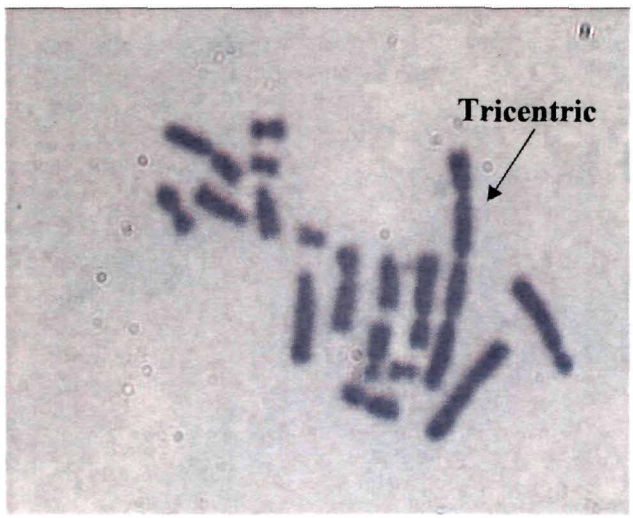


Fig 3.7: Chromosome exchange in V33 cell line after X-irradiation

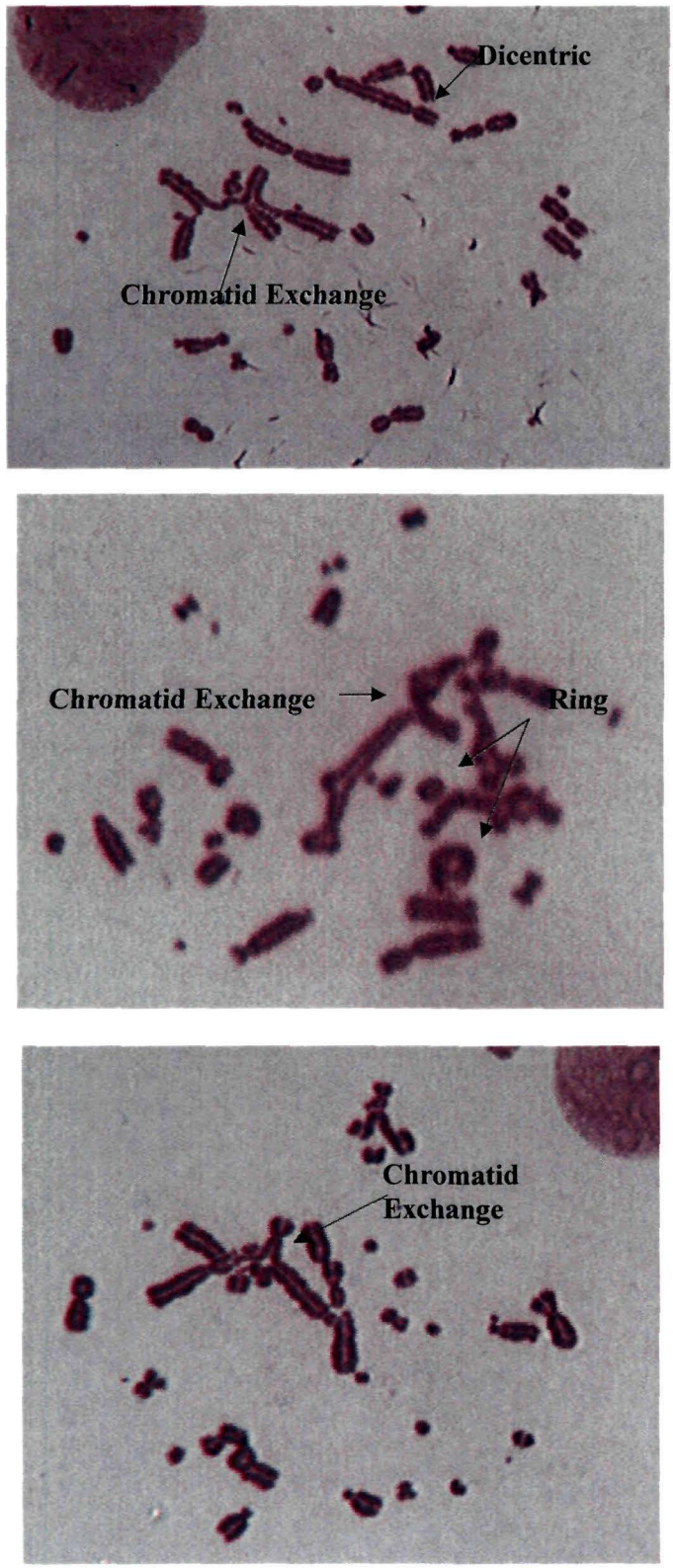


Fig 3.8: Chromosome and chromatid type of aberration in V33 cell line after X-irradiation in presence of exogenous GSH

Result:

Table 3.1: Induction of CAs by BLM or X-rays (0.68 Gy/min) alone or in combination with or without GSH in HPBLs at RT or 4°C.

Donor	*Experimental Condition*	*Aberrant Metaphase %*	*Total Metaphase*	*Exchange %*	*Deletion %*	*Chtd. Break %*
1	Untreated	0	80	0	0	1
	BLM	32	119	14	19	9
	2Gy	44	105	16	45	5
	BLM+2Gy	60	111	23@	72#	5
	BLM+2Gy+GSH	67	120	38	65	5
	BLM(4°C)	40	114	7	28	4
	2Gy(4°C)	52	103	7	53	9
	BLM+2Gy(4°C)	69*	100	10@	114$	9
	BLM+2Gy+GSH(4°C)	68	147	37#	87$	4
2	Untreated	1	106	0	1	0
	BLM	29	106	13	25	8
	2Gy	44	108	16	44	5
	BLM+2Gy	62	103	21	86#	9
	BLM+2Gy+GSH	75	93	43$	73$	12
	BLM(4°C)	36	116	6	36	6
	2Gy(4°C)	51	115	10	67	9
	BLM+2Gy(4°C)	66*	100	12	107$	10
	BLM+2Gy+GSH(4°C)	74	176	34#	91	7
3	4Gy	80	128	23	82	13
	BLM+4Gy	74	115	110$	104	17
	BLM+4Gy+GSH	87**	173	127#	99#	23#
	4Gy(4°C)	86	110	22	99	20
	BLM+4Gy(4°C)	81	106	92$	118	23
	BLM+4Gy+GSH(4°C)	86	128	121	117	16
4	Untreated	2	129	0	0	2
	BLM	36	170	16	23	14
	4Gy	75	110	42	79	14
	BLM+4Gy	77	121	101$	93	17
	BLM+4Gy+GSH	95***	163	122$	82$	11
	BLM(4°C)	42	142	9	37	12
	4Gy(4°C)	83	115	33	90	13
	BLM+4Gy(4°C)	90*	102	57@	99	12
	BLM+4Gy+GSH(4°C)	97*	110	99#	92	14

* Significant at P<0.05, ** Significant at P<0.01, *** Significant at P<0.001 2X2 $\chi 2$ Contingency test. @ Significant at P<0.05, # Significant at P<0.01, $ Significant at P<0.001 simple $\chi 2$ test (compared to respective control).

Table 3.2: Pooled data of induction of CAs by BLM or X-rays (0.68 Gy/min) alone or in combination with or without GSH in HPBLs at RT or 4°C.

Experimental Condition	*Aberrant Metaphase %± SEM*	*Total Metaphase*	*Exchange % ± SEM*	*Deletion %± SEM*	*Chromatid Break %± SEM*
Untreated	1±0.6	315	0	0.3±0.3	0.7±0.7
BLM	32±2	395	14±1	22±2	10±2
2 Gy	44±0	213	16±0	45±1	5±0
BLM+2 Gy	61±1	214	22±1	79±7$^{@}$	7±2$^{\$}$
BLM+2Gy+GSH	75***	93	43$^{\$}$	73$^{\$}$	12
4Gy	78±3***	238	33±10	81±1	14±1
BLM+4 Gy	76±2	236	106±5$^{\$}$	99±6	17±0
BLM+4Gy+GSH	91±4	336	125±3$^{\$}$	91±9$^{\$}$	17±6
BLM(4°C)	39±2	372	7±1	39±3	7±2
2Gy(4°C)	52±1	218	9±2	60±7	9±0
BLM+2Gy(4°C)	68±2*	200	11±1$^{\#}$	111±4$^{\$}$	10±1
BLM+2Gy+GSH(4°C)	71±3	323	36±2$^{\$}$	89±2	6±2
4Gy(4°C)	85±2	225	28±6	94±5	17±4
BLM+4Gy(4°C)	86±5	208	75±18$^{\$}$	109±10$^{\$}$	18±6
BLM+4Gy+GSH(4°C)	92±5	238	110±11$^{\#}$	105±13$^{@}$	15±1

* Significant at P<0.05, *** Significant at P<0.001 2X2 $\chi 2$ Contingency test; @ Significant at P<0.05, # Significant at P<0.01, $ Significant at P<0.001 simple $\chi 2$ test(compared to respective control).

Table 3.3: Pooled data of cell cycle kinetics of HPBL treated with BLM or X-rays (0.68 Gy/min) alone or in combination with or without GSH in HPBLs at RT or 4°C.

Experimental Condition	*M1%±SEM*	*Total Metaphase*	*AGT±SEM*
Untreated	31±4	1024	35.56±1
BLM	55±5	804	44.91±2
2 Gy	65±4	361	50.38±1[a]
BLM+2 Gy	85±1**	297	59.76±1[b]
BLM+2Gy+GSH	94**	192	67.92
4Gy	71±5	470	53±3
BLM+4 Gy	88±1***	275	64±0.3
BLM+4Gy+GSH	98±1***	359	70.59±1
BLM(4°C)	68±9	649	51.98±3
2Gy(4°C)	81±1	310	57.60±1
BLM+2Gy(4°C)	90±0**	265	64.87±1
BLM+2Gy+GSH(4°C)	94±1*	374	67.59±0.3
4Gy(4°C)	79±5	414	59.83±2
BLM+4Gy(4°C)	92±0***	266	66.46±0.4
BLM+4Gy+GSH(4°C)	97±1*	263	69.57±0.3

* Significant at P<0.05, ** P<0.01, *** P<0.001 2X2 $\chi 2$ Contingency test;

@ Significant at P<0.05, # P<0.01 simple $\chi 2$ test, $ P<0.001 simple $\chi 2$ test (compared to respective control).

Human lymphocytes exposed to combined treatment of BLM and radiation in presence of exogenous GSH:

Data obtained in the experiments provide information regarding the role of GSH in DNA DSB interaction of the lesion induced by BLM and radiation. We treated the HPBLs with Bleomycin (BLM) and X-radiation of dose 2 and 4 Gy. Soon after radiation, the cells are treated with GSH and incubated for one hour before setting up the culture. As mentioned in the protocol, the experiment is done at 4°C and at RT till

the culture is set. The rationale for such treatment at 4°C is based on the premise that the DNA DSB produced by BLM and radiation must not be repaired until GSH is present in the cell system. Our intention is to study the exchange frequency in GSH treated cells, which can explain its involvement in DNA DSB repair / misrepair.

BLM+Irradiated samples at 4°C were studied as positive controls for the GSH post treated samples. From the data presented in Table 3.1 and 3.2 we can perceive that the combined treatment of BLM and radiation induces significantly increased frequency of exchange and deletions. Such increase in the frequency of aberrations was found in both the experimental condition i.e. at 4°C as well as at room temperature. However, the degree of increase in the frequency was more at room temperature than at 4°C. After addition of GSH exogenously, the frequency of exchanges was increased significantly. The degree of increase in the frequency of exchanges was more at 4°C than at room temperature. It was found that the frequency of exchanges after GSH addition in the sample treated with BLM+ 2Gy and BLM+4Gy at 4°C was increased within the range of 2.83 – 3.7 fold and 1.32 – 1.74 fold, respectively whereas it was 2 fold and 1.15 – 1.21 fold in the samples treated at 37°C.

With respect to the frequency of deletions, it was decreased after addition of GSH in both the samples treated at 4°C and at room temperature. The degree of reduction is similar in both the conditions. It is also noted that the frequency of aberrant metaphases was increased wherever GSH was added after the treatment.

In order to study the cell cycle kinetics, the frequency of M1% was scored from FPG-stained slides. It was observed that the frequency of M1 was increased on treatment with either BLM or radiation. It is also noted that the degree of increase was more in the samples treated at 4°C than 37°C. The combined treatment with BLM and radiation showed significant increase in the frequency of M1 with respect to positive control. However, further increase in the frequency of M1 was observed after GSH addition, but such increase was not significant.

Table 3.4:Effect of vanillin (1.5m M) on chromosome exchangeaberration formation induced by BLM and radiation with or without GSH.

Donor	*Experimental Condition*	*Aberrant Metaphase %*	*Total Metaphase*	*Exchange %*	*Deletion %*	*Chtd Break %*
1	Untreated	2	98	0	2	0
	4Gy	75	104	46	71	7
	BLM+4Gy	71	120	104$^{\$}$	93$^{\$}$	10$^{\$}$
	Van+BLM+4Gy	76	105	89	133	14
	Van+GSH+BLM+4Gy	88*	114	96	122	11
2	Untreated	1	83	0	1	0
	4Gy	73	124	52	73	4
	BLM+4Gy	71	121	118$^{\$}$	97$^{@}$	5
	Van+BLM+4Gy	72	116	102	115	8
	Van+GSH+BLM+4Gy	83	96	111	113	7
3	Untreated	3	70	0	1	1
	Van	6	127	0	2	3
	4Gy	77	77	51	114	6
	BLM+4Gy	72	72	112	108	14
	Van+4Gy	80	75	55	137	3
	Van+BLM+4Gy	79	73	98	137	7
	Van+GSH+BLM+4Gy	82	110	106	123	11
4	Untreated	0	12	0	0	0
	Van	5	187	0	2	4
	4Gy	78	135	45	85	10
	BLM+4Gy	78	105	93	99	13
	Van+4Gy	82	111	39	95	11
	Van+BLM+4Gy	84	100	87	141	13
5	Van	6	193	0	2	5
	4Gy	72	174	56	76	11
	BLM+4Gy	87***	143	100$^{\$}$	77	11
	Van+4Gy	78	186	48	89	11
	Van+BLM+4Gy	92	166	84$^{@}$	96	12
	Van+GSH+BLM+4Gy	97	121	90	90	20

* Significant at P<0.05, *** Significant at P<0.001 2X2 $\chi 2$ Contingency test. @ Significant at P<0.05,

Significant at P<0.01, $ Significant at P<0.001 simple $\chi 2$ test (compared to respective control).

Table 3.5: Pooled data of Effect of vanillin (1.5m M) on chromosome exchange aberration formation with or without exogenous GSH.

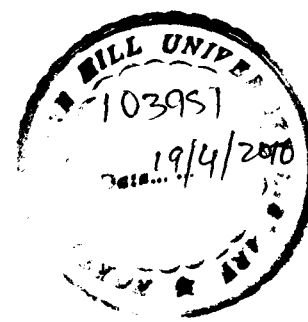
Experimental Condition	*Aberrant Metaphase %± SEM*	*Total Metaphase*	*Exchange % ± SEM*	*Deletion %± SEM*	*Chtd. Bk. % ± SEM*
Untreated	2±0.7	263	-	1±0.41	0.3±0.3
Van	6±0.3	507	-	2±0	4±0.6
4Gy	75±1	614	54±5	82±6	8±1
BLM+4Gy	74±2	430	105±4$^{\$}$	82±6	11±2
Van+4Gy	80±1	372	54±11	104±12	8±3
Van+BLM+4Gy	81±3	560	92±3$^{@}$	124±8$^{\#}$	11±1
Van+GSH+BLM+4Gy	88±3	441	103±4$^{\#}$	112±8	13±3

@ Significant at P<0.05, # P<0.01 simple $\chi 2$ test, $ P<0.001 simple $\chi 2$ test (compared to respective control).

Table 3.6: Pooled data of Effect of vanillin (1.5m M) on cell cycle kinetics with or without exogenous GSH.

Experimental Condition	*M1%±SEM*	*Total Metaphase*	*AGT±SEM*
Untreated	37±9	684	39.55±2
Van	52±3	1038	43.55±1
4Gy	81±3	805	57.84±2
BLM+4Gy	88±3***	737	63.7±2
Van+4Gy	83±7	504	60.19±4
Van+BLM+4Gy	88±5	666	65.99±2
Van+GSH+BLM+4Gy	96±1***	523	68.60±1

*** P<0.001 2X2 $\chi 2$ Contingency test (compared to respective control).



Human lymphocytes exposed to combined treatment of BLM and radiation in presence of Vanillin and or exogenous GSH:

In order to corroborate the role of GSH on DNA DSB interaction, there are 5 set of lymphocyte cultures where the cells were treated with DNA PKc inhibitor, vanillin, before treatment with BLM + X-irradiation. All these treated samples were studied as positive controls for the Vanillin + BLM + X-irradiation (Table 3.4, 3.5 and Fig. 3.11 and 3.12). It is observed that treating the cells with vanillin before BLM + radiation, result in decrease in the frequency of exchanges and increase in the frequency of deletions. In fact in all the 5 cultures, vanillin treatment before BLM+Radiation showed ~16% reduction in the frequency of exchange aberrations. Even presence of GSH increased the frequency of exchanges only marginally when vanillin was there.

The frequency of 1st cycle metaphases was increased significantly on treating the cells with radiation and BLM alone and also in combination. Presence of vanillin did not alter the frequency of M1 in any significant manner.

Table 3.7: CA in AA8 cells and V33 Cells exposed to X-radiation (33hrs)

Exptal. Condn.	*Aberrant Metaphase %*	*Total Metaphase*	*Chrom. Exch. %*	*Chtd. Exch %*	*Del %*	*Chtd. Bk %*	*TM*	*MI %*
Untreated								
AA8	12	50	6	0	6	2	165	20
V33	18	113	4	4	18	4	133	24
0.5 Gy								
AA8	42***	50	16#	0	34$	0	183	21
V33	66***	302	21$	43	75$	40$	441	26

*** Significant at P<0.001 2X2 $\chi 2$ Contingency test (compared to respective control)

@ Significant at P<0.05, # Significant at P<0.01, $ Significant at P<0.001 simple $\chi 2$ test (compared to respective control). Chrom. Exch.= Chromosome Exchange; Chtd. Exch.= Chromatid Exchange; Del.= Deletion; Chtd. Bk.= Chromatid Break, TM= Total Metaphase.

Table 3.8: CA in AA8 and V33 cells exposed to X-rays with respect to GSH status (harvested at 33hrs).

Experimental Condition	*Aberrant Metaphase %*	*Total Metaphase*	*Chromosome exch. %*	*Chromatid exchange %*	*Deletion %*	*Chtd. Break %*
Untreated						
AA8	8	129	2	0	3	1
V33	21	193	2	0	11	6
2 Gy						
AA8	57***	47	36$	0	43$	2
V33	87***	68	43$	99$	212$	133$
2 Gy						
AA8	46	114	35	0	45	0
V33	90	51	31	94	196	106
BSO+2 Gy						
AA8	74	76	16@	0	58	3
V33	100	59	46	88	222@	127
BSO+2 Gy						
AA8	37	63	17	3	58	0
V33	95	19	26	95$	258	189
4 Gy						
AA8	89*	184	85$	3	111$	18
V33	100***	120	40$	323$	346$	122$
4 Gy						
AA8	82	213	86$	2	110$	11
V33	100	132	32	336$	334$	128#
GSH+4Gy						
AA8	81	278	108	2	100	1
V33	100	151	51	399	534	239
GSH+4Gy						
AA8	84	286	111	0	100	13
V33	100	120	48	405	543	232

*Significant at P<0.05,*** Significant at P<0.001 2x2 $\chi 2$ contingency test; @ Significant at at P<0.05,# Significant at at P<0.01,$ Significant at at P<0.001 simple $\chi 2$ test(compared to respective control)

Table 3.9: Induction of CA in AA8 and V33 cell line irradiated with X-rays with/ without BSO(harvested at 28 hrs).

Exptal. Condn.	*Abb. Metaphase %*	*TM*	*Chrom. Exch. %*	*Chtd. Exch. %*	*Del. %*	*Chtd. Bk %*	TM	M1 %
Untreated								
AA8	0	25	0	0	0	0	145	26
V33	18	38	0	0	11	13	198	28
BSO (1mM)								
AA8	17***	113	1	1	15	4	257	51
V33	30***	107	3	10$	39$	11	233	50
2 Gy								
AA8	69***	86	33$	7	57$	7	128	76***
V33	91***	88	15$	85$	200$	100$	111	93***
2 Gy+BSO								
AA8	75***	120	23	3	76	10	193	72
V33	91	122	18	73	213	76	131	95
4 Gy								
AA8	91***	146	53$	18$	135$	4	191	83
V33	100*	70	21	280$	343$	100	83	99*
4 Gy+BSO								
AA8	95	114	41	10	151	4	135	84
V33	99	73	16	268	360	115	77	96
Untreated								
AA8	5	63	2	0	3	3	280	27
V33	5	63	1	0	3	3	334	35
BSO(0.5mM)								
AA8	15	81	4	0	17#	0	358	29
V33	30	124	1	1	29#	6	234	54
1Gy								
AA8	57***	183	27$	3	50$	15$	436	46***
V33	80***	244	13$	52$	139$	54$	279	91***
1Gy+ BSO								
AA8	59	133	19	1	59	14	310	50
V33	82	139	11	45	145	50	197	89
2 Gy								
AA8	71**	162	32#	2	70#	6	277	62***
V33	92**	152	24#	92$	213$	101$	172	95
2 Gy+BSO								
AA8	81*	130	27	5	86	12	248	59
V33	92	126	13	84	214	108	135	96

* Significant at P<0.05, ** Significant at P<0.01, *** Significant at P<0.001 2X2 $\chi 2$ Contingency test. @ Significant at P<0.05, # Significant at P<0.01, $ Significant at P<0.001 simple $\chi 2$ test (compared to respective control).

Results with DNA-repair deficient V33 cell line and normal AA8 cell line

In order to understand the pattern of aberration induction and cell proliferation in these two DNA-repair deficient cell lines, initially one experiment was performed and the data were presented in Table 3.7. The frequencies of spontaneously occurring CA in these two cell lines are presented in Table 3.7. Both chromatid and chromosome types of aberrations were observed in these two cell lines. Cell line deficient in NHEJ had higher frequencies of aberrations, particularly the frequency of chromatid types of aberrations was very high. The induction of exchanges around 6% was observed in these two cell lines and interestingly 4% chromatid-exchanges was also scored in NHEJ-deficient cell lines. The frequency of deletions was more in V33 cells than AA8 cells.

The induced frequencies of CA are presented in Table 3.8 and 3.9. When these cells were irradiated with 0.5Gy of X-rays, the frequency of chromosome and chromatid types of exchanges, deletions and chromatid breaks was significantly more in V33 cells than AA8 cells. We can observe high frequency of deletion on irradiation in both the cell lines, however, the frequency of deletion is much higher in V33 cells than AA8, indicating higher radio-sensitivity of V33 cells. In case of V33 cells, the exchange type is dominated by chromatid-type. Quite a good number of chromosome exchanges is also observed. In normal AA8 cell line, chromosome-type exchange is higher in frequency than chromatid- type of exchanges.

In order to evaluate the involvement of GSH on exchange aberration, the cells were treated with exogenous GSH and BSO. GSH was treated before the radiation exposure and such pretreatment increased the frequency of chromosome and chromatid type of exchanges in AA8 and V33 cells. Pretreatment of GSH increased the frequency of chromosome type exchanges and marginally reduced the frequency of deletions in AA8 cells whereas the frequencies of both chromosome and chromatid type exchanges and deletions were increased in V33 cells. There were no changes observed in the frequencies of aberrant metaphases induced by radiation in GSH- pretreated cells.

When these two cell lines were irradiated in presence of BSO, the frequency of aberrant metaphase was increased significantly in AA8 cells whereas for V33 cells there was no further increment. The data clearly showed that the frequency of exchanges was reduced in BSO-treated sample in AA8 cells, but in case of V33 cells the frequencies of both types of exchanges was reduced marginally. However, the frequencies of other aberrations induced by radiation were increased in both the cell lines while treated with BSO before irradiation.

It is also noted that fixation at 33hrs, only 22% cells were in the first cycle metaphases in both the cell lines. However, after radiation exposure with 1Gy and above, a significant delay in cell cycle was induced in both the cell lines. The extent of delay was more in V33 cells than AA8 cells. At 0.5Gy of radiation the induction of delay was not observed in both the cell lines.

Discussion:

Repair of cellular DNA is the principal process that protects cells and organisms from the deleterious effect of ionizing radiation. Glutathione (GSH) is the most abundant intracellular non protein thiol and involved in a wide variety of metabolic processes as well as in the enzymatic repair machinery of the cell. In this chapter we have made an effort to address the apparent role of GSH in DNA DSB repair pathway.

Two major pathways of DSB repair, NHEJ and HR, have been proposed to operate in mammalian cells. NHEJ is active throughout the cell cycle (predominating in G_0/G_1- phase cells) and is an error-prone process that does not require extensive homology at the site of repair. Repair of DSB by HR, on the other hand, is an error-free mechanism that occurs primarily in late S and G_2 phases of the cell cycle. In quiescent mammalian cells, repair has been reported to take place prevalently by the NHEJ rather than HR. It is acknowledged by the earlier workers that NHEJ is the most active repair pathway involved in the G_0 human lymphocytes. Dutta A *et al.* (2005) reported that NHEJ pathway might be involved in misrejoining of DNA DSB induced by radiomimetic chemical and γ-radiation, as indicated by the increased frequency of exchanges and interstitial deletions. NHEJ involves DNA end-binding heterodimer Ku70/80, the catalytic subunit of the DNA-PK the XRCC4 gene product and ligase IV. It was shown that on exogenous addition of GSH to BLM and radiation treated cells, the frequency of exchange aberrations was increased significantly. Such increased frequency of exchange aberration was reduced drastically

when combined treatment was given to BSO pre-treated cells. It seems that DNA lesions induced by BLM are subjected to interact or illegitimately unite with radiation-induced DNA-DSB and such interaction depends on the level of GSH in the cells. The consistent level of Ku70 protein in all the treated samples, with undetectable level of Rad51 in the G_0 lymphocytes indicates the involvement of NHEJ pathway in misrejoining of DNA DSBs.

The statement that GSH has a role to play in the interaction of DNA DSB to form exchanges is strengthened by the observation of increased frequency of exchange aberrations and decreased frequency of deletion in GSH post-treated samples treated with BLM and X-radiation at RT and 4°C. It has already been reported earlier that the increase of the endogenous GSH levels by treating lymphocytes with GSH or GSH-ester reduces the Fe(III).BLM to Fe(II).BLM and generates more radicals which might be responsible for the increased potentiation of BLM action (Chatterjee A *et al.* 1989; Chattopadhyay A *et al.* 1998). Therefore, it seems practicable that radiation induced lesions interact efficiently with the increased BLM-induced lesions under the influence of increased GSH level. If the present increased DNA DSB interaction took place in the above-mentioned condition then it is likely that NHEJ is involved in the production of higher exchanges under the influence of higher level of GSH.

In another approach, DNA PKc was inhibited by vanillin (3-methoxy-4-hydroxy benzaldehyde in order to disrupt the NHEJ repair pathway. It has been shown that vanillin affects DNA repair processes in bacteria

(Ohta *et al.* 1988) and disrupt the DNA rejoining in mammalian cells (Imanishi *et al.* 1990; Tamai *et al.* 1992). On treating the cells with vanillin, it was observed that the frequency of exchanges in the BLM and radiation treated cells was reduced, although not a very significant level, with respect to respective control. The diminution in the occurrence of exchanges reflects the inhibition of DNA PKc activity which is considered to be the key component of NHEJ pathway.

In spite of the presence of vanillin, the considerable frequency of exchanges was observed. Vanillin inhibits DNA repair by NHEJ and is a selective inhibitor of DNA-PK activity. It has been seen that checkpoint protein ATM is not effected by non-toxic concentration of vanillin. Ionizing radiation-induced phosphorylation of the down stream effector chk2, which is dependent on functional ATM/ATR after DNA damage, is not inhibited by vanillin (Durant and Karran 2003). This provide evidence that vanillin does not significantly impair ATM/ATR- dependent kinase activity. Vanillin is not observed to reduce the frequency of Rad51/RPA foci in agreement with its selective inhibition of NHEJ. It seems that besides NHEJ, there are some other means by which the DNA DSBs could interact and induce a good number of such exchange aberrations. Recently Iliakis *et al.* (2004, 2007) have proposed the existence of two types of NHEJ pathways: D-NHEJ (DNA-PK-dependent) and B-NHEJ (back up non-homologous end joining). While D-NHEJ is presumably involved in the fast DSB repair component with low level of DSB misjoining, B-NHEJ is involved in the slow DSB repair component with high level of

DSB misrejoining. Therefore, it seems that vanillin, an inhibitor of DNA-PK, could predominantly be interfering the D-NHEJ pathway and blocked the low level of DNA misjoining. Not significant increase in exchange aberration is noted on GSH addition to the above treated cells. Thus, the mere increase in the frequency of exchanges on GSH addition is unable to provide any conclusive remark on the contribution of GSH in B-NHEJ pathway, that is contributing to the formation of exchanges despite of blocking D-NHEJ pathway by Vanillin.

Another attempt is made to study the role of GSH on exchange aberration formation in the cells that are mutant in NHEJ repair pathway. We carried out our experiment in NHEJ deficient V33 cell line. The experiment is compared with wild-type AA8 cell line i.e the parental line of CHO. Cell line V33, deficient in NHEJ pathway, shows higher frequency of CA compared to their repair proficient cell line (Darroudi and Natarajan 1987). In the present study, the V33 cells showed higher frequency of CA than AA8 cells particularly after irradiation. High frequency of chromatid breaks and chromatid-type exchanges was induced by radiation in V33 cells. In general, it has been seen that NHEJ and HR deficient cell lines show a combination of chromosome and chromatid type aberration following irradiation of G_0/G_1 cells, very similar to that found in irradiated peripheral G_0/G_1 lymphocytes deficient in ataxia telangiectasia mutated (ATM) and Ku80 gene product (Taylor 1978; Darroudi and Natarajan 1987). Savage and coworkers have studied the pattern of radiation-induced aberration in different stages of cell cycle, by labeling cells with

tritiated thymidine (Vicia faba) or BrdUrd (Syrian hamster cells). They observed very few cells in late G_1 or early S phase containing both chromosome and chromatid types of aberration. In earlier studies it was proposed that the occurrence of chromatid type aberrations observed in CHO Xrs mutant cells following X-irradiation at G1 stage is due to the persistence of lesion other than DSB such as SSB and base damage, which are misrepaired during S phase (Darroudi and Natarajan 1987; Natarajan *et al.* 1993). Since then, the two major DSB repair pathways, NHEJ and HR have been recognized and the relative importance of these two pathways in DSB repair as a function of cell cycle have been evaluated using chicken DT40 cells (Takata *et al.* 1998). It has been suggested that repair deficient cell lines lack an efficient G_1 checkpoint that allows the carryover of the unrepaired DSB and other lesion into S phase. Misrepair of these lesions in S phase can lead to chromatid type of aberrations.

The present high frequency of chromatid type aberrations following irradiation in V33 cells could be due to accumulation of unrepaired DSB which could enter into S phase without G_1 arrest since repair deficient cells have inefficient G_1 checkpoint. Hence, a combined deficiency of DNA DSB repair and cell cycle regulation can account for the increased exchange type aberrations in NHEJ deficient cell lines (Natarajan *et al.* 2008). We also observed a high frequency of chromosome exchange in irradiated V33 cells. This exchange aberration could be formed by B-NHEJ pathway which is involved in slow DSB repair component with high level of DSB misrejoining. The

present V33 cells, having DNA-PK mutant, are unable to join DNA DSB by D-NHEJ pathway and therefore observed chromosome-type exchanges mainly formed by B-NHEJ pathway. Poor influence of BSO on chromosome-type exchanges observed in V33 cells indicates the less involvement of GSH in B-NHEJ pathway. As a whole, it seems that the presence of GSH before radiation increased the frequency of chromosome type exchanges in AA8 and chromatid type than chromosome type exchanges in V33 cells. Whereas in the presence of BSO during irradiation, the frequency of chromosome type exchanges was decreased in AA8 cells but not in V33 cells where the frequency of chromatid type exchanges was reduced marginally. The present data indicate the involvement of GSH in DNA DSB joining irrespective of NHEJ or HR pathway. It is also true that in BSO-mediated GSH-deficient cells induce higher number of DNA lesions due to lack of radioprotective ability of GSH either by hydrogen donation or by chromatin-shielding and therefore it shows higher number of deletions and chromatid breaks in both AA8 and V33 cells with respect to BSO-untreated cells. The question is that, why such increased DNA lesions are unable to interact to form higher frequency of exchange aberrations in GSH-deficient cells? It seems from the present and earlier studies that the presence of GSH is required for DNA DSB joining and such requirement is not particularly very specific to any one type of DNA DSB repair pathway.

In the absence of NHEJ pathway, cells with many unrepaired DSBs can reach the S phase, which may eventually be repaired by HR during replication. Though HR is considered to be error-free process, however, present high frequency of chromatid exchanges induction, suggest that HR is not error free in processing ionizing radiation induced DSB. Efficiency of HR may be impaired in the absence of functional NHEJ apparatus. This assumption stems from the finding that some of the protein complexes implicated in NHEJ pathway such as MRN (Mre11, Rad50 and Nbs1) complex also play important roles in HR pathway. B-NHEJ pathway may also be involved which is highly error prone (Iliakis G *et al.* 2004; Iliakis *et al.* 2007).

Formation of chromosome and chromatid type aberrations in one configuration has been observed earlier in about 0.4% of human lymphocytes of healthy donors irradiated with heavy ions (Loucas *et al.* 2004). Since we observed this type of aberration in DSB repair deficient cell lines, we have to consider that this mixed type of aberration in a single cell is due to the repair defects. It is possible that clustered DNA damage, some single strand breaks (requiring short patch repair), base damages induced in G_1 persists till S phase. A dicentric chromosome with an unrepaired lesion entering S phase can interact with persisting lesions in other chromosomes and generate complexes containing both chromosome and chromatid exchanges.

Significant increase in cell cycle delay is observed in AA8 and V33 cells on X-irradiation compared to untreated cells. However, very marginal difference in the cell cycle delay is observed after BSO administration to the cells as indicated by M1%. The statement is justified by the investigation that similar population of cells are being scored.

CHAPTER: 4

Glutathione: Protector or Modifier?

Literature Review:

Many of the damaging effects of ionizing radiation are mediated by free radicals. The effects are in general enhanced in the presence of molecular oxygen. Oxygen is known to enhance cell killing by ionising radiation, most likely by the fixation of potentially reversible radical damage to cellular DNA. This oxygen enhancement of cell killing has been shown to require the presence of reduced thiols. Reduced glutathione (GSH) plays a key role in cell protection against radiation, reactive oxygen species.

Endogenous thiols, especially the tripeptide-reduced glutathione (GSH) are versatile protectors (Meister 1983) and several distinct mechanisms of radioprotection by GSH have been proposed. These include radical scavenging, restoration of damaged molecules by hydrogen donation, reduction of peroxides, maintenance of protein thiols in the reduced state and partly as a chemical participator in biochemical repair processes of damaged DNA (Revesz *et al.* 1984; Vos 1992). Variations in non-protein thiol levels, and GSH in particular, are observed throughout the cell cycle (Sinclair 1969). The radiobiological K value (the constant K; oxygen enhancement ratio = $(m[O_2]+K) / (O_2 + K)$) is lower for GSH-deficient cells than for GSH-proficient ones, indicating a change in this constant, supporting the idea that thiols repair radiation induced damage by hydrogen donation in competition with oxygen (Solen *et al.* 1989). It has been shown that exogenous addition of GSH can effectively reduce radiation-induced micronuclei (Mazur 2000) and

chromosome aberrations (CA) (Chatterjee and Jacob-Raman 1986) in mammalian cells. Likewise depletion of endogenous GSH by buthionine sulfoximine (BSO) increases the frequency of CA that is induced by ionizing radiation in mammalian cells (Chattopadhyay *et al.* 1999; Ray and Chatterjee 2006).

However, the role of GSH is controversial in the field of radioprotection. Available evidence suggests that GSH may not be an efficient protector of DNA due to its -1 net charge, which on the basis of counter-ion condensation and co-ion depletion phenomena, may allow its dissociation from DNA (Fahey *et al.* 1991). Also, it has been proposed that GSH within the cell nucleus and in particular its close proximity to DNA is critical for conferring cellular radioprotection (Edgren and Revesz 1987; Prise *et al.* 1992). Furthermore, DNA-bound proteins may be more effective in protecting the DNA, in comparison to soluble compounds (Ljungman *et al.* 1991). Prise *et al.* (1992) demonstrated that there is a residual chemical repair capacity in eukaryotic cells that is not dependent upon GSH. This suggests that other reducing agents, such as protein thiols, may be more effective in ionizing radiation induced free radical scavenging of genomic DNA.

Oxygen is known to enhance radiation induced cell killing most likely through fixation of potentially reversible radical damage to cellular DNA (Revesz 1985). This oxygen-enhancement of cell killing has been shown to require the presence of reduced thiols (Bump *et al.* 1982). It was previously demonstrated that for each of the GSH concentrations (0.5 to 5 mM) used, the oxygen enhancement ratios (OERs) for single

strand breaks (SSBs) and double strand breaks (DSBs) were very similar (Ayene *et al.* 1995). This study reported that for the highest GSH concentration (20mM) in anoxic condition, the induction efficiencies for either SSBs or DSBs either increase or became saturate when compared to the corresponding values of 5 mM GSH. This indicates that in addition to its protective effects, GSH at high concentrations may also induce DNA strand breaks.

Poor protection of CA induced by 4 Gy in GSH-pretreated lymphocytes has been observed earlier (Chatterjee and Jacob-Raman 1986). It has also been shown that higher level of GSH in irradiated cells decreased the frequency of deletions but increased the frequency of exchange-type aberrations (Chattopadhyay *et al.* 1999). The role of GSH is controversial in the induction of apoptosis which is dependent on cell types and pro-apoptotic stimuli (Yang *et al.* 2000; Hentze *et al.* 2003).

GSH plays an important role in mammalian cells when they are irradiated in hypoxia, however, this role is generally slight when the irradiation is made in aerobic or in oxic condition. This role of GSH is attributed to competition between GSH and oxygen for the free radicals in target molecules (Hodgkiss *et al.* 1984). It is observed that several GSH deficient cell lines have shown considerable depletion of endogenous GSH levels (to 5 or 6% of control values) is accompanied by a considerable decrease of the OER. The three parameter of clonogenic survival, yield of DNA SSB and presence of micronuclei interpret similar result. The radioprotective role of GSH in irradiated

hypoxic cells can be interpreted by competing between GSH and oxygen for radioinduced radicals in target molecules, and also by GSH-mediated repairing the lesion by hydrogen donation while oxygen fixes the damage irreversibly (Malaise 1983). The assumption that GSH might play a critical role in determining radiosensitivity of living cells as well as radiosensitizing effect of oxygen has received further support by finding that human cells from subjects affected by an inborn deficiency in glutathione synthetase exhibit an extremely low GSH content and a value of the OER very close to 1 for both survival and DNA SSB production after irradiation (Edgren *et al.* 1981).

The conflicting results in the literature therefore, do not provide a definitive conclusion for the role of GSH either in radiosensitization or radioprotection. Under these circumstances, the present study's objective is to determining whether exogenous GSH pre-treatment protects or potentiates the amount of chromosomal damage induced by ionizing radiation. To address this issue, the current work attempts to confirm the role of GSH in radioprotection and to show the influence of GSH in a quantitative manner on radiation induced DNA damage shortly after irradiation, using a comet assay.

Materials and Methods

Chemicals

DL-Buthionine-S,R-sulphoximine (BSO), 5,5'-dithiobis(2-nitro benzoic acid) (DTNB), Hoechst 33258, 5-bromodeoxyuridine (BrdU), GSH-reductase, NADPH, Ficoll-hypaque, dimethyl sulfoxide (DMSO),

TritonX-100 and low melting Agarose (LMA) were obtained from Sigma Chemical Company (St. Louis, MO, USA). The culture medium RPMI 1640, Foetal calf serum, antibiotic penicillin and streptomycin and mitogen phytohaemagglutinine (PHA) were obtained from Gibco, USA. Giemsa stain was obtained from BDH chemicals Ltd., UK. All Other chemicals used in this study were of analytical grade.

Determination of GSH-level in human PBLs

The level of total GSH in peripheral blood lymphocytes was estimated by the method of Akerboom and Sies (1981). A total of five blood samples were collected from a healthy male donor, and all the five samples were as a control and for a single treatment with BSO, whereas three samples were used for a single treatment with GSH and also with X-rays. The study was performmed with full compliance with "Ethical Guidelines for Biomedical Research on Human Subject" formulated by Indian Council of Medical Research, India. Lymphocytes were separated out from heparinized whole blood in a Ficoll-hypaque (McFee *et al.* 1997) after 5 hours of BSO-treatment and 1 and 3 hours after GSH treatment (since GSH takes time to increase its level endogenously) or 1hr after X-irradiation. Freshly collected lymphocytes were washed with ice-cold 0.1M phosphate-buffered saline solution (pH 7.4) and the volume was made up to 1ml with PBS. Cell number was estimated using a haemocytometer and processed for determination of total GSH level (Chattopadhyay *et al.* 1999). After deproteinization

with 10% ice-cold 5-sulfosalicylic acid, 50µl sample suspension was taken and added to 1ml buffer (0.1M EDTA phosphate buffer, pH7.0), 50µl NADPH (4mg ml^{-1}), 20µl DTNB (1.5mg ml^{-1}) and followd by 20µl GSH reductase (6 units ml^{-1}). The optical density of the samples was measured at 412nm using the UV-visible spectrophotometer (Beckman model DU-640). A standard curve was prepared from a stock solution of 10mM GSH (3.1 mg ml^{-1}) in 5% 5-SSA diluted to 100 - 1000 nmol.

Treatment with X-radiation

Heparinized peripheral blood was drawn from three healthy male donors aand lymphocytes were isolated by density gradient centrifugation in Histopaque as described by McFee et al (1981). The monocyte cell layer was washed in phosphate-buffered saline, and about 1 x 10^6 viable lymphocytes were added to 1ml RPMI culture medium in a sterilized small flat bottom 25ml glass beaker. BSO was dissolved in phosphate buffer solution (pH 7.4) and 5mM was added into the lymphocytes and kept at 37°C for 5hrs before irradiating with 1-5 Gy using Faxitron Cabinet X-ray Systems (Model No. 43855D, 110kVp, 3mA, Beryllium window thickness 0.76mm) at the dose rate of 1.5 Gy min^{-1}. For one experiment, GSH (15mM) was added and kept at 37°C for 1h (in one case 3h); since the level of GSH was more than 1h) before irradiation. All the irradiated samples were kept at 37°C for an hour to allow normal cellular repair before setting up cultures.

Culture procedure and cell fixation

Cultures were set up in RPMI 1640 medium supplemented with 10% heat-inactivated Foetal Calf Serum. Lymphocytes were stimulated with PHA. BrdU (6μg ml^{-1}) was added to each culture during its initiation of all cultures. All cultures were incubated at 37^0C and harvested at 72hrs. Colcemid was added at a concentration of 0.01μg ml^{-1} during the last 3h of all cultures. Hypotonic treatment was done for 18 min and cells were fixed in acetic acid and methanol (1:3) and slides were prepared.

Differential staining for sister chromatids

The method of Goto *et al.* was followed. Slides were incubated for 10min with Hoechst 33258 (50μg ml^{-1}) at room temperature in dark, rinsed in distilled water, mounted in 2 x SSC (NaCl-Na-Citrate, pH 6.8) and kept in sunlight for 30-40 min. After rinsing in distilled water, slides were stained in 2% Giemsa for 4 min.

Protocol of Comet Assay:

Materials and Methods:

Glutathione Reduced, DL-Buthionine-S,R-Sulfoximine (BSO), Dimethyl Sulfoxide (DMSO), Disodium EDTA, Ethidium Bromide, Triton X-100, Low Melting Agarose (LMA), Sigma Chemical Company, USA, Tris-HCl, SRL, India.

Lysis Solution: pH 10

NaCl	2.5M
Na_2EDTA	100mM
Tris- HCl	10mM
DMSO	10%
Triton X-100	1%

Electrophoresis Buffer: pH >13

NaOH	300 mM
Na_2EDTA	1 mM
DMSO	0.2%

Neutralisation Buffer: **pH 7.4**

Tris	0.4 M

DNA strand breaks in the blood lymphocytes were measured by comet assay based on the method of Singh et al (1988). Lymphocytes were isolated from heparinised peripheral blood and 1×10^6 viable lymphocytes were added to 1 ml of RPMI 1640 culture medium. GSH or BSO was dissolved in PBS (pH 7.4) and added to the lymphocytes which were then kept at 37°C for 1 and 5 hr before irradiation with 2,4 and 6Gy using a Faxitron Cabinet X-ray system (Model no. 43855D, 100kVp, 3 mA, beryllium window thickness 0.76mm) at a dose rate of 1.5 Gy/min. Immediately after irradiation, an aliquot of the cell suspension was processed to evaluate initial damage. DNA strand rejoining was allowed to proceed by incubating another aliquot of treated cells for 2 and 4hr in RPMI 1640 with 10% foetal calf serum in the presence of penicillin/streptomycin at 37°C in 95% humidified air and 5% CO_2. Fully frosted microscope slides were covered with 200 µl of 1% agarose in PBS at 45° C, immediately cover-slipped, and kept at

4°C for 10 min to allow the agarose to solidify. The removal of the cover slip from the agarose layer was followed by the addition of the second layer of 200 µl of 0.5% LMA containing approximately 10^5 cells at 37°C. The slides were placed into a chilled lysis solution and incubated at 4°C in reduced light for 1hr. The slides were placed into a horizontal electrophoresis tank filled with freshly prepared alkaline buffer for 20 min and then electrophoresed at 25 V, 300 mA for 15-20 min. After electrophoresis, the slides were gently washed for three times with neutralization buffer and stained with ethidium bromide at a concentration of 20 µg/ml in PBS.

Comets capture and tail moment analyses were performed with a computerised image system on a Aristoplan (Leitz Wetzler, Germany) fluorescence microscope coupled with a charge-coupled device camera. DNA strand breaks were expressed as a migration coefficient (or tail moment) calculated by measuring the resulting comets. At least 100 randomly selected cells pooled from three independent experiments and three slides per experimental point were analyzed.

Scoring and statistical analysis:

Slides were coded randomly and studied. First cycle metaphase (M1) were considered for CA scoring. Types of CA included exchange with dicentrics and rings (with or without fragments), deletion and chromatid breaks. For scoring cell cycle kinetics, metaphases were catagorized as first, second or subsequent cycles based on their differential staining patterns. The statistical significance of the difference between control

and treated groups for the frequency of aberrant chromosomes and M1 cells was evaluated using 2×2 contingency χ^2-test and for different types of aberrations, a simple χ^2-test was used. The statistical significance of the difference between different groups in the comet assay was evaluated by a Student's t-test.

Result:

Level of reduced GSH

The endogenous level of reduced GSH with or without BSO or GSH was measured and shown in Table 4.1. The concentration of GSH in normal lymphocytes ranged from 3.67 to 6.44 μmol in 10^6 cells with an average of 5.43 ± 0.48 μmol in 10^6 cells. This GSH concentration was depleted by 84% of the control value after 5hrs treatment with 5 mM BSO. The statistical difference between the mean GSH concentrations of these two groups was significant.

The level of reduced GSH was measured 1 and 3h after reatment with 15mM GSH was also shown in table 4.1. There was a very little increment (within ~9%) after 1 hr of treatment but this increased (within ~21%) 3 hrs after the treatment. However, the endogenous level of GSH either did not increase or increased marginally after 1 and 4Gy of X-rays, measured 1h after radiation.

Table 4.1 ***Levels of GSH in human blood lymphocytes after a single treatment of BSO or GSH or X-rays.***

Sample No.	BSO/GSH/X-rays (mM)	Total GSH (μmol/10^6 cells)	Mean ± SEM (-reduction / + increment %)
1	0	3.67	5.43 ± 0.48
2		6.44	
3		5.57	
4		5.04	
5		6.41	
1	5 BSO	0.85	0.86 ± 0.05[a]
2		0.78	(-84%)
3		0.98	
4		0.79	
5		0.92	
1	15 GSH	3.92	5.90 ± 0.89
2	(1hr)	6.78	(+8.6 %)
3		6.62	
1	15 GSH	5.05	6.57 ± 0.65[a]
2	(3hrs)	6.96	(+ 21 %)
3		7.20	
1	X-rays 1Gy	3.70	5.33 ± 0.84
2	(1hr)	6.48	
3		5.80	
1	X-rays 4Gy	4.00	5.51 ± 0.77
2	(1hr)	6.64	
3		5.95	

[a]$P<0.05$ a student's t-test.

Table 4.2: Effect of BSO or GSH pretreatment on radiation induced CA and First cycle metaphases in human blood lymphocytes.

Donor No.	*Exptal Condn*	*TM*	*Abb. Metaphase*	*Aberration per cell*		*M1%/Total Metaphase*
				Exchange	*Deletion*	
01	Untreated	110	02	0	0	46/245
	2Gy	156	54	0.29	0.20	63[¶] /310
	GSH+2Gy	136	48	0.26	0.10*	54[¶] /296
	BSO+2Gy	130	60	0.20*	0.41*	60/316
	3Gy	178	72	0.46	0.34	76/262
	GSH+3Gy	115	70	0.52	0.26*	68/237
	BSO+3Gy	130	80[¶]	0.25*	0.47*	73/217
	4Gy	118	78	0.57	0.52	81/250
	GSH+4Gy	110	80	0.68*	0.30*	70[¶] /259
	BSO+4Gy	116	84	0.36*	1.02*	78/219
	5Gy	122	86	0.67	0.92	87/234
	GSH+5Gy	102	87	0.74	1.10*	77[¶] /246
02	Untreated	118	02	0	0.01	37/348
	2Gy	204	51	0.32	0.26	52[¶] /394
	GSH+2Gy	157	47	0.26	0.20	43[¶] /371
	BSO+2Gy	107	55	0.22*	0.50*	50/285
	3Gy	201	82	0.49	0.57	75/273
	GSH+3Gy	118	83	0.54*	0.33*	63[¶] /194
	BSO+3Gy	107	88	0.40*	0.70*	76/234
	4Gy	108	89	0.66	0.64	90/236
	GSH+4Gy	122	90	0.78*	0.56	84/265
	BSO+4Gy	112	90	0.30*	1.55*	88/216
	5Gy	136	92	0.81	1.04	93/235
	GSH+5Gy	145	92	0.87	1.33*	82[¶] /215
03	Untreated	115	02	0	0.01	28/400
	2Gy	132	48	0.25	0.25	42[¶] /364
	GSH+2Gy	152	38[¶]	0.22	0.20	36/450
	BSO+2Gy	147	52	0.10*	0.62*	38/416
	4Gy	144	74	0.49	0.53	69/273
	GSH+4Gy	117	75	0.55	0.42*	62/208
	BSO+4Gy	121	75	0.32*	1.06*	70/202
	5Gy	130	83	0.61	0.78	84/195
	GSH+5Gy	122	87	0.66	0.74	77/188

[¶] $p<0.05$ 2 x 2 contingency X^2-test; * $p<0.05$ X^2-test at d.f.= 2 compared with respective positive control.

Table 4.3: Effect of radiation with or without GSH or BSO on DNA Damage in human blood lymphocytes assayed by Comet assay under the alkaline condition.

Experimental Condition	Fixation Time (hrs)	Mean Tail Length Micron± SEM	Tail moment ± SEM
Unirradiated	0	26.5± 1.4	0.4±0.02
2Gy	0	44.0±4.7	3.7±0.9
	2	33.3±3.3	2.2±0.4
	4	29.6±3.2	0.9±0.3
	24	24.9±0.5	0.5±0.02
GSH+2Gy	0	44.6±5.7	2.8±0.6*
	2	34.6±2.7	1.5±0.4
	4	30.4±3.1	0.6±0.1
	24	25.0±0.9	0.5±0.05
BSO+2Gy	0	44.6±5.7	4.9±0.7*
	2	34.6±2.7	8.1±0.9*
	4	30.4±3.1	5.8±0.8*
	24	25.0±0.9	2.7±0.2*
4Gy	0	60.7±9.4	5.2±0.8
	2	44.1±5.4	4.1±0.8*
	4	33.4±3.6	1.9±1.1
	24	28.4±0.9	1.3±0.6
GSH+4Gy	0	62.0±7.2	5.8±0.8
	2	43.3±8.2	3.8±1.9*
	4	39.6±6.1	3.1±1.8
	24	30.9±3.0	2.1±1.2
BSO+4Gy	0	44.6±5.7	8.0±1.3*
	2	34.6±2.7	8.7±1.8
	4	30.4±3.1	4.5±0.8
	24	25.0±0.9	2.9±0.3*
6Gy	0	80.8±1.6	9.0±1.5
	2	60.8±8.3	11.4±0.8*
	4	56.2±2.4	10.9±0.9*
	24	33.7±3.0	6.6±0.5*
GSH+6Gy	0	80.7±2.8	10.8±1.8*
	2	68.3±9.2	11.7±1.6
	4	58.5±3.2	12.7±0.5*
	24	44.0±3.5	9.5±0.2*

Significance with respect to positive control is indicated by * ($P<0.05$; t-test).

Figure 4.1. The effect of X-rays with or without GSH (15 mM) or BSO (5 mM) pretreatment on the frequency of (A) aberrant metaphases, (B) deletions and (C) exchanges in human peripheral blood lymphocytes. The values shown are the means ± standard errors.

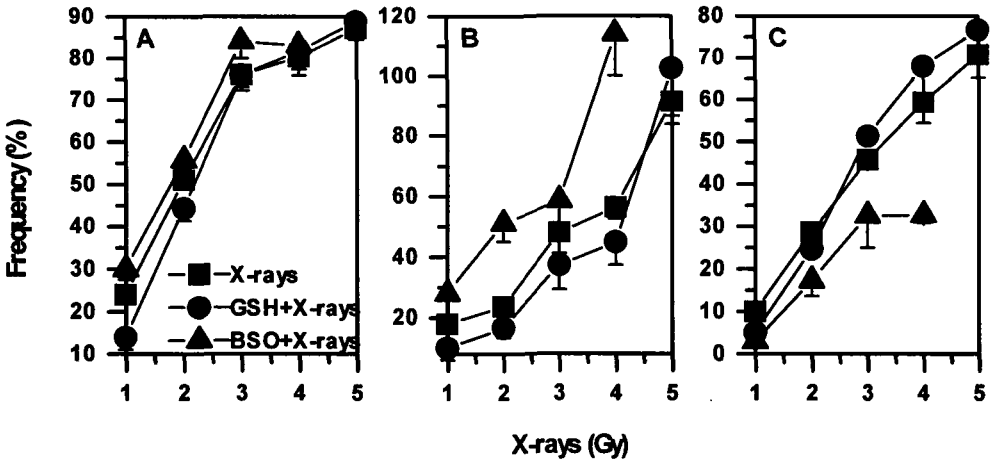


Figure 4.2. The effect of X-rays with or without GSH (15 mM) or BSO (5 mM) pretreatment on the frequency of 1st cycle metaphases in human peripheral blood lymphocytes. BSO was given 5h and GSH was given 1h prior to irradiation. The values shown are the means ± standard errors.

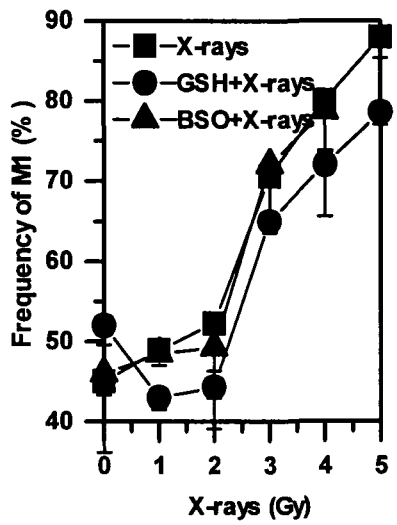
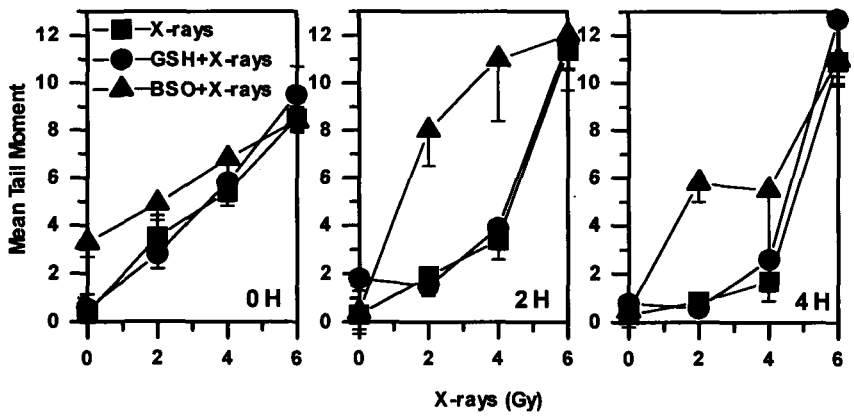


Fig. 4.3. Mean tail moment ± standard errors for comets of human lymphocytes irradiated with X-rays with or without GSH (15 mM) or BSO (5 mM) pretreatment. The cells were fixed at 0, 2 and 4 h after irradiation.



Effect of GSH or BSO pretreatment on CA in lymphocytes

Radiation-induced CA in BSO or GSH-treated and untreated lymphocytes are shown in Fig. 1 (A to C). The induction of CA and aberrant metaphases by radiation increased in a dose-dependant manner and the frequency of chromatid breaks was low at all radiation doses (data not shown).

Treatment with GSH for 1 hr before radiation reduced the frequency of aberrant metaphases and aberrations induced by 1 and 2 Gy of X-rays but this reduction did not improve at 3Gy and above. Even irradiation after 3 hrs of GSH pretreatment did not increase a reduction of the CA frequency (Table 4.2). GSH-mediated reduction in the frequency of deletions induced by radiation was observed untill 4Gy, after that no further reduction was seen. It is interesting to see that the frequency of exchanges induced by 3 to 5 Gy of X-rays was enhanced by GSH pretreatment.

The frequency of CA and aberrant metaphases increased significantly when radiation was given to BSO-treated cells: however, the frequency of exchange aberrations was reduced.

Cell Cycle kinetics

The radiation induced delay in cell kinetics was measured in terms of the increase in the frequency of first-cycle metaphases (M1) following treatment compared to that of the untreated controls. Although the basic cell cycle progression varied among all the three donors, the pattern of effects exerted by radiation with or without GSH or BSO

was similar. The data presented in Fig. 2 shows that the induced delay in cell cycle progression by radiation was significant and increased in a dose-dependent manner. The presence of GSH before irradiation reduced the frequency of M1, while irradiation to BSO-treated cells did not show any significant change in the frequency of M1 with respect to radiation alone.

Measurement of DNA damage by Comet Assay

An *ex vivo* exposure to X-rays induces damages to the DNA of human peripheral blood lymphocytes, as can be seen from the comet assay data presented in Fig. 3. Exposure of human leucocytes to 2, 4 and 6 Gy of X-rays resulted in an increase of tail-moments. The presence of BSO during irradiation enhanced these tail-moments compared to radiation alone at all the three fixation points (0, 2 and 4 hrs).

Pretreatment with GSH before exposure to 2 Gy decreased the tail moment compared to 2 Gy alone, however, this trend was not seen with cells irradiated with 4 and 6 Gy. It was observed that the tail moment at 2 and 4 Gy exposed cells was gradually reduced from 0 to 4 hr fixed samples, but not at 6 Gy, where the tail moment was higher.

Discussion

Data shown here indicate that GSH pretreatment failed to protect radiation induced CA uniformly. This failure in protection by GSH-pretreatment was also observed in the comet assay under alkaline conditions. Interestingly, in BSO-mediated GSH-depleted cells, the frequency of radiation-induced CA increased in a nonuniform manner. The concentration of GSH (15 mM) used in this study was chosen from earlier studies that showed protection against a delay in cell cycle and CA induction by 2 Gy in mammalian lymphocytes (Chatterjee and Jacob-Raman 1986). Incubation of freshly drawn blood with BSO for 5 hrs was done since in cultured cells, >75% depletion of GSH was achieved within 4-5 hrs using 500 μM to 10 mM BSO (Shrieve *et al.* 1985). The concentration of BSO used was 5 mM since significant sensitization was observed at this concentration with respect to radiation induced CA (Chattopadhyay *et al.* 1999). A modest increase in endogenous GSH level was observed after 3 hrs of treatment with exogenous GSH. Earlier, it was reported that a marked increase in intracellular cysteine concentration occurred after GSH-treatment and an increase of GSH by ~20% was achieved after 6-8 hrs of GSH-treatment in Chinese hamster V79 cells (Wardman *et al.* 1992). Therefore, we assume that our GSH-pretreated cells might contain ~10% more endogenous GSH and a higher level of cysteine than control cells during treatment with radiation.

The present results show that radiation alone did not increase the level of endogenous GSH in unstimulated lymphocytes. This elevation of endogenous GSH was reported in irradiated as well as chemically-treated cells in different cell lines and tumor cells (Ahmad *et al.* 1987; Lee and Siemann 1989). It has been shown that unstimulated G_0-lymphocytes do not synthesize DNA and show lower physiological and metabolic activity than cells in the divisional phases (Kay *et al.* 1975; Torelli *et al.* 1981). This could be a factor in the inability to raise the endogenous GSH level after radiation exposure.

Pretreatment with GSH reduced the frequency of all types of CA and aberrant metaphases induced by 1 and 2 Gy of X-rays and also decreased the tail moment, which suggests radiation protection. Such uniform protection by GSH pretreatment was not seen when cells were exposed to 3Gy or above even at higher level of endogenous GSH. As reported earlier (Chattopadhyay *et al.* 1999), it was observed that the frequency of radiation (3Gy and above) -induced exchange aberrations was increased and the frequency of deletions was reduced in the presence of GSH. Exchange aberration formation is thought to arise as a consequence of illegitimate reunion (misrejoining) of free ends involving different DNA DSBs (Cornforth and Bedford 1993). The involvement of GSH in exchange aberration formation is strengthened by the observation of an increased frequency of exchange aberrations and decreased frequency of deletions in GSH/GSH-ester-treated human lymphocytes irradiated at 4°C (Chattopadhyay *et al.* 1999). It was also shown that combined treatment of bleomycin and radiation induces

higher frequencies of CA, particularly, exchange aberrations and interstitial deletions, whose frequency was reduced if the cells were pretreated with BSO (Dutta *et al.* 2005). Therefore, it seems that endogenous GSH could modulate the efficiency of DSB repair. These observations are important since the role of GSH in DNA synthesis under certain conditions (Holmgreen 1979) and as a cofactor in enzymatic repair processes in the cell (Xue *et al.* 1988) have been demonstrated. Furthermore, it has been shown that the inhibition of unscheduled DNA synthesis in a BSO-treated ovarian carcinoma cell line and replenishment of GSH in BSO-treated cells with GSH monoethyl ester resulted in a complete recovery of DNA repair activity (Lai *et al.* 1989).

The present data show that GSH failed to reduce deletions induced by 5 Gy of radiation. Similarly, in the comet assay, the tail moment was more prominent in 4 and 6 Gy irradiated samples with GSH than without GSH. This indicated that exposure to 4 and 6 Gy of X-rays on GSH-treated samples resulted in higher DNA damage and higher residual un-repaired damage than those irradiated without GSH. Although the precise molecular reason for this observation is unclear, an interaction between the trace amounts of DNA-bound iron or copper ions with GSH or cysteine could play an undefined but critical role in these effects. Such increased DNA damage generally requires the simultaneous presence in a solution of a thiol of one of the bivalent metal ions- (copper or iron), and a reduced form of the metal ion to interact with peroxides to form hydroxyl radicals via a Fenton reaction

(Reed and Douglas 1991). A similar observation was made by Ayene *et al.* (1995) when 20 mM of GSH treatment induced additional DNA damage after radiation exposure to superhelical SV40 DNA in the presence of nitrogen.

It has been thought that DNA protection in cells is due to the scavenging of hydroxyl radicals by soluble intracellular compounds (Revesz 1985). However, it has also been stated that both the structural arrangement of the chromatin and the presence of DNA-bound proteins offer more efficient protection against radiation-induced DNA strand breaks than intracellular scavengers of hydroxyl radicals (Nygren *et al.* 1995). If so, then it is likely that complex damage to intracellular DNA could be induced by multiple radical attacks on local sites producing clustered DNA lesions at higher doses of radiation that have more DNA double strand breaks (Ward 1994; Sutherland *et al.* 2002). Such an increase in complex DNA DSBs makes them less capable of being repaired by the cell. It has also been demonstrated that the cellular microenvironment does play an important role in the induction of such unrepairable clustered DNA lesions (Bennet *et al.* 2008). Moreover, it was suggested that eukaryotic DNA-radical precursors are structurally different and therefore, make them less reactive towards thiol (Prise *et al.* 1992). Thus, it may be presumed, that exogenous GSH unable to reduce such clustered DNA lesions induced by higher doses of radiation.

It was shown here that GSH pretreatment reduces the radiation-induced delay in the cell cycle at all radiation doses irrespective of

protection from CA, as was shown earlier (Ray and Chatterjee 2006). The question may arise whether protection obtained for deletions and not for exchanges, really does show a protective effect of GSH or is simply due to scoring of different cell population as the result of cell cycle shifts. In fact, in this study, GSH pretreated cells showed a 6 to 9% reduction in the M1 frequency with respect to 3, 4 and 5 Gy irradiated cells and such a minor shift likely does not produce different subpopulations of significantly different radiosensitivity. Moreover, it was demonstrated earlier that a 1 hr / Gy lag period in irradiated cultures helps the cell-cycle delay without reducing the frequency of CA (Chatterjee and Jacob-Raman 1986). Therefore, the present effect of GSH pretreatment on CA is likely to be independent of its cell cycle delay- reducing effect.

Thus the present investigation indicates that exogenous GSH may not be a strong radioprotector against DNA damage induced by X-rays, but rather that it acts as a modulator of DNA repair capacity.

SUMMARY

Ionizing radiation induces a large number of different types of molecular damage in mammalian cells which can subsequently lead to diversity in cellular response, such as inactivation, chromosomal rearrangement and mutation. It is well established that the efficiency of producing biological damage varies with quality of radiation. Ionization density influences the damage distribution in the genome. On the cellular level, low LET radiation such as X-rays and gamma radiation, induces sparsely ionizations which are typically randomly distributed within the nucleus. For such radiation, the predominant indirect action via free radicals can cause cluster of ionization that lead to complex DSB critical for cell survival. However, for most of the DNA damage induced by low LET radiation consists of SSB and base damages that are relatively easily repaired. In contrast, high LET radiation leads to induction of more complex and highly localised DNA damage along particle tracks. Thus, high LET radiation induces DNA damage in a clustered lesion consisting of multiple strand breaks, base alteration etc. produced by direct interaction between DNA and charged particle, which deposits its energy densely along its path.

The Pelletron at the Inter University Accelerator Centre (IUAC), New Delhi, provided the accelerated ions. Two different ions ^{12}C and ^{7}Li were extracted from the ion source and accelerated to energies of 85 and 50 MeV respectively. Beam properties are listed in the Table.

Beam properties:

Ion Species	Energy (MeV)	LET (KeV/μm)	Fluence (particles/cm^2)	Dose Equivalent (Gy)
Carbon Ion (^{12}C)	85	287	2.3×10^6	1.06
			6.9×10^6	3.17
Lithium Ion (^7Li)	50	60	1.1×10^7	1.06
			3.2×10^7	3.07

Sulfhydryl agents such as cysteine, glutathione, cysteamine and other antioxidants have been seen to protect against lethal effects of radiation. Glutathione (GSH), the major non-protein thiol in mammalian cells, is involved in many cellular functions. This tripeptide plays a role in protection against tissue damage produced by oxidative stress, radiation and chemotherapy. Modification of GSH metabolism has been postulated as being useful in cancer therapy. Indeed, the introduction of agents that can either increase or decrease GSH concentrations in cells opened up the possibility of modulating the cellular response to different anticancer treatments. GSH has been suggested as potential regulator of protein synthesis, DNA synthesis and cell proliferation.

It has been found that endogenous GSH is one of the factors playing an important role for cellular sensitivity towards radiation and chemicals. However, fragmentary report exists in literature that could explain the

role of GSH as radioprotector against high LET radiation. Studies have shown that chemical agents that bring change in the low-LET radiation sensitivity of mammalian cells are less effective against high-LET radiations. The extent of DNA damage and delay induced in cell cycle proliferation by low LET radiation with respect to GSH status of the cell has been explored much extensively. There is no such information available on the role of GSH on high LET radiation induced CA and cell cycle delay in mammalian cell system. Therefore, it will be interesting to compare the influence of GSH on the effect of low and high LET radiation induced DNA damage and delay in cell proliferation. To examine the mechanistic basis for the LET-specific cytogenetic changes we determined the frequency and type of CA induced by ^{7}Li ion, ^{12}C ion and X-radiation.

We made an attempt to modulate the level of GSH and to evaluate its role in low and high LET radiation induced chromosome damage and cell cycle proliferation.

The important aspects of these investigations are:

- CA and delay in cell cycle is LET and dose dependent. ^{12}C beam (LET 287 KeV/μm) is inducing higher percentage of CA and delay in cell proliferation than ^{7}Li beam (LET 60 KeV/ μm) in CHO cell line. Low LET X-radiation induces comparatively lesser percentage of CA and cell cycle delay than high LET ^{12}C and ^{7}Li beam. Due to clustering of ionisation by high LET particles the DSB induced by

such radiation are believed to be more severe and less repairable, thus producing more chromosome DNA damage and division delay.

- It is observed that the frequency of CA increases with increase in sampling time. Such increase is more with high LET radiation. This is due to the inhomogenous energy deposition by the ion particle. The cell with less particle hit induces less damage, enter mitosis earlier than cells with more chromosome damage, thereby showing higher aberration at later harvest period. Unlike, X-radiation deposits energy uniformly leading to homogenous distribution of aberration and delay within the exposed cell population.

- Spectrum of aberration is dominated by deletion in both high and low LET radiation exposed cells. Chromosome as well as chromatid type of exchanges is also observed. On comparing the type of exchange aberration, it is observed that the frequency of chromatid exchange is higher in high LET irradiated cells than low LET X-radiation exposed cells, though the cells are being irradiated at G_1 phase. The SSB when unrepaired at G_1, can be converted into DSB by replication and repair thereby leading to chromatid type aberration in subsequent mitosis.

- From the present data, it seems that GSH showed mild protective effects against low-LET radiation. Protection of GSH depends on the ability to reduce the intracellular concentration of free radicals and

reactive oxygen species. However, GSH is unable to act as radioprotector against high LET radiation. Damage from high LET radiation is primarily due to direct interaction, and because the relative yields of water radiolytic products and reactive oxygen species decreases with increasing LET, protection against high LET radiation by GSH is more difficult to achieve.

It is known that low LET and high LET radiations act differently on DNA because of the differing degrees of spatial clustering of ionisations and DSBs. In contrast to sparsely ionising radiation, high LET radiation leads to the induction of more complex and highly localized DNA damage along particle tracks. Thus, high LET radiation induced DNA damage is a clustered lesion consisting of multiple strand breaks, base alterations action produced by the direct interaction between DNA and the charged particle, which deposits its energy densely along its path. Both the localisation and complexity of high LET radiation induced DSB may influence the cellular capacity to repair such damage. The DNA damage induced by charged particles is associated with slower rejoining of DSBs (Lobrich *et al* .1998). Till date, synergistic effect of BLM and high LET radiation on chromosome aberration is not known. Because of the difference in the molecular nature of the damage induced by low and high LET radiation, it is interesting to study and compare the pattern of interaction of the DNA lesion induced by BLM and heavy ion. It was demonstrated by Preston (1982) that if the DNA damage produced by

two agents is repaired at very different rates then the probability of producing a synergistic effect on aberration frequency is low. On the other hand, if the damage from both agents is repaired rapidly, then there is a high probability of producing a synergistic or interactive effect. Therefore, the present study considered these possibilities with an aim to investigate the pattern of interaction of DNA DSBs induced by high or low LET radiation with bleomycin (BLM) induced DNA lesions.

- Combined treatment of BLM and X-radiation induces more exchange aberration compared BLM and high LET (^{12}C and ^{7}Li) radiation in CHO cells. Since DNA damage induced by BLM and X-radiation is repaired rapidly, it seems that in this synergistic process misrejoining and misrepair of DNA DSBs induced by both BLM and radiation may take place with a high frequency and thus increased the frequency of exchange aberrations. Due to the complex and clustered DNA damage produced by ^{12}C and ^{7}Li beam, the rate of interaction of DNA DSB lesions produced by BLM and high LET radiation is less than that of the interaction of DNA DSB lesions induced by BLM and X-radiation.

- Huge elevation in the frequency of exchange aberrations induced by combined treatment of BLM and X-rays but not with BLM and high LET radiation, to GSH-pretreated cells. It indicates that the better interaction of DNA lesions induced by BLM and X-rays is possible

in the presence of GSH which failed to improve such interaction between DNA lesions produced by BLM and high LET radiation.

From our data, we have seen that exogenous supply of GSH to the CHO cells, induces higher frequency of exchanges. Thus, to verify the role of GSH in interaction of DNA lesion in mammalian system, we have taken three approaches:

1. Allow the interaction of lesions induced by X-rays and BLM at 4°C in presence of GSH and compare it with the similar treatment at 37°C.
2. Allow the interaction of lesions induced by X-rays and BLM in presence of agent that selectively blocks DNA repair pathway.
3. Allow the interaction of lesions induced by X-rays and BLM in DNA-Repair deficient cell lines.

- Increased frequency of exchange aberrations and decreased frequency of deletion is observed in GSH post treated HPBL treated with BLM and X-radiation at RT and 4°C, suggesting involvement of GSH in NHEJ that is predominant repair pathway involved in G_0 lymphocytes. It can be said that NHEJ is involved in the production of higher exchanges under the presence of higher GSH level.

- On inhibiting the activity of DNA PKc by Vanillin (3-methoxy-4-hydroxybenzaldehyde) in HPBL treated with BLM and X-radiation, the frequency of exchange aberrations decreased insignificantly. Therefore, it seems that vanillin could be predominantly interfering the D-NHEJ

pathway and blocked the low level of DNA misjoining, not interfering with other components of NHEJ pathway. On GSH addition to the above treated cells, exchange aberration does not seem to increase significantly. Mere increase in the exchange frequency did not provide any strong evidence of involvement of GSH in B-NHEJ pathway.

An attempt was made to assess the role of GSH in NHEJ pathway, by using mammalian cells deficient in NHEJ pathway. The cell lines used in this study are as follows:

Genotype and characteristics of CHO cell line used in this study

Cell Line	Genotype	Defect	Human homolgues
AA8	Wild-type	-	-
V33	XRCC7$^-$	Deficient in NHEJ	DNA-PK

- A very high frequency of chromatid type of exchange is observed in DNA-PK deficient, V33 cell line. Besides this, a good number of chromosme exchanges are observed as well. This exchange aberration could be formed by B-NHEJ pathway which is involved in slow DSB repair component with high level of DSB misrejoining. Poor influence of BSO on chromosome-type exchanges observed in V33 cells indicate the less involvement of GSH in B-NHEJ pathway.

- Presence of GSH before radiation increased the frequency of chromosome type exchanges in AA8 and chromatid type than chromosome type exchanges in V33 cells. Whereas in the presence of BSO during irradiation, the frequency of chromosome type exchanges was decreased in AA8 cells but not in V33 cells where the frequency of chromatid type exchanges was reduced marginally. The present data indicate the involvement of GSH in DNA DSB joining irrespective of NHEJ or HR pathway.

- From cytogenetic study as well as through comet assay, it is observed that there is still higher damage even in the presence of GSH at dose beyond 3Gy of X-radiation. Increase in exchange aberration is noted on GSH pre-treatment to cells exposed to X-rays even at higher dose of 5Gy. It is also observed that GSH pre-treatment reduces the radiation-induced delay in cell cycle at all radiation doses irrespective of protection from CA. Thus, GSH is seen not be efficient radioprotector at higher dose of radiation but it acts as a modulator of DNA repair capacity.

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Influence of glutathione levels on radiation-induced chromosomal DNA damage and repair in human peripheral lymphocytes

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ABSTRACT

Endogenous thiols, especially the tripeptide-reduced glutathione (GSH), are known to play an important role in cellular defense against radiation. However, there are evidences that suggest that GSH may not be an efficient protector of DNA. The present study will determine whether modulation of endogenous GSH levels protects or potentiates the amount of chromosomal damage induced by ionizing radiation (IR). Human lymphocytes were isolated and then treated with GSH (for 1 h) or buthionine sulfoximine (BSO; GSH-depleting agent for 5 h) before X-irradiation. DNA damage was analyzed by scoring chromosome aberrations (CAs) and by comet assay. The level of endogenous GSH was measured in lymphocytes treated with GSH, BSO or X-rays. A roughly 20% increase in endogenous GSH level was observed after a 3-h treatment with exogenous GSH and this reduced the frequency of all types of CA and aberrant metaphase chromosomes induced by 1 and 2 Gy of X-rays and also decreased the tail moment as determined by comet assay, suggesting radiation protection. Such uniform protection by GSH pretreatment was not visible while cells were exposed to 3 Gy or higher. Interestingly, in GSH-depleted lymphocytes, the frequency of radiation-induced CA was increased in a non-uniform manner. Therefore, an increase in the level of endogenous GSH in lymphocytes was unable to reduce chromosomal damage induced by 3 Gy or above, whereas decrease in the level of GSH enhanced the frequency of CA at all radiation doses in a non-uniform manner. It seems that GSH did not act as a radioprotector against DNA damage induced by higher dose X-rays rather it acts as a modulator of DNA repair activity.

1. Introduction

Endogenous thiols, especially the tripeptide-reduced glutathione (GSH) are versatile protectors [1] and several distinct mechanisms of radioprotection by GSH have been proposed. These include radical scavenging, restoration of damaged molecules by hydrogen donation, reduction of peroxides, maintenance of protein thiols in the reduced state and as a partial chemical participator in biochemical repair processes of damaged DNA [2–4]. Variations in non-protein thiol level, and GSH in particular, are observed throughout the cell cycle [5]. The radiobiological K value (the constant K; oxygen enhancement ratio (OER) = $(m[O_2]+K)/(O_2+K)$) is lower for GSH-deficient cells than for GSH-proficient ones, indicating a change in this constant, supporting the idea that thiols repair radiation-induced damage by hydrogen donation in competition with oxygen [6]. It has been shown that the exogenous addition of GSH can effectively reduce radiation-induced micronuclei [7] and chromosome aberrations (CAs) [8] in mammalian cells. Likewise depletion of endogenous GSH by buthionine sulfoximine (BSO) increases the frequency of CA induced by ionizing radiation (IR) in mammalian cells [4,9].

However, the role of GSH is controversial in the field of radioprotection. Available evidence suggests that GSH may not be an efficient protector of DNA due to its −1 net charge, which on the basis of counter-ion condensation and co-ion depletion phenomena, may allow its dissociation from DNA [10]. Also, it has been proposed that GSH within the cell nucleus and in particular its close proximity to DNA is critical for conferring cellular radioprotection [11,12]. Furthermore, DNA-bound proteins may be more effective in protecting the DNA, in comparison to soluble compounds [13]. Prise et al. [12] demonstrated that there is a residual chemical repair capacity in eukaryotic cells, that is not dependent upon GSH. This suggests that other reducing agents, such as protein thiols, may be more effective in IR-induced free radical scavenging of genomic DNA.

Oxygen is known to enhance radiation-induced cell killing, most likely through the fixation of potentially reversible radical damage to cellular DNA [14]. This oxygen-enhancement of cell killing has been shown to require the presence of reduced thiols [15].

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