Radiation and Bleomycin induced cell cycle delay, apoptosis and DNA damage in mammalian cells with respect to the endogenous glutathione status

By

Aparajita Dutta

Submitted

In
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Of
North-Eastern Hill University
Shillong - 793022

Mailing Address: Balimath
Kazirhat-Battala
P.O. Lakurdi
BURDWAN
WB 713102

DR. SUDHANGSU K. GHOSAL
M. Sc. (Cal.), Ph. D. (McGill), F. Z. S. (Cal.), M. S. D. B.
Retired PROFESSOR
Formerly :
Asst. Prof., New York Med. Coll.
Res. Assoc., Univ. Saskatchewan
Cytogeneticist, R. I. Hosp., Brown Univ.

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ZOOLOGY DEPARTMENT
THE UNIVERSITY OF BURDWAN
BURDWAN, WEST BENGAL
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North-Eastern Hill University
Shillong 793 022

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V Tandon 27.10.03
Head

Head
Department of Zoology
North Eastern Hill University
Shillong - 793022

Supervisor

Candidate

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Contents

Abbreviations used

Abt.M	Aberrant Metaphases
AGT	Average Generation Time
ALP	Alkaline Phosphatase
ATP	Adenosine triphosphate
BLM	Bleomycin
BMC	Bone marrow cells
BrdU	5'-bromodeoxyuridine
BSO	D, L-Buthionine- (S, R)-sulfoximine
CAs	Chromosomal aberrations
Chtd. Bk	Chromatid break
Del	Deletion
DL	Dalton's Lymphoma cells
DNA	Deoxyribonucleic acid.
DPX	Distrin phthalate in xylene
Dsb	double strand break
DTNB	5-5'-dithiobis 2'-nitrobenzoin acid
EDTA	Ethylene diamine tetra actic acid.
Exch.	Exchanges
FPG	Fluorescence plus Giemsa staining
GGT	γ-glutamyl transpeptidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
Gy	Gray
HDR	High dose-rate
HPBLs	Human Peripheral Blood Lymphocytes
i.p.	Intraperitoneal
IAEA	International Atomic Energy Association
IgG	Immunoglobulin G
isochtd. Bk	Isochromatid break
LDR	Low dose-rate
LET	Linear energy transfer
M1	First cycle metaphase
M2	Second cycle metaphase
M3	Third cycle metaphase
MI	Mitotic Index
NADPH	Nicotinamide adenine dinucleotide phosphate

NHEJ	Non-homologous end joining
NHR	Non-homologous recombination
NPSH	Non-protein thiols
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
RI	Replicative Index
RIPA	Radio Immuno Precipitation Assay buffer
RPMI	Rosewell Park Memorial Institute
SCU	Sister chromatid union
SDS	Sodium dodecyl sulfate
SSA	5-sulfosalicylic acid
Ssb	single strand break
SSC	Saline sodium citrate
tGSH	Total GSH
TNB	5-thio-nitrobenzoic acid
UV	Ultra-violet
WBC	White blood corpuscles

Introduction

Radiation biology had its start in the year 1895 with the discovery of X-rays by Prof W.C. Roentgen. Almost simultaneously, within a period of about 4 months of their discovery, the ability of radiation to damage cells had been detected. In 1897, Dr Freund first used radiation for therapeutic purposes. In the recent context, radiobiology had become a very complex science because of its ambition to understand every step leading from the absorption of energy to death or final injury.

Ionizing radiation of every type is a form of energy. In order to act upon living or non-living systems, it has to be absorbed. Thus the way in which the various types of ionizing radiations are absorbed constitutes the first step in the process of interaction of radiation with matter.

An entire spectrum of electromagnetic radiation, which consists of oscillating electric and magnetic fields, is present in our environment in the form of radio waves, infrared visible, UV, X and gamma rays. The radiations of wavelengths in the visible regions are important to life. UV, too, is important for a few biological processes and produces its effects largely by the process of excitation. The electromagnetic radiations with shorter wavelengths produce both ionization and excitation in the media through which they travel. The biological effects of this radiation apparently result from the ionizations produced largely however. There are essentially three mechanisms by which energy can be transferred from these radiations to their surrounding media: the Photoelectric effect, the Compton effect and Pair production.

Ionizing radiation is detrimental to life. The energy absorbed from them induces changes at the molecular level. Most cell constituents including macromolecules (like DNA and enzymes) or even small molecules (like ATP or co-enzymes) are changed by radiation. The biological effects of radiation are the end products of a long series of phenomenon, which are set in motion by the passage of radiation through the cell. A time scale of the radiolytic events is outlined in table 1.

Radiation damage involves two kinds of mechanisms, which however cannot be separated in the living system. The two mechanisms by which radiation produces it's effects on the cell are a) the ***direct action*** where the molecular damage occurs by the

absorption of energy in the molecule itself directly without the involvement of any intervening medium and, b) ***indirect action*** where the energy is first absorbed by the surrounding water molecules leading to the generation of highly reactive free radical species. These free radicals then react with the tissue molecules. Absorption of radiation energy takes place within $10^{-18} - 10^{-16}$ seconds followed by ionization or excitation ($10^{-15} - 10^{-13}$ seconds) and free radical generation ($10^{-13} - 10^{-11}$ seconds). Since cells consist of about 85% water, to a great extent, the biological effects are mainly mediated through the action of radiation on water. The damage due to the indirect effect of radiation is supposed to be caused by the free radicals produced in the water sheath around the DNA molecule containing the bound water. The width of this water sheath is assumed to be several nm (Hutchinson, 1985; Ward, 1988). In cells, the DNA is partly protected from diffusible water radicals (Warters and Childers, 1982) and this protection generally diminishes the lifetime of the free radicals in a cellular environment.

Interaction	**Time (sec)**	**Events and processes**
Physical	10^{-18}-10^{-15}	Energy absorption, excitation and ionization.
Physico-chemical	10^{-15}-10^{-10}	Rearrangement of ionized or excited molecules, formation of diffusible radicals such as $H^{\cdot}$, $OH^{\cdot}$, $e^{-}_{aq.}$
Chemical	10^{-10}-10^{-3}	Free radical reactions molecular alterations, formation of bioradicals by indirect action, long lived lesions in macromolecules.
Biochemical	10^{-3}-10^{4}	Enzymatic reactions, recognition of lesions, repair, fixation of damage.
Cellular level	10^{4}-10^{7}	Cell death, cell loss, division kinetics, mutation.
Systemic	10^{8}-10^{10}	Hormonal effects, immune reactions, Vascular changes, functional impairment, adaptation, carcinogenesis, ageing and death.

Table 1. Time scale of radiolytic events in biological system

The yields of the free radicals depend on the density of ionization events and it can be expected to decrease with increasing ionization density as experiments (Appleby and Schwartz, 1969; Sauer *et al.*, 1977) and theoretical predictions show (Magee and Chatterjee, 1980). The radiolytically formed free radicals and molecular products from the reactions outlined above react with biomolecules and bring about the changes in their structure and function. Therefore, free radical formation processes are considered to play a very important role in radiation induced cellular lethality. The free radical species are very reactive and short lived. It is important that most of the free radical action in irradi- ated cells is fast and completed within a fraction of second leading to the fixation of damage which could be expressed immediately within hours, days or years depending on its mode and magnitude. Thus the free radical products formed initially, quickly decreases and an equilibrium concentration is set up which does not alter as the amount of radiation is increased. The magnitude of this equilibrium values however depends on the intensity (dose-rate) at which the radiation is delivered (Bacq and Alexander 1961). When considering the radical combination process, it is in general only necessary to deal with radicals formed within the same track since the distance between tracks is much greater than the distance between ionization (or excitation) within the track. For this reason, the dose-rate does not influence the number of primary chemical events. Consequently, only the radiochemical reactions in which the dose-rate effect can be determined are those in which a relatively long-lived intermediary is produced which is capable of reacting with other radicals. When dose-rate becomes extremely high, then the instantaneous concentration of radicals produced will go up since the different tracks overlap.

From the biological point of view, the effects produced by ionizing radiation depends on the energy absorbed by the system, and among other factors, largely, this depends on the rate at which this energy is deposited (Hall, 1991). This dose-rate effects covers significant areas in radiobiology applied to radiation therapy as well as radiation protection. Dose-rate effects have been widely studied and numerous examples quote the effect of variation of dose-rates of radiation on different endpoints within the biological system.

Deoxyribonucleic acid is a critical cellular target for the cytotoxic, mutagenic and carcinogenic effects of ionizing radiation (Alper, 1979, Grosch and

Hopwood 1979, Biaglow, 1981). Radiation induced chemical modifications of DNA involve the formation of ionic, radical and excited intermediates as a result of the deposition of energy within the biopolymers and indirect process involving water radiolysis species (Adams and Jameson, 1980). The radiation chemistry of DNA is extremely complex. One reason for this is the fact that ionizing radiation is not selectively absorbed by the molecule alone, but the solvent and other solutes, so that indirect reactions may interfere. In DNA, radiation induced alterations may be broadly classified as:

1. *Single strand breaks* – The scission in the sugar-phosphate backbone in one strand of DNA double helix causes single strand breaks.
2. *Double strand breaks* – Breaks in both the strands of DNA double helix, which may be caused either by single energy deposition event or by the interaction of two single strand breaks formed individually in close proximity.
3. *Base damage and base loss* – Alterations of the nitrogenous bases or loss of the bases by breakage of the glycosidic bonds upon irradiation. Some of these base damages even may result in the rupture of the sugar-phosphate backbone.
4. *Denatured zones* – This consists of breaking of the hydrogen bonds between the between the base pairs of the two strands of the double helix.
5. *Crosslinks* - Radiation induces the formation of various types of crosslinks. Intermolecular crosslinks between two molecules of DNA, or intramolecular crosslinks within the same molecule of DNA can be formed.

Despite the diversity of damages in DNA quoted by ionizing radiation as outlined above, there is evidence that the double strand breaks (dsb) is the lesion which if unrepaired or misrepaired is most likely to lead to cell death (Frankenberg *et al*, 1981, Iliakis, 1991). Many studies have pointed out a good correlation between dsb induction and radiosensitivity in human tumor cell lines (Ruiz de Almodovar 1994c, Whitaker *et al* 1995). There is evidence, however, that a correlation between remaining dsb and radiosensitivity may be found after exposure of human cells to radiation at 37°C at different dose-rates in human cells (Blocher *et al* 1991, Wurm *et al* 1994). From several studies it seems that the dsbs remaining after exposure to radiation at 37°C predict intrinsic cell radiosensitivity more accurately than measurements of dsb induction or

rejoining after an acute irradiation. Using alkaline unwinding technique, Dikomey (1988) had shown that DNA singe-strand breaks repair kinetics do not differ when cells were irradiated at 37°C or at 4°C. However, there are no similar studies on dsb, and results with all the strand breaks may not be directly extrapolated to dsb. The level of DNA dsbs immediately after exposure of a non-transformed human fibroblast cell line (HF-19) to γ-rays (0-40Gy) at four dose-rates (10, 1, 0.1 and 0.01 Gy min^{-1}) at 37°C was demonstrated using clamped homogeneous electric field gel electrophoresis (Forray *et al* 1996). This study showed that the rate of dsb rejoining changes continuously with repair time and that it is independent of dose and dose-rate in the range 10-40 Gy.

There is an interest in the development of assays that might predict response to radiotherapy is based upon observations that there is a range of radiation responses of cultured tumour and normal cells from different individuals. So far, there is no simple method has been devised that can rapidly and accurately predict patient sensitivity to ionizing radiation. Because measurements of rejoining of DNA strand breaks can be performed rapidly, they are appealing for use in a predictive assay. While a correlation between radiosensitivity and dsb repair capacity for tumour cells has been observed by some workers (Zaffaroni *et al* 1994, Schwartz *et al* 1996), others have failed to find one (Olive et al 1994, McKay and Kefford 1995). Moreover, the normal tissue response limits the total radiation dose that can be administered to a patient.

The ability of human peripheral lymphocytes to repair DNA damage by ionizing radiation has been shown to vary with the individual. This variability may depend on many factors including individual age (Licastro *et al* 1982, Mayer et al 1991, Fenech *et al* 1993) and DNA repair-status (McCurdy *et al* 1997). Rejoining of radiation induced ssb and dsb in HPBLs is slow relative to that observed for proliferating cells or stimulated lymphocytes (Tobi and Itzhaki 1993, Chukhlovin *et al* 1995). This observation can be explained at least in part by small pool sizes for deoxyribonucleotides (Green et al 1996) and low levels of at least some repair enzymes (Tashiro *et al* 1996). However, some rejoining always occurs, and perhaps the rate of rejoining in WBC may be indicative of repair capacity in other quiescent normal tissues that are radiation dose-limiting. In contrast, in proliferating cultured cell lines, kinetics of rejoining of ssb generally fail to account for differences in radiosensitvity (Szumiel 1981).

The idea of using PBL cells in the present study is largely because of the ready accessibility of these cells and well established and suitable for the assessment of cytogenetic effects (Adler 1984). Several laboratories have reported inter-individual differences in lymphocyte response to radiation damage when measured using clonogenicity (Geara *et al* 1992, Elyan *et al* 1993), or using the micronucleus assay (Ban *et al.,* 1993, Floyd and Cassoni 1994). However, whether there are substantial inter-individual differences in the ssb or dsb rejoining rates and whether observed differences are related to intrinsic radiosensitivity are still uncertain. At least a part of the difficulty in addressing these questions is that methods used to measure DNA repair may not be completely reliable since WBC are susceptible to rapid radiation-induced apoptosis and necrosis.

Apoptosis and necrosis are two distinct forms of cell death, which are triggered among other insults by ionizing radiation. These two forms of cell death have different morphological and molecular features and implications for the surrounding tissue. Apoptosis is an active process of cellular destruction characterized by cell shrinkage, chromatin aggregation with extensive genomic fragmentation and nuclear pyknosis (Kerr *et al.*, 1972, Wyllie *et al.,* 1980). In vivo, phagocytes normally sequester antigenically modified apoptotic cells, preventing inflammation and damage to the surrounding tissue (Savill *et al.,* 1995). Necrosis is instead characterized by passive cell swelling, intense mitochondrial damage with rapid energy loss and generalized disruption of internal homeostasis. This swiftly leads to membrane lysis and the release of intracellular constituents, which evoke an inflammatory reaction with local cellular infiltration, vascular damage, edema and injury to surrounding tissues.

As mentioned above, a number of factors may be mediating the differences in radiosensitivity found amongst both normal and tumour cell lines. The first one is the efficiency with which the external beam low-LET ionizing radiation induced DNA dsb, thought to be the critical lesion leading to cell death, may differ between cell types. The dose-response for DNA dsb could vary markedly between cell lines, mirroring differences in survival response, and suggesting a common relationship between the level of response in the non-denaturing filter elution assay and the level of cell killing for the cell lines examined (Radford 1986). Although there has been support for the conclusion that

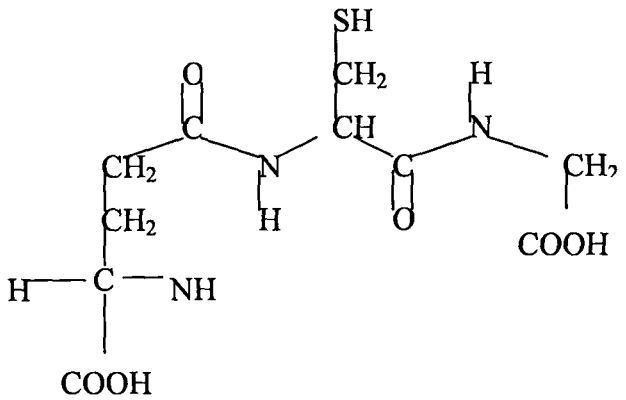
differences in the dose-response for induction of dsb can be important cause of differences in radiosensitivity (Peacock *et al.* 1989), there has been questioning of the validity of the non-denaturing filter elution assay as a measure of DNA dsb (Hutchinson, 1989). The second factor that may contribute to differences in radiosensitivity is mode of cell death after irradiation. It has long been known that some normal cell populations (eg. Lymphoid cells), which undergo apoptosis after irradiation, can show extreme radiosensitivity. It has been shown that non-lymphoid (predominantly fibroblast-like) cell lines underwent necrosis whereas lymphoid and myeloid lines tested showed evidence consistent with apoptotic death (Radford 1994). These differences in radiosensitivity appeared to correlate with the rapidity with which apoptosis was induced after lethal radiation insult, which in turn appeared to reflect differences in the pathways used to induce apoptosis in different cell lines (Radford and Murphy 1994).

Apoptosis is in fact required to destroy errant cells that pose a threat to the organism. It eliminates cells that have accrued genetic mutations and may thereby become cancerous. An important paradigm of the ontogeny of cancer came from the discovery of the roles of dormant proto-oncogenes that mutate into growth promoting oncogenes typified by such genes as *src, ras* and *raf*. However, on the other hand there is another class of genes known as tumor suppressors or anti oncogenes that suppress oncogenesis. For example, cell fusion experiments demonstrated that the malignant potential of cells could be abrogated by fusing them with normal cells. Some tumor suppressors are able to counteract the proliferative consequences through their ability to induce apoptosis. Our present understanding of cancer pivots on the understanding of these opposing influences and how this knowledge may be used for therapeutic intervention. Indeed induction of apoptosis is the mode of action of many anti tumor therapeutic agents that are currently in use.

Thiols dominated the field of radiation protection from the 19540s through to the 1980s. In recent years, studies on radioprotectors appeared to be a turning point: only a few new candidate drugs have been proposed and non-thiol protectors, including protease inhibitors, vitamins, metallo-elements, and calcium antagonists are actually playing a much larger role in radioprotection. In the 1990s, interest increased in endogenous

protective systems as opposed to chemical radioprotectors. Some of these biological agents act best when given prior to radiation; others can modulate radiation injury when given after irradiation. Aminothiols represent the most important group of radioprotective compounds. The endogenous aminothiol which has far-out the highest intracellular concentration is glutathione (GSH) (Vos,1992). GSH (L-γ-glutamyl-L-cysteainyl-glycine) is widely distributed in animal tissues and body fluids and is found also in many plants and microorganisms. Its concentration in most animal tissues is in the range of 0.5 – 10 mM (Meister, 1988) and is thus substantially higher than many other intracellular metabolites. GSH is characterized by its reactive thiol group –SH and its γ-glutamyl bond that makes it resistant to normal peptidase activity (Moldeus and Jiang 1987).

Features of GSH intimately associated with its diverse and important functions include: (1) protection of cells against oxidative damage, (2) existence as an important component of a system using pyridine nucleotides to provide a reducing atmosphere essential for the integrity of the cell membranes; (3) a key role in amino acid transport and multiple metabolic pathways such as synthesis of proteins, nucleic acids and leukiotrienes; (4) regulation of enzyme activation and the immune response and (5) acting as a reservoir of cysteine. GSH is also proposed to be involved in the homeostasis and detoxification of metal ions in biological systems (Freedman *et al* 1989, Gardener *et al* 1993, Kang *et al* 1988)



STRUCTURE OF GSH

Reaction of GSH with oxidants converts it to either glutathione disulfide (GSSG), an oxidized glutathione form or to a mixed disulfide (RSSG) (Moldeus and Jiang 1987). Thus three forms of GSH may exist in a cell. The reduced glutathione/oxidized glutathione (GSH/GSSG) complex is the major buffer in the cell (Meister 1988). Under normal physiological conditions, the intracellular environment is highly reducing due to a high GSH/GSSG ratio of 30:1 to 100:1 (Hwang *et al* 1992). Thus most of the cellular glutathione (90%) is present in its reduced form, while GSSG and RSSG constitute ~5% each. The bulk of intracellular glutathione is found in the cytosol but the existence of a minor mitochondrial pool has also been demonstrated (Moldeus and Jiang 1987). Tirmenstein and Reed (1988) found high concentrations of GSH in the nucleus, the values of the pool being similar to those of cytosolic pool. GSH is present in various body fluids including plasma, bile, glomerular filtrate, and in the lung bronchoalveolar lavage fluid (Moldeus and Jiang 1987). The distribution of GSH in different tissues is different. GSH is transported into the extracellular space and cleaved to its constitutent amino acids, which are subsequently utilized for the synthesis of GSH. This complete cycle is known as the γ- glutamyl cycle. Regulation of GSH biosynthesis may occur through feedback inhibition mechanism (Rahman *et al* 1999).

Information about the physiological functions of GSH is obtained by the observations on *in vivo* systems in which there is marked deficiency of glutathione. One of the many ways in which such expectation has been realized is by the use of selective inhibitors of glutathione synthesis. Depletion of glutathione in biological systems has been achieved by the use of oxidants such as hydroperoxides (Flohe *et al.* 1974, Sies and Wendel 1979) and diamides (Kosower and Kosower 1976). However, the effects of these compounds are generally short lived and are associated with markedly increased concentrations of glutathione disulfide. Since these reagents are non-specific, oxidation of other cellular components may occur, and also their effects may be of short duration because the rate of glutathione synthesis may increase sharply after the depletion of glutathione (Meister 1983). An alternative way intitially developed was by inhibition of glutamine synthetase by L-methionine-S-sulfoximine (Meister 1968, Meister 1974, Meister 1978), however, this was not found to be suitable since this compound was found to inhibit both glutamine synthetase as well as γ-glutamylcysteine synthetase, but

it's interaction with glutamine synthetase offers severe complications on studies in glutathione metabolism. Later on, prothionine sulfoximine and buthionone sulfoximine were found to selectively inhibit γ-glutamylcysteine synthetase, and it was notable that buthionine sulfoximine was much more effective than the other compounds in the depletion of glutathione levels (Meister and Anderson, 1983).

Since GSH constitutes approximately 90% of the non-protein thiols in the cell, considerable research has been directed to the understanding of its role in radiation response. It has already been proved that endogenous thiols of which GSH is the most prevalent one are important in cellular radiosensitivity (Edgren *et al.*, 1980, Clark *et al.*, 1984, Vos et al, 1986) and in general it was found that low intracellular GSH concentration cause sensitization especially under anoxic or hypoxic condition (Revesz, 1985). There is variation in the GSH content between different cell lines however, and this was reported by Mitchell *et al.* (1986) as well as Philips *et al.*(1986). The variation of GSH in different phases of the cell culture (Post *et al.*,1983) and in different culture conditions (Mitchell and Russo, 1983), have been reported.. GSH levels have also been reported to vary with different phases of the cell cycle (Ohara and Terasima, 1969) and that resistance to X-ray result synchronously with GSH content has been reported by Sinclair (1969). The role of glutathione in protecting the cells against radiation damage has been studied extensively by a number of workers. Most workers have used BSO for such studies. BSO inhibits GSH synthesis and very low levels of GSH can be obtained over a relatively short time periods without appreciable toxicity. When GSH levels are depleted very rapidly, cells are sensitized to radiation to a small extent (Mitchell *et al.*, 1983, Biaglow *et al.*, 1983). Prolonged exposure to BSO with subsequent chronic GSH depletion leads to greater radiosensitization (Biaglow *et al.*, 1986). However, it has been suggested that the compartmentalization of GSH within the cells take place and the GSH within the nucleus and in particular close to the DNA is important in determining cellular radiosensitivity (Edgren, 1987). GSH has also been hypothesized to act as a repair agent. Mitchell *et al.* have reported the role of GSH as a chemical repair agent in mammalian cells in 1986. In fact, a reduction in the chemical repair rate by about 2-3 fold has been determined on treatment with BSO. That GSH also plays a role as a cofactor in the enzymatic repair processes in the cell has also been outlined by Xue *et al.*, (1998).

The participation of GSH in the repair of single strand breaks in oxic conditions has been reported by Edgren *et al.* (1981) Thus on the basis of these background information, the **objectives** of the present investigation were:

- To evaluate the influence of different dose-rates of radiation on chromosome aberrations and cell proliferation in mammalian cells with respect to the endogenous GSH status.
- To study the influence of endogenous GSH on the interaction of the lesions produced by bleomycin and radiation at different dose-rates.
- To evaluate the apoptotic cell death by the combined treatment of bleomycin and radiation in mammalian cells.
- To examine the antitumour activity of bleomycin in combination with radiation at different dose-rates.

For the above-mentioned studies, the two model systems used were the mice bone marrow cells (BMCs) for the *in vivo* portion of the investigation and the human peripheral blood lymphocytes (HPBLs) for the *in vitro* portions. Both are well-established systems for the kind of work that was undertaken for the following investigations. The mice bone marrow cells are pleuripotent stem cells, which are in the continuous process of division. These cells are loosely attached to one another and therefore it is easy to prepare cell suspensions with them. However, the HPBLs are all quiescent cells i.e they are all in the G_0 phase. The HPBLs are a readily available source of cells (Adler, 1984) and they can be made to divide, by the addition of a mitogen, to obtain cells in large numbers and also the studies done on them could be easily correlated to humans *in vivo* (Evans and Buckton, 1982).

Chapter I

Endogenous glutathione and the dose-rate effect

Literature Review

The biological effects of ionizing radiation correlates closely with the energy absorbed per unit mass (i.e. the dose). For a given dose the biological effects depends *on the pattern of energy deposition at the microscopic level i.e. the quality of radiation* expressed in terms of Linear Energy Transfer (LET) and the rate of energy deposition i.e. the dose-rate. These latter two factors are not independent variables since the dose-rate effect varies qualitatively and quantitatively with LET.

Radiation has a variety of possible effects on a living cell, the simplest being to kill it. The death of a single cell is unlikely however to cause any harm to an exposed person since we have huge numbers of cells in our bodies that are dying and at the same time being replaced continuously. However, if too many cells get killed at one time, they may impair the various functions of the body resulting maybe in the ultimate death of the exposed individual within a few days or weeks. These acute health effects are subject to a threshold dose above which the severity of the effect increases with increase of dose. Below this threshold there may still be adverse health effects because cells may be damaged but not killed. If the DNA has been damaged it may ultimately result in cancer in the exposed individual or hereditary effects in their descendants. The fundamental characteristic of radiation damage that can lead to a risk of cancer in living organism is damage to the DNA. The UK National Radiological Protection board argues cogently that even the lowest possible dose and dose-rate might result in DNA damage that could be incorrectly repaired (International Nuclear Societies Council Action Plan 1997-98). DNA repair systems exist primarily for the reasons of repairing not only radiation induced damage but those induced by other agents as well, however there is now considerable evidence that low doses and low dose-rates of radiation may induce or activate additional cellular DNA repair capacity.

The biological effects of low dose-rate radiation exposure are of importance to both cancer radiotherapy and also in radiation protection. The effects of radiation on mutation and cell transformation are perhaps more relevant to human risk estimates. *These endpoints are thought* to be indicators of damage and repair mechanisms that

result in long-term effects such as heritable genetic change or cancer. Earlier on, the effects of dose fractionation on cell survival, a parameter relevant to radiotherapy was focused on, and it was found that splitting an X-ray dose into a number of fractions resulted in an increase in cell survival (Elkind and Sutton, 1959). The effect was observed both *in vitro* as well as *in vivo*, and it was hypothesized that the time between the fractions to repair a subset of radiation induced damage known as the 'sub-lethal damage' was important (Elkind *et al.*, 1967). One test of the repair hypothesis was to compare the effect of changes in dose-rate on survival of normal cells and radiation sensitive mutants or other cell lines deficient in DNA repair. The mouse lymphoma cell line LY-R showed increased survival at low dose-rate, while its X-ray sensitive derivative, LY-S manifested only a slight dose-rate effect (Evans *et al.*, 1985). Similarly CHO-K1 cells had about 2.5 fold increase in survival at low dose-rate compared with acute exposure while their X-ray sensitive mutants xrs-5, xrs-6 (Nagasawa *et al.*, 1989) and irs-20 (Stackhouse and Bedford, 1993), exhibited no dose-rate effect at all. These sensitive cells are all believed to be deficient in double strand break repair. Human skin fibroblasts, derived from am ataxia telengiectasia (AT) patient have also been found to have no low dose rate effect in comparison with normal human fibroblast, which showed enhanced survivality at low dose rates (Nagasawa *et al,* 1992) in contrast with the repair deficient mutants. Studies performed on transformed rodent cells suggest that these cells may exhibit an even greater dose-rate effect than normal cells. Transformed 10T ½ cells for example cycled more rapidly than non transformed C3H 10T ½ cells and showed greater sparing by fractionated gamma-rays exposures (Zemen and Bedford, 1985). Rat embryo cells transfected with Ha-*ras* oncogene also showed a greater dose-rate effect for the survival than non-transfected cells (Ong *et al.*, 1993). Low dose rate ^{137}Cs irradiation in mice produced fewer hprt mutations in their T lymphocytes than did acute exposures (Lorenz *et al.*, 1994). It has been suggested by Amundsen and Chen (1996) that the differences in the responses of the different cell lines may be due to different capacities in error-prone repair perhaps involving recombination mechanisms. However, even though low dose-rate induction of mutation has been studied in several mammalian cell lines, but a coherent picture of the effects is yet to emerge.

The dose-rate used for most radiobiological studies on cells and tissues tend to be in the range of 1-5 Gy/min, as are the dose-rates used clinically for external beam radiotherapy. As dose-rate is lowered, the time taken to deliver a particular

radiation dose increases; it then becomes possible for a number of biological processes to take place during irradiation and to modify the observed radiation response. These processes are the 4 Rs of radiobiology: recovery (repair), reassortment (dell-cycle proliferation), repopulation and reoxygenation. The range of dose-rates over which each has an effect depends on its speed. Repair is the fastest of these processes and when the exposure duration is of the order of one hour, considerable repair will take place. Even in the clinical external-beam dose rates, small effects on tolerance may arise form changes in dose-rate. Repopulation however, is a much slower process. Only when the exposure duration becomes a considerable fraction of a day will significant repopulation occur during irradiation. Reassortment as well as reoxygenation also modify responses but over an intermediate range of dose-rates.

The ability of GSH to protect cells against damages induced by ionizing radiation was reported more than 50 years ago (Laterjet *et al*, 1948,). Revesz *et al*, (1963) and Ohara and Terasima (1970) reported that reduced GSH is an inherent radioprotector. The increased NPSH content reported by Revesz *et al.*, (1963) represented an increased thiol concentration in some of the cell lines observed which could be related to reduce radiosensitivity. Ohara and Terasima (1970) established the cyclic variation of cellular NPSH content during the cell cycle in HeLa S3 cells and it's correlation to the cyclic variation of X-ray sensitivity. The observation of an association of radioresistence with increased cellular thiol levels were subsequently confirmed on a number of other cell systems such as mouse lymphoma cell lines (Alexander *et al.*, 1965), bacterial stains (Bruce and Malcham, 1965) and yeast cells (Brunborg, 1977). Therefore, in order to know the role of cellular thiols in the irradiated cells an approach was taken to manipulate intracellular thiol concentrations by treating with several chemical compounds. During this search for such compounds, it was determined that Buthionine sulfoximine (BSO) was the best chemical that could be used for this purpose which blocks GSH synthesis by selectively blocking the enzyme gamma glutamine cysteine synthetase (Bump *et al.*,1982; Griffith and Meister,1979). GSH has also been hypothesized to act as a repair agent. Mitchell *et al.* have reported the role of GSH as a chemical repair agent in mammalian cells in 1986. In fact, a reduction in the chemical repair rate by about 2-3 fold has been determined on treatment with BSO. The GSH also plays a role as a cofactor in the enzymatic repair processes in the cell has also been outlined by Xue *et al.* (1998). It has been shown that such depletion of endogenous GSH

by BSO increases the frequency of CA induced by arecoline (Deb and Chatterjee 1998) and mitomycin C (Dev-Giri and Chatterjee 1998). It was demonstrated in our earlier study that BSO-mediated GSH depletion increased radiation-induced CAs except exchange aberrations. This could be due to reduction in the DNA shielding effect of GSH and failure in rejoining of DNA double strand breaks (dsbs) (Chattopadhyay *et al.*, 1999).

A central issue in radiation biology is to explain the marked differences in sensitivity to cell killing by low LET ionizing radiation shown by both normal and tumor derived cell types. For example, haemopoietic cell types are generally very radiation sensitive whilst hepatocytes are relatively radiation resistant. Likewise, differences in *in vitro* radiation response are also found between tumor types. For example, neuroblastomas are generally radiosensitive whereas melanomas are generally radioresistant (Steel, 1989). Such differences often correlate with *in vivo* responses. Although generalizations can be made about the relative radiosensitivity of the different tissues and tumor types, individual tumors form a given category, or normal tissue samples from different individuals can show a broad range of responsiveness. Increased knowledge of the mechanisms leading to radiation induced cell death would be expected to improve our understanding of the causes of such cell type-dependent differences in radiosensitivity.

Apoptosis is the term used to describe the terminal morphological and biochemical events seen in programmed cell death. Apoptosis has recently been a subject of great interest because it has been clearly been demonstrated to mediate cell death not only during the process of development, but also in neoplasia in response to cancer chemotherapy and radiation. Increased interest in apoptosis is also the result of finding several genes that regulate the apoptotic process in cells. An understanding of the mechanisms involved in apoptotic cell death will clearly have a major impact on the therapy of cancer and other pathological conditions that involve active cell death. The process of apoptosis is very different from necrosis. Necrosis results from the death of cells as a result of injury. Necrotic cells exhibit early lysis of the plasma membrane preceded by swelling before lysis. Besides this, nuclear swelling, is also observed with different surface features in necrosis. On the other hand, apoptosis is characterized by cell shrinkage, chromatin aggregation, ordered fragmentation of the chromatin with nuclear condensation and blebbing of the surface (Kerr *et al.* 1972, Wyllie *et al.* 1980). Unlike necrosis, apoptosis is generally not associated with any inflammation. Apoptotic

cells are somehow recognized and engulfed by neighboring cells and macrophages, leaving the tissue with minimal damage. One model delineates the process into five sequential steps: precondensation, condensation, fragmentation, phagocytosis and degradation. In this model, the precondensation phase is initiated by a tissue specific stimulus that engages cell-directed transcription and translation of a series of regression-selected genes (Bursch et al 1990). It is now widely recognized that apoptosis is required for normal tissue turnover because it allows for precise regulation of the cell numbers, but most importantly, it is now clear that apoptosis also serves as a defense mechanism and it eliminates cells potentially dangerous for the genomic integrity such as virus infected cells and cells insulted by the treatment with toxins, radiation, or other adverse environmental conditions (Thompson *et al.*, 1995, Meyn *et al.*, 1996). The ability of cells to maintain genome integrity is critical to ensure the long-term survival of the individual. Repair, growth arrest and cell suicide through apoptosis are therefore all possible strategies in response to DNA damage although each different even will depend on cell type, location, environment and extent of damage. Apoptosis may be the prudent option in heavily damaged cells that retain substantial replicative potential and therefore constitute a neoplastic risk. Investigation of the factors regulating the apoptotic cell death program is important both to optimize cancer therapy and to design strategies to improve therapeutic outcomes. Furthermore, the role of apoptosis has to be taken into account to correctly evaluate the DNA damage induced by cell exposure to physical and chemical agents.

Keeping all these points in mind, the rational behind the present investigation was to see the role of endogenous GSH on determining the cellular radiosensitivity. It is known that A-T cells are very sensitive to the lethal and chromosome damaging effect of ionizing radiation (Taylor, 1984). The differences in radiosensitivity (cell killing) between AT and control fibroblasts can be magnified by using low dose rate of radiation rather than high dose rate irradiation. This is because whereas there is a sparing effect of LDR in normal cells, this is absent for AT cells and has been interpreted as a repair defect (Cox, 1982). Therefore, in the present study we would like to see whether the level of endogenous GSH does show any effect on the dose-rate sparing.

Materials and Methods

Materials: This study was done both *in vivo* as well as *in vitro.* The two cells systems used were mouse bone marrow cells for the *in vivo* study, and human peripheral blood lymphocytes for the *in vitro* study. The BMCs are rapidly proliferating cells while the lymphocytes during the treatments given, were all in the differentiated G_0 stage.

Mouse bone marrow cells (BMCs):

Inbred Swiss albino mice of 6-8 weeks age weighing about 20-25 gms were used for all *in vivo* experiments (2n=40). The mice were maintained in communal cages, with sterile bed under controlled temperature (20° ± 2°C) and lighting condition (12 hours light and 12 hours dark). Standard animal feed (NMC Oil Mills Ltd. Pune, India) and water was provided *ad libitum.* For all the experiments, the femur bones were dissected out from each mouse and the bone marrow cells were isolated from them just before use. Minimums of 3 mice were used per point in these experiments.

Human Peripheral Blood Lymphocytes (HPBLs):

All *in vitro* experiments were conducted on HPBLs. The heparinised whole blood was collected by venipuncture of four healthy adult donors (25-30 years of age) mostly males.

Reagents: The following reagents were used for the experiment.

- DL–Buthionine-(S,R)-sulfoximine (BSO, Sigma, USA). BSO was freshly prepared in phosphate buffered saline (PBS), pH 7.4. For the in vivo experiments, BSO was injected intraperitoneally whereas for the in vitro experiments, BSO was added directly to the freshly collected blood.
- Phytohaemagglutinin M (Gibco, USA).
- RPMI (Rosewell Park Memorial Institute) 1640, culture medium (Hyclone, USA) with Penicillin and Streptomycin (Hyclone,USA) and Amphotericin B.
- Heat inactivated Newborn Calf Serum (Hyclone, USA).

- 5-bromo-2-deoxyuridine powder (BrdU) (Boehringer Manheim, Germany). For the in vitro study the BrdU powder was dissolved in RPMI 1640 to prepare a working solution of 100μg/ml.
- Colchicine (Sigma, USA).For the HPBLs, the colchicine powder was dissolved in RPMI 1640 to make a working solution of 100μg/ml. For the in vivo study, a working solution of 1mg/ml was used for injection intraperitoneally.
- Bis–benzimide (Hoechst 33258) (Sigma, USA). This was used at a concentration of 50μgml^{-1} in double distilled water.
- Giemsa, 3% (BDH Chemicals Ltd, UK) was used for staining the slides for aberration study while for the study of apoptotic cell death, the slides were stained with Eosin and Haematoxylin (SD's Fine Chemicals, India).
- Ficoll Hypaque (Sigma, USA) 1077 gm ml^{-1}.

All other chemicals used were of analytical grade.

Irradiation: Both the cell systems were irradiated with γ-radiation from a ^{60}Co source. Irradiations were carried out at the Saha Institute of Nuclear Physics, Kolkata and at North Eastern Hill University, Shillong. The dose-rates used for the aberration study were in the range of 0.7-7.7Gy min^{-1} for the BMCs whereas the dose-rates of 0.61-7.1Gymin^{-1} were used for HPBLs. The dose-rate variation was achieved with the help of lead cylinders of varying thickness placed within the irradiation chamber. The dose-rates were determined by Fricke dosimetry. The two doses used for all the investigations here were 2 and 4Gy.

Treatment:

In vivo - In case of BMCs, the mice were injected with BSO (200 mg kg $^{-1}$) 10 hours prior to irradiation. Irradiation of 2 and 4Gy were given at dose-rates of 0.7, 1.1, 2.2 and 7.7Gymin^{-1}.10 hours after irradiation, the mice were injected with colchicine (10mg kg^{-1}), and after 3 hours, they were sacrificed by cervical dislocation. The bone marrow cells were isolated from the femurs.

In vitro – 1ml of the whole blood was taken in a small 25ml flat-bottomed sterilized beaker for each treatment. When BSO was added, 5mM of the chemical was administered 5 hrs before irradiation and kept at 37°C. Irradiations were done at doses of 2 and 4 Gy at the dose-rates of 0.61, 1.91 and 7.1Gymin^{-1}.One hour after irradiation, cultures were set up in medium containing RPMI 1640 with antibiotics and supplemented

with 10% heat inactivated Newborn calf serum. To stimulate the G_0 lymphocytes into division, 0.2ml of PHA (M) per ml of blood was added to each culture. To each vial, BrdU was added at a concentration of 6μg ml^{-1}, and the cultures were incubated at 37°C. After every 12 hrs, each culture was shaken gently. The cultures were terminated at 56hrs, three hours prior to which, colchicine was added to each culture at a concentration of 0.01μg ml^{-1}.

Metaphase preparation and differential staining:

For each mouse, the femur bones were dissected out and the bone marrow was flushed out from each by injecting them with prewarmed 0.075M KCl with the help of a 26g needle. A single cell suspension was made in this hypotonic solution and the cells were incubated in this KCl solution for 15 mins at 37°C. The cells were then centrifuged at 1200 rpm for 5 mins and when the supernatant was discarded, the pellet containing the cells, were fixed in chilled acetomethanol (methanol:acetic acid :: 3:1) for 30 mins. The cells were centrifuged again and washed in two changes of the fixative. Finally they were resuspended in 1ml of the fixative and dropped onto grease free chilled glass slides and flame dried. The slides were then stained in 3% Giemsa, air-dried, and mounted in DPX.

For whole blood , upon termination of the cultures, they were centrifuged at 1200rpm for 5 mins, and the cells were given the hypotonic treatment for 18mins. After 18mins, the cells were centrifuged at 1200rpm for 5 mins and then the pellet was fixed in chilled acetomethanol for 30 mins. The pellet was washed with acetomethanol until a clear solution was obtained and the pellet was resuspended in 1ml of the solution. Slides were prepared by the flame drying method as described above.

For differential staining of the slides, the method of Goto *et al.* (1975) was followed. The slides were treated with Hoechst 33258 at a concentration of 50μg ml^{-1} for 15 mins in dark at room temperature. The slides were then rinsed in distilled water and mounted in 2 X SSC (Saline-sodium citrate, pH 6.8) and kept if the sunlight for about 30-40 mins depending upon the intensity of the light. At the end of that time, the slides were then rinsed in distilled water and stained in 3%Giemsa for 4-6 mins, followed by rinsing in distilled water. The slides were then air dried and finally mounted in DPX.

The fluorescence plus Giemsa (FPG) technique was outlined by Perry and Wolff in 1974. By this procedure the BrdU, which is a thymine analogue, present on the newly

syhtheiszed 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 lightly stained. This helps in distinguishing between the first, second and subsequent cell division cycles. This technique was used for the study of the lymphocytes.

Evaluation of the apoptotic cell death:

For the light microscopic observation of apoptotic cell death, the method of Vral *et al*, (1998) was followed. The mice were given BSO treatment in the same way as has been described above. 10 hrs after BSO treatment, irradiation with 2 and 4 Gy at dose rates of 0.68 and 6.33Gymin^{-1}, were done followed by samplings at 6 and 24 hrs. The BMCs were flushed out and washed twice in PBS (pH 7.4). The supernatant was thoroughly decanted and the cells were fixed in acetomethanol (1:20). After 30 mins fixation, the cells were concentrated in a small amount of the acetomethanol and dropped gently onto slides with a Pasteur pipette. The slides were stained with haematoxylin/eosin at 40°C over 40 mins.

When HPBLs were used, the lymphocytes were first separated from the whole blood in Ficoll hypaque and washed in RPMI 1640. After irradiation with 2 and 4 Gy dose of radiation at 0.2 and 6Gy min^{-1}, cultures were set in the absence of PHA and BrdU in RPMI 1640 supplemented with 10% serum only. Samplings were done at 24 and 48 hrs and after the cells were isolated, the same procedure as mentioned above, was followed for fixing and staining the cells. After staining, the slides were all air dried and mounted in DPX.

Scoring and statistical analysis:

Slides were coded at random. At least 100 well spread first cycle metaphase plates were selected for aberration study from each mouse as well as from the HPBLs. Normal as well as aberrant cells were scored. For the BMCs the chromosome aberrations scored were chromatid breaks, isochromatid breaks, various exchanges and sister chromatid unions. For the HPBLs, the aberrations scored were chromatid breaks, dicentrics and deletions. All aberrations were scored from first cycle metaphases only, 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. The AGT is given by the ratio of the BrdU duration (hrs) and the Replicated Index (RI) of the cells where RI= (1 x M1 + 2 x M2 + 3 x M3) / Total number of cells scored. The BrdU duration in the case of HPBLs here was 56hrs.

In the case of BMCs, the fixation of cells for aberration study was carried out 13hrs after irradiation since the unirradiated mice have been found to show a high frequency of M1 cells (96.5%) at this hour. The cell proliferation kinetics was determined in this case by the study of the Mitotic Index (MI). The Mitotic Index (%) is given by the ratio of the **(Number of cells in metaphase / Total number of cells) X 100**.

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.

The degree of sparing was expressed as the percentage reduction in chromatid break (for mouse BMC) deletion (for HPBLs) yield (Y) between LDR and HDR exposure, which is given by the following formula:

Sparing effect = $(1 - Y_L/Y_H)$ X 100

Where Y_H and Y_L are the yields of deletions / chromatid breaks per 100 cells at HDR and LDR respectively.

The yields of the exchange aberrations from the HPBLs were further analysed since they are one of the important classes of aberrations. From the data obtained after study of the exchange pattern after irradiation at LDR (0.68 Gy min^{-1}) and HDR (7.1 Gy min^{-1}), the regression coefficients were determined using the following formula:

$$r = \frac{\Sigma xy - (\Sigma x\, \Sigma y) / n}{(\Sigma x^2 - (\Sigma x)^2/n)\,(\Sigma y^2 - (\Sigma y)^2/n)}$$

Where x = the doses used in Gy; y = the yields of the exchanges corresponding to each of the doses; n = the number of observations.

The regression coefficients showed values that were close to 1 but they were all less than 1. Therefore, for a further analysis, the linear regression was used. For this, the graphs of the exchange aberrations obtained against each dose at LDR and HDR were

plotted and each graph was then fitted with a linear fit. The equation used for the linear fit was Y = a + bD where 'a' and 'b' are the components of linear regression and D = the dose of radiation (Gy). The curve fitting was done with the help of Origin 3.8 in a computer. The 'a' and the 'b' values were obtained from the analysis of the graphs on the computer.

The apoptosis in cells at different dose rates in normal and GSH-depleted cells was scored using the technique of Vral *et al.* (1998). The cells with pyknotic nuclei were scored as apoptotic as opposed to normal cells with dispersed chromatin. The percentage of apoptotic cell death was statistically tested by the 2 x 2 χ^2- contingency test.

Results

Effect of BSO on in vivo-induced aberrations

Gamma-ray induced CA in BSO-treated and untreated mouse BMC are shown in table 1.1 and 1.2. The figure 1.1a shows the microphotograph of a normal plate and figure 1.1b shows some of the aberrations that had been scored for mice BMCs. The induction of aberrations by radiation is clearly dose-dependent at both LDR and HDR of irradiation. Isochromatid break and chromatid break were the most frequent type of aberrations and increased most consistently in the presence of BSO. While BSO was added prior to irradiation the frequency of aberrant metaphases was increased from 38 to 54% by 2 Gy at 0.7 Gy min^{-1} and from 58 to 71% by 2 Gy at 7.7 Gy min^{-1} irradiation. The frequency of exchange aberrations including sister chromatid union (SCU) was not enhanced in the BSO-treated sample although there was a marked reduction in their frequency induced by 4 Gy at 0.7 Gy min^{-1} (0.14 to 0.09 per cell) and at 7.7 Gy min^{-1} (0.41 to 0.33 per cell). The effect of BSO itself on the formation of spontaneous CA was studied in the unirradiated mouse. A significant increase in the frequency of aberrant metaphases and chromatid breaks was observed with 200 mg kg^{-1} treatment.

In order to see the sparing effect of LDR we considered both chromatid-break and isochromatid break since both are the most predominant type of aberrations and showed significant increase in the frequency in BSO-treated cells at all radiation doses. From the data in table 1.2, it is clear that the extent of sparing effect was less for BSO-treated

samples for both the aberrations at LDR while comparing with radiation doses at 7.7 Gy min^{-1}.

The scoring of mitotic index gives an idea about cell proliferation. Each unirradiated mouse bone marrow cells show consistent mitotic index with a mean value of 4.78%. This value was reduced after 2 Gy and such reduction was increased from LDR to HDR. It indicates that there was a delay in cell proliferation after irradiation. While radiation was given to BSO-treated cells the mitotic index value was improved with respect to irradiated normal cells in the most cases (Table 1.1 and 1.2). Even BSO-treatment alone showed significant reduction of mitotic index with respect to untreated control.

Effect of BSO on in vitro-induced aberrations

The induction of aberrations by radiation is clearly dose dependant at both low and high dose-rate, however, the frequency of chromatid breaks was very low at all radiation doses (Table 1.3 and 1.4). The figures 1.7a and 1.7b show some of the aberrations obtained upon irradiation, while the figures 1.6a and b show normal first and second division cycles respectively. Radiation at HDR shows higher frequency of aberrations than radiation at LDR. Distinct increase in the induction of deletions and aberrant metaphases were observed in all BSO treated samples at all radiation doses and some are significant. Interestingly, the frequency of exchanges did not increase at all in BSO treated samples rather a tendency of reduction was observed in most cases. The effect of BSO (5mM) itself on the spontaneous CAs was also studied. No significant effect was observed.

Deletion is the type of aberrations that is the most prominent type and showed significant increase in the frequency in BSO-treated cells at all radiation doses. From the data it is clear that the extent of sparing effect was less for BSO-treated samples at both the radiation doses in the individual samples (except at 4 Gy in donor 1 and at 2 Gy for donor 2). The mean yield of sparing effect of BSO-treated samples shows less at 2Gy and at 4Gy it shows similar to BSO-untreated samples.

Effect on cell cycle kinetics

The fluorscence plus Giemsa staining technique facilitated the scoring of cell cycle delay in terms of reduction in the frequency of second and subsequent division metaphases following Gamma-ray treatment. The percentage of M1 was higher in Gamma-ray treated samples with respect to unirradiated control indicating a delay in cell proliferation. Irradiated at HDR showed more delay in cell kinetics than LDR radiation exposure. This is consistent in each individual sample (Table 1.5). The AGT was significantly increased

after treatment with radiation compared to control. Treatment with BSO prior to radiation reduced the delay in cell cycle since the frequency of M1 and the AGT both were improved.

Linear Regression fitting

The fig 1.11 shows the graphs for the linear fit to the exchange data (Table 1.7a and 1.7b). After analysis of the regression coefficients, the values obtained for the exchange aberrations at HDR were 0.99232 when cells were irradiated without BSO while a value of 0.96214 was obtained for the regression coefficient when BSO was added to the HPBLs prior to irradiation at HDR. At LDR, in the absence of BSO, the r-value (r = regression coefficient) was found to be 0.99458 whereas in the presence of BSO, the regression coefficient was found to be 0.99760. In all the cases the values of the r was close to 1, but never more than 1. Therefore, it was clear that the exchange aberrations were following a linear pattern. When the graphs had been fitted by linear regression, the values of the components $\pm$ SD were generated by the computer were a = -3.43 $\pm$ 3.7 and b = 15.8 $\pm$ 1.1 for the samples that had been irradiated at HDR in the absence of BSO. When BSO was added to the samples prior to irradiation, it was observed that a = -3.77 $\pm$6.6 and b = 12.3 $\pm$ 2.5. For the graph obtained at LDR, the values obtained for a and b were a = -0.68 $\pm$ 1.8 and b = 9.03 $\pm$ 0.5 in the absence of BSO whereas a = -0.54 $\pm$ 6.6 and b = 6.69 $\pm$ 0.3 when BSO was added prior to irradiation.

Cell death by apoptosis

The apoptotic cell death was evaluated in both mouse BMCs and human PBLs. Cells with pycnotic nuclei were scored as shown in figure 1.12. The two dose rates at which apoptotic cell death was studied were 0.68 and 6.33 Gy min^{-1} for mouse BMCs and 0.2 and 6.0 Gy min^{-1} for human PBLs. For mouse BMCs the sampling were done at 6 and 24h after radiation and the frequency of apoptotic cell death was increased with increasing dose and time although not significantly (Table1.8 and 1.9). However, HDR radiation induced significantly higher frequency of apoptotic cell death than LDR. Treatment with BSO marginally increased the frequency of apoptotic cell death at both LDR and HDR radiation.

For human PBLs the samplings were done at 24 and 48h after radiation and the frequency of apoptotic cell death was increased with the increasing dose and time (Table 1.10 and 1.11). The significant increase in the frequency of apoptotic cell death was

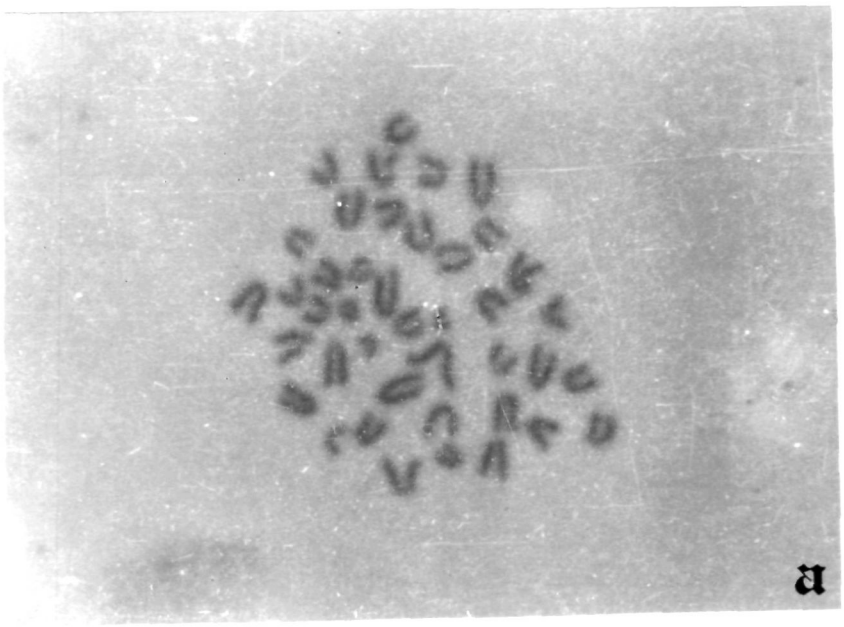
observed by HDR of radiation with respect to LDR exposure. BSO treatment prior to radiation did not make any significant changes to the percentage of apoptotic cell death. At the higher dose-rate, when BSO was added, a slight decreasing trend in the percentage of apoptosis was seen.

In both the cases BSO alone did not increase the apoptotic cell death with respect to untreated control.

Fig 1.1: Microphotographs showing metaphase plate in BMCs.

A. Normal.

B. Aberrant showing exchange (e) and chromatid breaks(c).


a

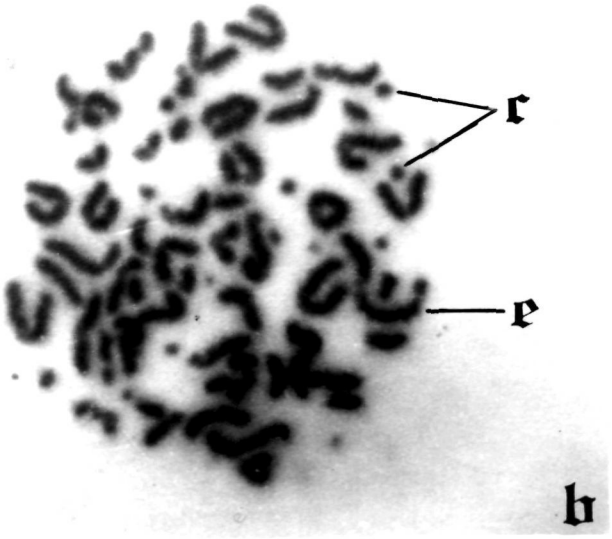
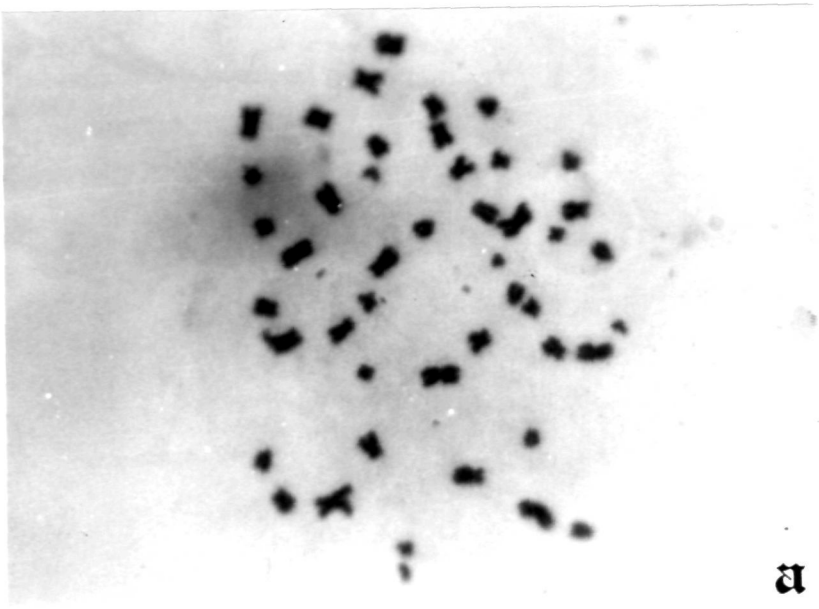

r
e
b

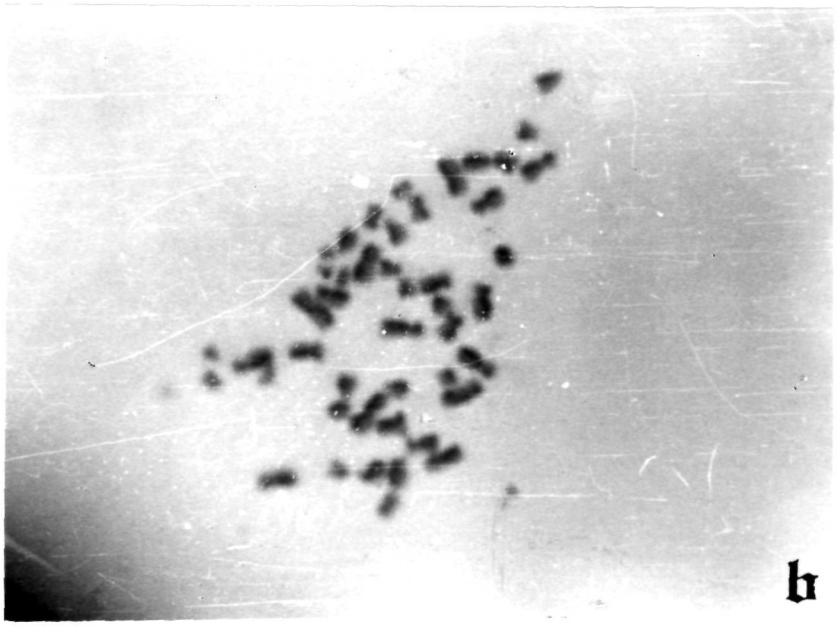
Fig 1.6: Microphotographs showing normal metaphase plates in HPBLs.

A. First division cycle.

B. Second division cycle.



a

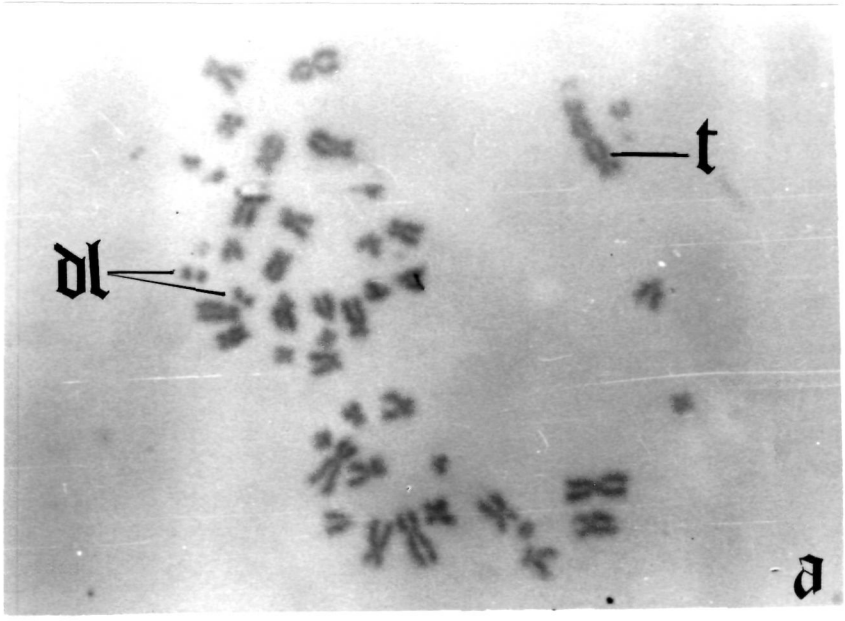


b

Fig 1.7: Microphotographs showing aberrant metaphase plates in HPBLs induced by radiation.

A. Showing tricentric (t) and deletion (dl).

B. Showing ring (r) and fragment (f).


t
dl
a

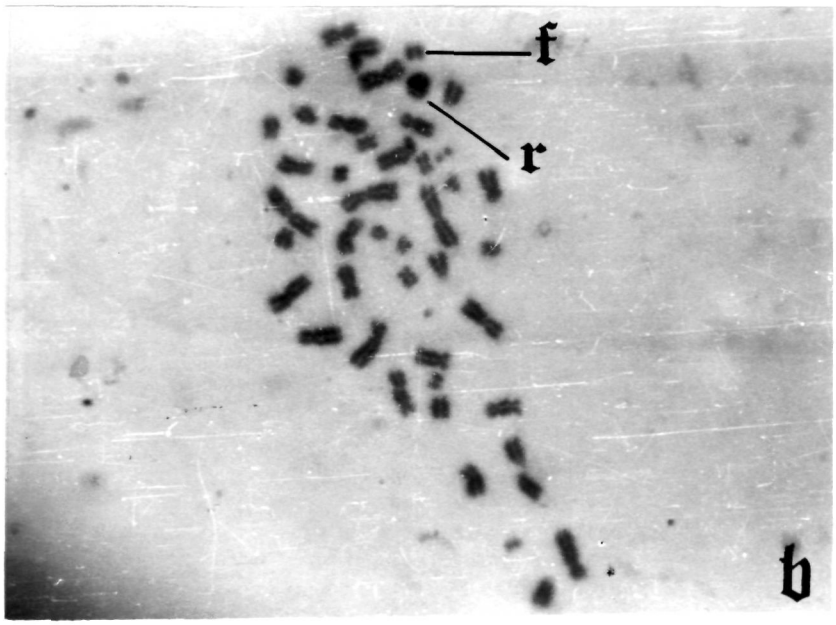

f
r
b

Fig 1.12: Microphotograph of apoptotic cell death in BMC. Cells with pyknotic nuclei (→) are scored as apoptotic as compared to normal cells with dispersed chromatin.

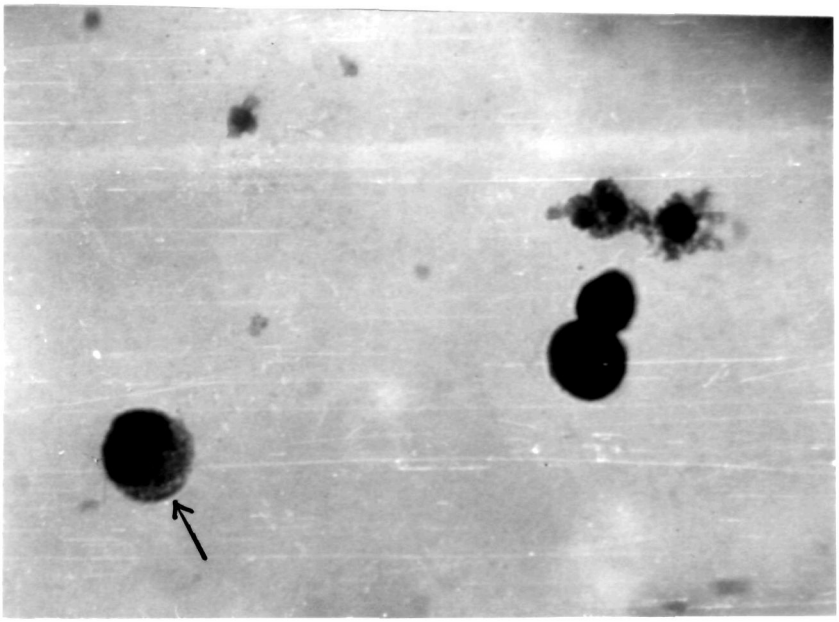


Table 1.1: Individual data on the effect of radiation at different dose-rates in mice bone marrow cells with and without BSO (200mg kg^{-1}).

Dose rate ($Gymin^{-1}$)	Exptal. Condtn.	TM	AbM%	Aberrations per cell				MI%
				Exchs.	Chtd.Bk.	Isochtd.bk	SCU	
0	0	152	1	0	0.01	0	0	4.59
		144	1	0	0.01	0	0	4.79
		132	2	0	0.01	0.01	0	5.12
		122	2	0	0.01	0.01	0	4.63
0	BSO	167	9	0	0.09	0.01	0	1.85
		120	6	0	0.1	0.02	0	2.3
		133	6	0	0.13	0.02	0	2.4
		164	7	0	0.08	0.01	0	3.0
0.7	2Gy	152	35	0.04	1.7	0.1	0.01	2.80
		126	34	0.03	1.9	0.11	0.03	2.60
		138	44	0.08	1.3	0.07	0.02	2.50
		133	40	0.02	1.7	0.08	0.01	2.80
	BSO+2Gy	183	59	0.01	1.7	0.16	0.01	2.85
		135	54	0.03	2.3	0.11	0.01	2.60
		140	52	0.02	1.6	0.07	0.02	2.90
		152	51	0.04	2.2	0.09	0.01	2.86
	4Gy	104	72	0.07	2.1	0.37	0.11	2.10
		120	71	0.1	2.1	0.4	0.05	1.90
		141	66	0.04	2	0.59	0.1	2.06
		130	72	0.02	1.8	0.46	0.04	2.11
	B+4Gy	110	79	0.05	2.3	0.9	0.05	2.80
		134	73	0.04	2.6	0.8	0.08	2.70
		126	78	0.02	2.7	0.5	0.06	2.20
		128	77	0.03	1.2	1.3	0.02	2.00
1.1	2Gy	108	46	0.07	1.7	0.13	0.03	2.20
		127	49	0.05	2	0.12	0.01	2.40
		110	52	0.02	1.2	0.16	0.05	1.90
		196	54	0.06	1	0.13	0.02	2.10
	BSO+2Gy	102	61	0.03	2	0.21	0.02	1.97
		107	63	0.04	2.2	0.14	0.01	2.18
		119	69	0.03	2	0.18	0.02	2.33
		122	64	0.05	1.4	0.16	0.02	2.31
	4Gy	125	76	0.08	2.4	1	0.11	1.20
		158	71	0.06	2.1	0.61	0.1	1.80
		124	75	0.07	1.3	0.43	0.1	2.10
		114	72	0.03	2.3	0.52	0.09	1.60

	BSO+4Gy	131	73	0.05	2.6	0.3	0.04	1.70
		128	92	0.07	1.9	1.2	0.09	1.60
		135	86	0.01	2.2	1.5	0.08	1.80
		139	91	0.03	2.7	0.6	0.06	1.90
2.2	2Gy	160	56	0.04	1.2	0.15	0.02	1.92
		121	54	0.03	1.2	0.25	0.05	2.18
		132	48	0.08	2.1	0.1	0.06	1.80
		143	53	0.06	1.8	0.21	0.06	2.10
	BSO+2Gy	152	71	0.03	2.4	0.34	0.02	2.30
		148	63	0.04	2.6	0.21	0.03	2.60
		137	69	0.02	2.2	0.18	0.04	2.80
		176	68	0.03	2	0.25	0.03	2.60
	4Gy	133	77	0.06	2.5	2.5	0.12	0.63
		138	75	0.12	3.4	3.4	0.12	1.10
		120	71	0.02	1.4	1.4	0.15	1.90
		107	76	0.08	2.6	2.6	0.13	1.80
	BSO+4Gy	102	94	0.06	2.8	2.8	0.1	2.37
		111	92	0.04	2.9	2.9	0.09	2.21
		108	87	0.04	3.1	3.1	0.11	2.20
		113	91	0.03	2.9	2.9	0.12	2.33
7.7	2Gy	142	61	0.11	2.25	0.18	0.03	1.51
		122	55	0.10	2.16	0.23	0.04	1.61
		134	58	0.17	2.1	0.28	0.03	1.57
	BSO+2Gy	130	68	0.07	2.65	0.25	0.01	2.34
		127	75	0.06	2.13	0.43	0.02	2.28
		121	69	0.02	2.28	0.29	0.03	2.24
	4Gy	96	84	0.21	4.15	1.9	0.15	0.93
		102	82	0.34	3.12	1.6	0.17	0.62
		97	96	0.23	3.7	1.4	0.13	0.85
	BSO+4Gy	92	98	0.18	4.6	2.3	0.13	1.02
		116	93	0.21	3.31	2.7	0.1	0.93
		98	96	0.24	3.8	2.2	0.12	1.11

TM: Total metaphases; Abt M: Aberrant metaphases; Exchs: exchanges; Isochtd Bk.: Isochromatid break; Chtd.Bk: Chromatid break; SCU: Sister chromatid union; MI: Mitotic Index.

Table 1.2. Pooled data for the effect of variation of dose-rate of radiation in normal and BSO-treated (200mg kg^{-1}) Mice bone marrow cells.

Dose-rate (Gy min^{-1})	Exptal. Condtn.	TM (no.of mice)	AbtM% ± SEM	Aberrations per cell ± SEM				MI% ± SEM	Sparing effect	
				Exchs.	Isochtd.Bk	Chtd Bk.	SCU		Chtd bk.	Isochtd Bk
0	0	550(4)	2 ± 0.3	0±0	0.01±0	0.01± 0	0 ± 0	4.8 ± 0.1		
0	BSO	584(4)	7 ± 0.7	0±0	0.02±0	0.10±0	0 ±0	2.4 ± 0.2		
0.7	2Gy	549(4)	38 ± 2.3	0.04 ± 0.01	0.09 ± 0.01	1.6 ± 0.1	0.02 ± 0.01	2.7 ± 0.1	24%	61%
	BSO+2Gy	610(4)	54 ± 1.8 [b]	0.03 ± 0.01	0.11 ± 0.02	1.9 ± 0.2	0.01 ± 0	2.8 ± 0.1	21%	66%
	4Gy	495(4)	70 ± 1.4	0.06 ± 0.01	0.46 ± 0.05	2.0 ± 0.1	0.08 ± 0.02	2.0 ± 0.1	46%	82%
	BSO+4Gy	498(4)	77 ± 1.3	0.04 ± 0.01	0.88 ± 0.17 [a]	2.2 ± 0.3	0.05 ± 0.01	2.4 ± 0.2	44%	70%
1.1	2Gy	541(4)	50 ± 1.8	0.05 ± 0.01	0.14 ± 0.01	1.5 ± 0.2	0.03 ± 0.01	2.2 ± 0.1	29%	39%
	BSO+2Gy	450(4)	64 ± 1.7 [b]	0.04 ± 0.01	0.17± 0.01	1.9 ± 0.2 [a]	0.02 ± 0	2.3 ± 0.1	21%	46%
	4Gy	521(4)	74 ± 1.2	0.06 ± 0.01	0.64 ± 0.13	2.0 ± 0.3	0.10 ± 0	1.7 ± 0.2	46%	74%
	BSO+4Gy	533(4)	86 ±4.4 [b]	0.04 ± 0.01	0.90 ± 0.27 [a]	2.4 ± 0.2 [a]	0.07 ± 0.01	1.8 ± 0.1	38%	31%
2.2	2Gy	556(4)	53 ± 1.7	0.05 ± 0.01	0.18± 0.03	1.6 ± 0.2	0.05 ± 0.01	2.0 ± 0.1	24%	22%
	BSO+2Gy	613(4)	68 ±1.7 [b]	0.03 ± 0	0.25± 0.03	2.3 ± 0.1 [a]	0.03 ± 0	2.6 ± 0.1	4%	22%
	4Gy	498(4)	75 ± 1.7	0.07 ± 0.02	1.63 ± 0.10	2.5 ± 0.4	0.13 ± 0.01	1.4 ± 0.3	32%	34%
	BSO+4Gy	434(4)	91± 1.5 [b]	0.04 ± 0.01	2.40 ± 0.11 [a]	2.9 ± 0.1 [a]	0.11 ± 0.01	2.3 ± 0	26%	18%
7.7	2Gy	398(3)	58 ± 1.7	0.13 ± 0.02	0.23 ± 0.02	2.1 ± 0	0.03 ± 0	1.6 ± 0		
	BSO+2Gy	378(3)	71± 2.2 [b]	0.05 ± 0.02 [a]	0.32 ± 0.04 [a]	2.4 ± 0.2 [a]	0.02 ± 0	2.3 ± 0		
	4Gy	295(3)	87 ± 4.4	0.26 ± 0.04	2.48 ± 0.41	3.7±0.3	0.15 ± 0.01	0.8 ± 0.1		
	BSO+4Gy	306(3)	96 ± 1.5 [b]	0.21 ± 0.02	2.93 ± 0.06 [a]	3.9±0.4 [a]	0.12 ± 0.01	1.0 ± 0.1		

TM: Total metaphases; Abt M: Aberrant metaphases; Exchs: exchanges; Isochtd Bk.: Isochromatid break; Chtd.Bk: Chromatid break; SCU: Sister chromatid union; MI: Mitotic Index.

[a] Significant at $p<0.05$ simple $\chi 2$- test. [b] Signifcant at $p<0.05$ $\chi 2$ – contingency test.

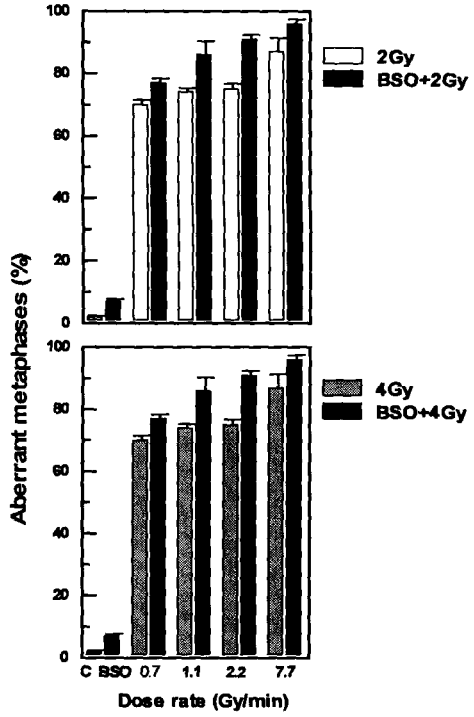


Fig. 1.2 Aberrant metaphases in normal and BSO-treated mouse BMCs upon irradiation of 2 and 4Gy at different dose-rates.

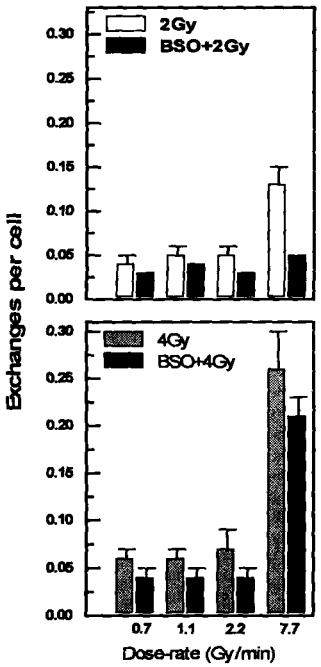


Fig. 1.3 Exchanges per cell induced by 2 and 4Gy irradiation at different dose-rates in normal and BSO-treated BMCs.

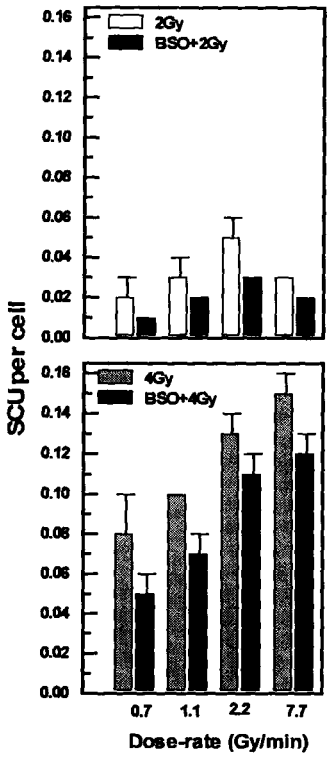


Fig. 1. 4 Sister chromatid union induced by different dose rates of irradiation in normal and BSO-treated BMCs

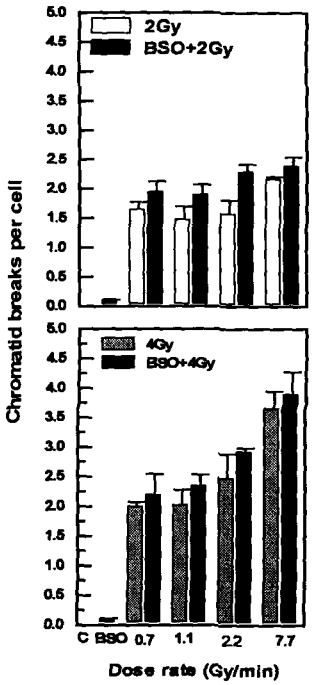


Fig. 1.5 Chromatid breaks induced by 2 and 4Gyγ-ray irradiation at different dose-rates in normal and BSO-treated BMCs.

Table 1.3. Individual data on the induction of chromosome aberrations by γ-ray dose-rate variation in Normal and BSO (5mM) depleted HPBLs.

Donor	Dose-rate (Gy/min)	Exptal. Condtn.	TM	AbtM %	Aberrations per cell			Sparing effect for deletions
					Del	Chtd.Bk.	Exchs.	
1.	0	0	94	03	0.01	0.01	00	-
	0	0	128	04	0.02	0.01	00	-
	0.61	2Gy	214	39	0.10	0.01	0.19	33%
		B+2Gy	219	48[$]	0.31	0.03	0.12	15%
		4Gy	185	80	0.94	0.14	0.32	51%
		B+4Gy	113	81	0.96	0.18	0.27	66%
	1.91	2Gy	185	46	0.17	0.02	0.21	-
		B+2Gy	226	50	0.30	0.04	0.14	-
		4Gy	104	91	1.56	0.17	0.54	-
		B+4Gy	154	92	1.93	0.24	0.48	-
	7.1	2Gy	173	66	0.15	0.06	0.22	-
		B+2Gy	234	71	0.37	0.08	0.14	-
		4Gy	186	93	1.93	0.21	0.57	-
		B+4Gy	101	96	2.83	0.27	0.46[*]	-
2.	0	0	147	02	0.01	0.01	00	
	0	0	117	03	0.02	0.01	00	
	0.61	2Gy	163	34	0.08	0.02	0.18	39%
		B+2Gy	133	46[$]	0.19	0.03	0.11	50%
		4Gy	152	82	0.92	0.12	0.35	42%
		B+4Gy	129	90	1.16	0.16	0.27	36%
	1.91	*2Gy*	*172*	*39*	*0.22*	*0.03*	*0.21*	-
		B+2Gy	174	50[$]	0.36	0.05	0.14	-
		4Gy	107	87	1.67	0.17	0.56	-
		B+4Gy	131	90	2.05	0.26[*]	0.49	-
	7.1	2Gy	211	62	0.13	0.09	0.26	-
		B+2Gy	220	68	0.38	0.13	0.18	-
		4Gy	192	87	1.59	0.27	0.63	-
		B+4Gy	133	92	1.81	0.31	0.55	-
3.	0	0	158	02	0.01	0	00	-
	0	0	109	03	0.02	0.01	00	-
	0.61	2Gy	123	35	0.13	0.02	0.15	52%
		B+2Gy	120	43[$]	0.20	0.04	0.12	28%
		4Gy	154	80	0.79	0.11	0.32	29%
		B+4Gy	142	89[$]	1.33	0.20[*]	0.29	52%
	1.91	2Gy	176	40	0.25	0.03	0.17	-
		B+2Gy	133	43	0.32	0.05	0.14	-
		4Gy	192	88	1.15	0.15	0.52	-
		B+4Gy	188	92	1.57	0.23[*]	0.45	-
	7.1	2Gy	176	60	0.27	0.05	0.19	-
		B+2Gy	132	65	0.29	0.06	0.12	-
		4Gy	183	90	1.91	0.20	0.62	-
		B+4Gy	110	93	2.75	0.28[*]	0.52[*]	-
4.	0	0	126	03	0.01	0.01	00	-
	0	0	194	04	0.03	0.01	00	-
	0.61	4Gy	170	86	1.19	0.17	0.32	-
		B+4	171	91	1.69	0.22	0.25	-
	1.91	2Gy	127	44	0.14	0.04	0.21	-
		B+2	104	53[$]	0.37	0.06	0.14	-

7.1	2Gy	157	64	0.25	0.07	0.29	-
	B+2Gy	270	70	0.39	0.09	0.19*	-
	4Gy	179	94	1.91	0.25	0.68	-
	B+4Gy	111	95	2.38	0.32	0.58*	-

TM: Total Metaphases; AtbM: Aberrant Metaphases; M1: First division cycle; AGT:Average Generation Time; Del: deletions; Chtd.Bk.: chromatid breaks; Exchs: exchanges.
* Significant at $p<0.05$ simple $\chi 2$- test. $ Signifcant at $p<0.05$ $\chi 2$ – contingency test.

Table 1.4. Pooled data on the induction of chromosome aberrations by γ-ray dose-rate variation in Normal and BSO (5mM) depleted HPBLs

Dose-rate	Dose	BSO (mM)	TM	AbtM% ± SEM	Aberrations per cell ± SEM			Sparing effect for deletions
					Del	Chtd.Bk.	Exchs.	
0	0	0	525	3±0.3	0.01±0	0.01±0	0	
0	0	5mM	548	4±0.3	0.02±0	0.01±0	0	
0.61Gy/min	2	0	500	36±1.5	0.10±0.01	0.02±0	0.17±0.01	41±6
	2	5mM	472	46±1.5$	0.23±0.04*	0.03±0	0.12±0	32±10
	4	0	661	82±1.4	0.96±0.08	0.16±0.01	0.33±0.08	51±5
	4	5mM	555	88±2.3	1.29±0.15*	0.19±0.01	0.27±0.01	51±9
1.91Gy/min	2	0	660	42±1.7	0.19±0.02	0.03±0	0.20±0.01	-
	2	5mM	637	50±1.0	0.34±0.01*	0.05±0	0.14±0	-
	4	0	403	89±1.2	1.46±0.16	0.16±0.01	0.53±0.01	-
	4	5mM	473	95±2.0	1.85±0.14*	0.24±0.01*	0.47±0.01	-
7.1Gy/min	2	0	717	63±1.3	0.20±0.04	0.07±0.01	0.24±0.02	-
	2	5mM	856	69±1.3	0.36±0.02*	0.09±0.02	0.16±0.02	-
	4	0	740	91±1.6	1.80±0.1	0.23±0.02	0.63±0.02	-
	4	5mM	526	92±1.4	2.40±0.2*	0.32±0.03*	0.59±0.08	-

TM: Total Metaphases; AtbM: Aberrant Metaphases; M1: First division cycle; AGT:Average Generation Time; Del: deletions; Chtd.Bk.: chromatid breaks; Exchs: exchanges.
* Significant at $p<0.05$ simple $\chi 2$- test. $ Signifcant at $p<0.05$ $\chi 2$ – contingency test.

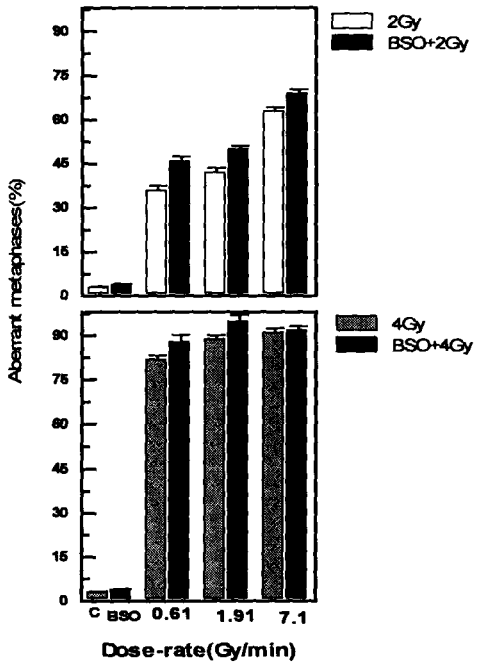


Fig. 1.8 Induction of aberrant metaphases by radiation at different dose-rates in normal and BSO-treated HPBLs

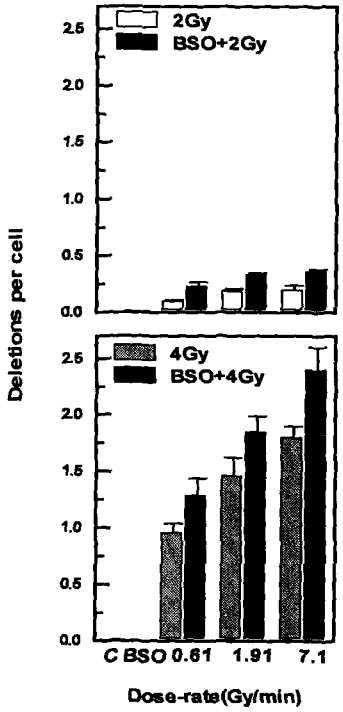


Fig. 1.9 Deletions induced by radiation at different dose-rates in normal and BSO-treated HPBLs.

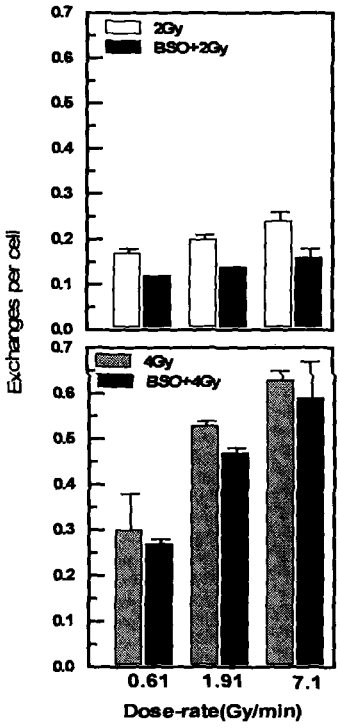


Fig.1.10 Exchange aberrations induced by radiation at different dose-rates in HPBLs.

Table 1.5: Cell proliferation kinetics in response to irradiation at different dose-rates of γ-radiation in HPBLs in the presence and absence of BSO(5mM)

Donor	Dose-rate (Gy/min)	Exptal. Condtn.	TM	M1%	AGT(H)
1.	0	0	132	69.0	42.9
	0	0	169	70.1	43.1
	0.61	2Gy	258	70.1	45.6
		B+2Gy	266	69.9	43.9
		4Gy	217	81.7	46.8
		B+4Gy	158	77.0	44.5
	1.91	2Gy	222	70.3	46.5
		B+2Gy	275	79.0	44.7
		4Gy	171	83.2	47.8
		B+4Gy	178	74.1	45.8
	7.1	2Gy	203	85.2	47.8
		B+2Gy	273	78.3	44.6
		4Gy	211	91.2	49.8
		B+4Gy	164	89.1	48.3
2.	0	0	191	68.1	42.1
	0	0	163	70.4	43.2
	0.61	2Gy	196	69.1	45.1
		B+2Gy	161	69.9	43.5
		4Gy	182	79.0	47.1
		B+4Gy	164	74.3	46.2
	1.91	2Gy	198	71.0	46.6
		B+2Gy	197	76.4	44.5
		4Gy	165	88.6	48.3
		B+4Gy	164	76.0	46.7
	7.1	2Gy	251	82.2	47.0
		B+2Gy	259	76.8	43.6
		4Gy	252	85.6	47.8
		B+4Gy	189	83.3	45.4
3.	0	0	187	67.3	42.4
	0	0	169	68.8	42.8
	0.61	2Gy	162	73.1	44.9
		B+2Gy	170	70.4	43.6
		4Gy	184	79.0	46.6
		B+4Gy	175	71.8	45.1
	1.91	2Gy	197	81.2	45.6
		B+2Gy	183	71.0	44.1
		4Gy	233	82.6	47.2
		B+4Gy	229	75.3	46.5
	7.1	2Gy	211	83.6	46.3
		B+2Gy	158	65.1	42.2
		4Gy	224	88.3	49.1
		B+4Gy	161	85.3	47.6
4.	0	0	159	69.3	43.2
	0	0	238	70.7	43.9
	0.61	4Gy	209	77.7	46.1
		B+4	221	73.8	45.6
	1.91	2Gy	168	79.1	46.7

	B+2	147	71.8	45.7
7.1	2Gy	187	85.4	47.3
	B+2Gy	296	77.0	44.0
	4Gy	210	93.3	48.3
	B+4Gy	211	91.1	47.8

TM: Total Metaphases; AtbM: Aberrant Metaphases; M1: First division cycle; AGT:Average Generation Time

Table 1.6. Pooled data on cell proliferation kinetics by irradiation with different dose- rate of radiation with and without BSO in HPBLs.

Dose-rate	Dose	BSO (mM)	TM	M1% ± SEM	AGT (H) ± SEM
0	0	0	525	69±0.6	42.6±0.3
0	0	5mM	548	70±0.5	43.3±0.2
0.61Gy/min	2	0	500	71±1.2	45.2±0.2#
	2	5mM	472	70±1.7	43.7±0.1
	4	0	661	79±0.8	46.6±0.2#
	4	5mM	555	84±3.8	45.4±0.4
1.91Gy/min	2	0	660	76±2.7	46.3±0.3#
	2	5mM	637	75±1.9	44.8±0.4
	4	0	403	85±1.9	47.8±0.3#
	4	5mM	473	77±0.6	46.8±0.3#
7.1Gy/min	2	0	717	84±0.8	47.1±0.3#
	2	5mM	856	74±3.1	43.6±0.5
	4	0	740	90±1.7	48.8±0.4#
	4	5mM	526	87±1.8	48.1±1.2#

TM: Total Metaphases; AtbM: Aberrant Metaphases; M1: First division cycle; AGT:Average Generation Time. # Significant at $p<0.01$ as compared to control by students t-test.

Table 1.7a. Effect of BSO and different dose-rates (Gy min $^{-1}$) of radiation on exchange frequency in HPBLs.

Donors	Treatment	Dose-rate	TM	Abt M%	Exchs
	Unirradiated	*0.65*	94	3	0
1	BSO		128	4	0
	2Gy		185	39	0.19
	B+2Gy		226	48@	0.12
	4Gy		104	80	0.32
	B+4Gy		154	81	0.27
2	Unirradiated		147	2	0
	BSO		117	3	0
	2Gy		172	34	0.18
	B+2Gy		174	46@	0.11
	4Gy		107	82	0.35
	B+4Gy		131	90	0.27
3	Unirradiated		158	2	0
	BSO		109	3	0
	2Gy		176	35	0.15
	B+2Gy		133	43@	0.12
	4Gy		192	80	0.32
	B+4Gy		188	89@	0.29
4	Unirradiated		126	3	0
	BSO		194	4	0
	4Gy		170	78	0.32
	B+4Gy		171	74	0.25
5	Unirradiated		108	2	0
	2Gy		58	37	0.16
	3Gy		148	57	0.32
	5Gy		122	98	0.52
6	Unirradiated		145	3	0
	2Gy		140	37	0.14
	3Gy		127	62	0.30
	5Gy		138	80	0.40
7	Unirradiated		145	2	0
	BSO		76	3	0
	3Gy		146	58	0.28
	BSO+3Gy		134	62	0.19*
8	Unirradiated		103	3	0
	BSO		73	4	0
	3Gy		131	50	0.22
	BSO+3Gy		148	54	0.17

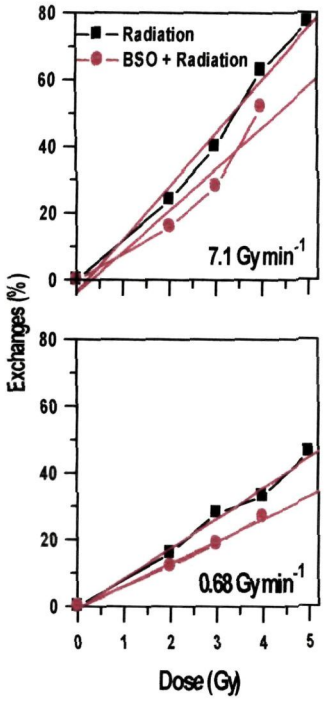
5	Untreated	*6.8*	221	3	0
	BSO		132	4	0
	2Gy		173	66	0.22
	B+2Gy		234	71	0.14 [b]
	3Gy		134	73	0.39
	4Gy		186	93	0.57
	B+4Gy		101	96	0.46*
6	Untreated		203	2	0
	BSO		228	3	0
	2Gy		211	62	0.26
	B+2Gy		220	68	0.18
	3Gy		104	76	0.43
	B+3Gy		175	77	0.30*
	4Gy		192	87	0.63
	B+4Gy		133	92	0.55[b]
	5Gy		121	93	0.76
7	Untreated		216	2	0
	BSO		202	3	0
	2Gy		176	60	0.19
	B+2Gy		132	65	0.12
	3Gy		128	70	0.31
	B+3Gy		129	74	0.25
	4Gy		183	90	0.62
	B+4Gy		110	93	0.52*
	5Gy		157	95	0.77
8	Untreated		203	3	0
	BSO		200	4	0
	2Gy		157	64	0.29
	B+2Gy		270	70	0.19*
	3Gy		102	75	0.44
	4Gy		179	94	0.68
	B+4Gy		111	95	0.58*

TM= Total Metaphases; AbtM =Aberrant Metaphases; Exchs.=Exchanges.
@ significant at $p< 0.05$ by χ^2 - contingency test.
* significant at $p<0.05$ by simple χ^2 - test. b = borderline of $p<0.05$

Table 1.7b ***Pooled data for exchanges in response to BSO and different dose-rates of radiation in HPBLs.***

Treatment	Dose rate (Gy min^{-1})	TM	Abt M%	Exchs. ± SEM
Unirradiated		1686	2	0±0
2Gy	0.65Gy/min	3092	36	0.16±0.01
3 Gy		552	57	0.28±0.02
4Gy		573	80	0.33±0.01
5 Gy		160	89	0.46±0.06
2Gy	6.8Gy/min	717	63	0.24±0.02
3Gy		468	74	0.40±0.03
4Gy		740	91	0.63±0.02
5Gy		278	95	0.77±0.01
BSO		1459	3	0±0
B+2Gy	0.65Gy/min	533	46	0.12±0
B+3Gy		282	58	0.18±0.01
B+4Gy		644	84	0.27±0.01
B+2Gy	6.8Gy/min	856	69	0.16±0.02
B+3Gy		304	66	0.28±0.03
B+4Gy		455	94	0.53±0.01

TM= Total Metaphases; AbtM =Aberrant Metaphases; Exchs.=Exchanges.



Radiation : a= -3.43 ± 3.7 b=15.8 ± 1.8 r = 0.99232
BSO + radiation: a= -3.77 ± 6.6 b=12.3 ± 2.5 r = 0.96214

Radiation : a = -0.68 ± 1.8 b = 9.03 ± 0.5 r = 0.99458
BSO + radiation : a = -0.54 ± 6.6 b = 6.69 ± 0.5 r = 0.99760

1.11 Induction of exchanges by radiation at two dose-rates in human PBLs

Table1.8. Individual data on apoptosis in Mice BMCs irradiated at different dose rates, with and without BSO treatment.

Dose-rate	Dose	BSO	%Apoptotic cells (total cells scored)	
(Gy/min)	(Gy)	Mg/kg	6H	24H
0	0	0	11 (1026)	-
			9 (1034)	-
0	0	200	11 (1396)	14 (1074)
			9 (1262)	13 (1030)
0.68	2	0	23 (1010)	33 (1540)
			25 (1325)	33 (1230)
			23 (1245)	30 (1221)
		200	24 (1361)	35 (1211)
			27 (1243)	36 (1294)
			28 (1151)	37 (1227)
	4	0	30 (1356)	38 (1017)
			28 (1244)	43 (1253)
			29 (1273)	49 (1200)
		200	32 (1143)	49 (1287)
			33 (1257)	50 (1256)
			32 (1345)	46 (1034)
6.33	2	0	38 (1063)	47 (1093)
			34 (1024)	47 (1328)
			38 (1341)	47 (1275)
		200	45 (1264)	54 (1138)
			42 (1287)	56 (1120)
			42 (1149)	51 (1158)
	4	0	38 (1541)	55 (1081)
			46 (1542)	52 (1019)
		200	56 (1132)	64 (1358)
			50 (1235)	60 (1625)
			55 (1316)	60 (1333)

Table 1.9. Pooled data for apoptotic cell death in normal and BSO treated mice bone marrow cells in response to radiation at different dose rates.

Dose-rate	ose	BSO	%Apoptotic cells ±SEM	
(Gy/min)	(Gy)	($mgkg^{-1}$)	6H	24H
0	0	0	10 ±0.95	-
0	0	200	10 ±0.60	14 ±0.75
0.68	2	0	26 ±0.88	32 ±0.96
		200	26 ±1.03	36 ±0.61
	4	0	29 ±0.62	43 ±2.97
		200	32 ±0.39	48.±1.25
6.33	2	0	37 ±1.47	47 ±0.23
		200	43 ±0.94	54 ±1.45
	4	0	42 ±4.00	53 ±1.05
		200	54 ±1.88*	61 ±1.38*

*Significant at $p<0.05$ $\chi 2$ – contingency test

Table 1.10 : Individual data on apoptotic cell death in normal and BSO treated HPBLs

Dose-rate	Dose	BSO	%Apoptotic cells.			
			Cells scored	24Hrs	Cells scored	48Hrs
0		0	1353	20.6	1236	22.8
			1396	19.02	1126	22.1
			1120	17.6	1010	21.3
			1101	18.5	0739	22.3
0		5mM	1573	21.9	1158	24.3
			1151	19.7	1478	23.5
			0996	19.11	1006	22.8
0.2Gy/min	2Gy	0	1365	20.1	1152	25.4
			1248	21.6	1312	25.1
		5mM	1129	24.4	1039	28.6
			1054	26.2	1017	29
	4Gy	0	1182	34.6	1257	46.7
			1296	33.2	1346	44.8
		5mM	1463	38.7	1009	48.9
			1200	36.3	1111	51.1
6Gy/min	2Gy	0	1391	48.1	1247	53.2
			1121	46.3	1361	55.1
		5mM	0897	43.5	1391	50.3
			1116	45.4	0986	49.8
	4Gy	0	1222	56.6	1219	61.1
			1001	54.6	1029	58.9
		5mM	1327	52.6	1337	55.3
			1282	52.1	1365	58.7

Table 1.11: ***Pooled data for apoptotic cell death in normal and GSH depleted Human PBLs.***

Dose-rate	Dose	BSO	Apoptotic cell death (%)			
			Cells scored	24H	Cells scored	48H
0	0	0	4970	18.93±0.63	4111	22.13±0.31
0	0	5mM	3720	20.24±0.85	3642	23.53±0.43
0.2Gy/min	2Gy	0	2643	20.85±0.75	2464	25.2±0.15
		5mM	2183	25.30±0.90	2056	28.8±0.20
	4Gy	0	2478	33.90±0.70	2603	45.75±0.95
		5mM	2663	37.50±1.20	2120	50.00±1.10
6Gy/min	2Gy	0	2512	47.20±0.90*	2608	54.15±0.95*
		5mM	2013	44.45±0.95	2377	50.05±0.25
	4Gy	0	2223	55.60±1.00*	2248	60.00±1.10*
		5mM	2609	52.35±0.25	2702	57.00±1.70

* significant at $p<0.05$ χ^2-contingency test.

Discussion

Buthionine sulfoximine has been used to evaluate the effect of GSH depletion on radiosensitization at different dose-rate of mouse BMCs *in vivo* and human PBL *in vitro*. The parameter like CAs has been considered because it seems that this could provide insight about the role of GSH in both physicochemical and biochemical processes. In order to score CAs, it is advisable to use BUdR-differential staining method to determine most suitable harvesting time to have optimum number of cells in 1st cycle metaphases (M1) from which CAs should be scored (Bianchi *et al.*, 1982). For this reason the present *in vivo* experiment was carried out by fixing the cells at 13h after irradiation since the unirradiated mouse shows very high frequency of M1 (96.5%) at 13h fixation time (Chattopadhyay *et al.*, 1999).

Our results indicate that the presence of BSO increased the cellular radiosensitivity in both mouse BMCs and human PBL at all dose-rate of irradiation and it could be due to depletion of endogenous GSH as it was shown earlier (Chattopadhyay *et al,*. 1999). Mice treated with BSO exhibit a rapid decline in GSH in kidney, liver, pancreas and muscle and after prolonged treatment also showed lowered concentration of GSH in other tissues (Griffith and Meister, 1979). The reduced-GSH estimation after 10h incubation with BSO (200 mg kg^{-1}) in the earlier study could deplete GSH level significantly with respect to control. It has been demonstrated that following a single dose of 556 mg kg^{-1} BSO, the GSH concentrations of various normal tissues were depleted in a time dependent manner (Lee *et al.* 1987). They had observed that the intermediate rates of depletion in the bone marrow with a nadir at 8-12h and the GSH content of the BMCs following depletion was 17% of the initial level. Therefore, in the present in vivo study the incubation period with BSO-treatment was 10h. In case of in vitro study the freshly drawn blood was incubated with BSO for 5h since in cultured cells more than 75% depletion was achieved within 4-5h duration by 500μm to 10mM of BSO (Shrieve *et al.*,1985, Edgren and Revesz, 1987). In the earlier study the concentration of BSO used was 5mM since significant sensitization by BSO was observed at this higher concentration with respect to radiation-induced CAs (Chattopadhyay *et al.* 1999).BSO mediated increase in frequency of radiation induced chromatid and iso-chromatid breaks in mouse BMC and deletions in human PBL in this study are not in agreement with the reported failure to

increase radiosensitivity with respect to DNA single strand breaks in BSO-treated, GSH depleted cells under aerobic condition (Edgren *et al.,* 1985) and also in aerated Chinese hamaster lung and A549 human lung carcinoma cells (Bump *et al.,* 1982; Clark *et al.,* 1984). However, equal sensitization of aerated and hypoxic cells using similar in vitro systems has been reported (Mitchell *et al.*, 1983; Koch *et al.*, 1984). Depletion of intracellular GSH by BSO sensitized V79-379A cells to X-irradiation at all oxygen tensions has been demonstrated by Shrieve *et al.,* (1985). Therefore, reports are available both in favour and against the view that BSO treatment increases the radiosensitivity of aerated cells and the present study also clearly indicates that depletion of GSH by BSO sensitized the cells to radiation with respect to CAs.

It has been thought that the protection of DNA in cells is due to scavenging of hydroxyl radical 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 a far more efficient protection against radiation induced DNA strand breaks than intracellular scavengers of hydroxyl radicals (Ward 1994, Nygren *et al.*, 1995). It has been claimed that at least two peptides, one being GSH, are bound to the nucleoproteins, as mixed disulphides (Modig, 1973). Moreover, aminothiol radioprotectors, in general, have been reported to bind with DNA and slow down strand separation for replication (Brown, 1976). Such inhibition of DNA synthesis could induce cell cycle delay as well as SCEs (Schneider *et al.*, 1978). It has been shown that reduced GSH could also induce both cell cycle delay (Chatterjee and Jacob-Raman, 1986; Chatterjee et al 1995) and SCEs (Speit *et al* .1980; Chatterjee *et al.* 1995). It may be presumed therefore, that a portion of cellular GSH binds on DNA or DNA-bound proteins and provide shielding effect against radiation. Thus BSO-mediated GSH depletion could reduce the shielding effect and enhance the DNA strand breaks induction probably by hydroxyl radicals because increase in damage by removing natural protection system is largely due to the effect of hydroxyl radical (Ljungman *et al.* 1991; Nygren *et al.* 1995).

In the present study we exposed the cells to radiation at different dose-rates. As the dose-rate is lowered, the time taken to deliver a particular radiation dose increases. Hence it becomes possible for a number of biological processes, specifically the repair of the sub-lethal damages (Elkind *et al.*, 1967) to take place during the period of exposure. The data from this study have also indicated the same trend. As the dose-rates were lowered, all the aberration frequencies were found to decrease, indicating that more repair of the damages were taking place as the dose-rates were lowered. Hence the

aberrant metaphase percentages at the lower dose-rates were also found to be lower in comparison to the aberrant metaphase percentages at the higher dose-rates indicating that the damages acquired per cell at the higher dose-rate were also more. One of the important classes of aberrations scored here were the exchanges that includes dicentrics and rings in human PBLs and different chromatid exchanges and sister chromatid union for mouse BMCs. These are aberrations that arise out of the interaction of lesions between two different chromosomes during the process of misrepair. In one report, Lobrich *et al.* (2000) have shown that fractionating a given dose of X-rays significantly reduced the dsb misrejoining frequency whereas there was an increase in the probability of correct rejoining of the dsb ends produced. In another study by Rothkamm *et al.* (2001), when 80Gy of radiation had been delivered to MRC-5 cells over a period of 14 days, effective and correct rejoining of the lesions took place at this low dose-rate. However, when the same dose was delivered at the rate of 23Gy/min i.e. at a high dose-rate, 50% of the induced dsbs were misrepaired in a period of 24 hrs. Therefore, it can be concluded that if the dsbs are separated in time and space, which is the case with irradiation at low dose-rates, then the broken ends are correctly rejoined.

The interesting observation in this study is the failure of increase in the frequency of exchange aberrations in BSO-pretreated cells in spite of significant enhancement of chromatid and deletion type of aberrations as it was shown earlier (Chattopadhyay *et al* 1999). An exchange aberration is thought to arise as a consequence of illegitimate reunion ('misrejoining') of free ends from different DNA DSB (Cornforth and Bedford, 1993). In an attempt to clarify the possible role of GSH in biochemical repair processes, the extent of rejoining of radiation induced single strand breaks (Ssbs) was determined upto 1h 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. Therefore, present failure in either restitution or illegitimate reunion soon after DSB induction by radiation could lead to increase in the frequency of deletion and chromatid aberrations in BSO-pretreated cells.

This assumption has further strengthened after observing an increased frequency of exchange aberrations and decreased frequency of deletions in GSH/GSH-ester posttreated human PBL irradiated at 4°C (Chattopadhyay *et al.*, 1998). The rationale for such posttreatment to irradiated cells at 4°C is based on the premise that increased endogenous GSH level could act on radiation induced unrepaired DNA lesions (due to

4°C incubation) after shifting the temperature from 4°C to 37°C. Therefore, GSH/GSH-ester was added soon after irradiation and kept for 3h at 4°C since GSH-ester is readily transported into cells and converted to GSH increasing the level of GSH within 3-4h (Wellner *et al.*, 1984). It was explained that the increase and decrease in frequency of exchange aberrations and deletions respectively could be due to enhancement in rejoining (both restitution and illegitimate reunion) of radiation induced DNA DSB under the influence of increased endogenous GSH. It has been demonstrated that mammalian cells require the enzymatic machinery for joining together non-homologous DNA ends (Thode *et al.*, 1990). Moreover, with in vitro assay by utilizing either 'naked' agarose embedded cellular DNA (Cheong *et al.*, 1996) or DNA organised in chromatin as found in cell nucleus (Ganguly and Iliakis, 1995), it has been shown that efficient rejoining of radiation induced DNA DSB require activities present in cell extracts. From the present result it seems that endogenous GSH could be one of the important component of enzymatic machinery which is needed for such DNA DSB joining. This consideration finds further support in the report where inhibition of unscheduled DNA synthesis in 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 were demonstrated (Lai *et al.*, 1989).

Generally exchanges are analyzed by either the linear quadratic model or the linear regression fit. In the present case when the regression coefficients were calculated, their values were all found to be close to 1, but less than 1. Therefore, a better fit for the presently obtained data was the linear regression. The data were fitted with the help of a computer using the software Origin 3.8. From the graphs 1.12 it can be seen that the slope for the data obtained for high dose-rate (HDR) was different from the slope obtained from the data for low dose-rate (LDR) of the curves generated by the computer. The values of the 'a' and 'b' components of the fitted curves obtained from the samples irradiated at the HDR were always higher than the values obtained from the curve at the lower dose-rate. This can be explained by the fact that during irradiation at low dose-rate, protracted exposure time allows for the repair of the sub lethal damages within the cell and as a result a number of damages get repaired at LDR. That however is not the case with HDR, and from the data it can be seen that that CA's are indeed more at HDR. Therefore, the values of the two components 'a' and 'b' would be expected to be higher at the HDR than at the LDR. Besides this, at HDR the average energy deposited in the cell nucleus by the secondary electrons produced by the HDR irradiation

is more and therefore the likelihood of it producing greater than one dsb by a single electron track is more and the damages are probably clustered. Thus the value of the 'a' term is much higher at HDR than at LDR. At both the dose-rates again, it can be seen that whenever BSO was added to the system, although the 'a' value was still almost the same, the 'b' value has changed. There was in fact a drop in the 'b' value whenever BSO was added in addition to irradiation at both the dose-rates. The explanation is given by the two- component model which outlines that the 'a' component arises out of the direct hits that are produced within the nucleus by the passage of radiation, and the 'b' component arises out of the indirect event. This means that the 'b' component arises from the production of the free radicals by the radiolysis of water within the biological system. Therefore, in the presence of BSO, it could be that when GSH is depleted, oxygen, which competes with GSH for the fixation of the damage, interacts with lesions and thereby fixes them. In the process probably oxygen could be altering the end chemistry of the lesions in such a way that they are being unable to interact and thereby the rejoining is affected. This could be the reason for increasing in the frequency of aberrations when BSO treatment was given.

The sparing effect gives an idea of how much the percentage differences in between the high and the low dose-rates irradiated cell were regarding the deletions or isochromatid breaks, with and without BSO. The sparing effect of LDR in BSO-treated cells was reduced at 2 and 4Gy in both mouse BMC and human PBLs. It shows similar to BSO-untreated normal samples with respect to deletions and chromatid and iso-chromatid breaks. Earlier it was shown that there is sparing effect of LDR in normal cells and is absent in AT cells and has been interpreted as a repair defect (Cox, 1984). Therefore, the present results indicate that BSO could interfere the DNA-repair mechanism and therefore overall DNA DSB-ends joining was affected. In the case of BMCs, where sparing effect had been calculated at all the three dose-rates in comparison to the highest dose-rate, it was seen that sparing effect was increasing with decreasing dose-rates. In the case of HPBLs, the sparing effect had been calculated between the highest and lowest dose-rates. When the means of these sparing effects were taken, it was seen that with BSO, the sparing effect was decreasing in the irradiated cells with respect to normal-irradiated cells. Therefore, as a whole, the sparing effects were never more in the presence of BSO, and this was obvious at all the dose-rates that were used in this study.

However, the presently observed increase in frequency of radiation induced CAs in BSO-treated cells could also be a manifestation of apoptosis since these cells are unusually responsive to radiation by apoptosis. The present data show that the apoptotic cell death at HDR was more in comparison to apoptosis at LDR. Increased apoptotic cell death in bovine lens epithelial cells irradiated at higher dose-rate than at LDR have been reported by Belkacemi *et al.,* (2000), and increased survivality of cells at LDR have also been reported by Evans *et al.,* (1985) in mouse lymphoma cells in addition to a 2.5 fold increase in survivality in CHO cells irradiated at LDR as compared to the cells exposed to acute exposure (Nagasawa *et al.,* 1989.)

It has been demonstrated that human PBLs show morphological characteristics of apoptosis following irradiation with γ-rays doses ranging from 0.1 to 5Gy (Vral *et al.*, 1998). It has been shown that the fraction of cells with aberrations will increase when the apoptotic response has been counteracted by the addition of phytohaemagglutinin (PHA) in irradiated Go human lymphocytes and suppression of the apoptotic process by the post-irradiation addition of PHA could be seen as late as 48h after irradiation (Harms-Ringdahl *et al.*, 1996). Reports are already there that GSH may also have a role in modulating the mode of cell death following toxic injury (Fernandes and Cotter, 1994). The role of GSH in modulating the cytotoxicity of platinum complexes by affecting DNA repair, apoptosis and free radical scavenging has also been demonstrated (Pendyala *et al.*, 1997). Therefore, it can be said that present increase in the frequency of CAs could be at the expense of the genomic fidelity of the population and if it is so then radiation induced apoptosis induction in BSO-treated cells is important from cancer therapeutic point of view. It has already been shown that tumours were sensitized to radio and chemotherapeutic modalities when the cellular GSH content was decreased by BSO (Hamilton *et al.,* 1985, Meister, 1991).

However, it is known that antioxidants usually prevent tissue damage under normal conditions, and the apoptosis inducing effects can be blocked by glutathione and N- acetylcysteine (Simon *et al.*, 2000). This fact could be used to explain the increase of apoptotic cell death when BSO was given to the cells irradiated at LDR. At HDR, when BSO treated cells were irradiated, there was found to be an insignificant but slight decrease in apoptosis in HPBLs, whereas the BMCs showed increased apoptosis when GSH was depleted in the presence of BSO preceding irradiation. Now at HDR the damages were found to be more on the chromosomes and these were further increased in the presence of BSO. Therefore, it could be expected that cells loded with such heavy

damages were probably die by necrosis rather than apoptosis in the HPBLs. This phenomenon of the preferred mode of cell death as necrotic cell death over apoptosis has been studied in cells irradiated with high doses of radiation produced by high LET radiation which produces extensive and complex damages to DNA (Harms-Ringdahl *et al.*, 1996). As a result of this, it could be that apoptotic cell death as scored by light microscopy was found to show a slight decreasing trend in the HPBLs when they were irradiated in the presence of BSO at HDR.

A novel aspect of the present study is the analysis of the influence of endogenous GSH-status on the radiation-induced cell cycle delay has been done simultaneously with the aberration scoring. Cell cycle control has widely been accepted to play an important role in the response of cells to DNA-damaging agents. Cells can arrest in the G_1 or G_2 (or both) phases of cell cycle following DNA damage caused by ionizing radiation in attempts to repair or recover from the induction of DNA-lesions (Jacks and Weinberg, 1996). When nuclear DNA is damaged normal cells initiate a response that includes cell cycle arrest, apoptotic cell death and transcriptional induction of genes involved in DNA repair (Goodrich *et al.*, 1991). In the present investigation there is increase of CAs with increasing dose-rate of radiation. Therefore, it is obvious that the damage caused at the HDR is more than at the LDR. In this study it was observed that the induction of delay in cell cycle was more at HDR radiation exposure than LDR and this could be equated with the increased frequency of CAs at HDR. When BSO was added to the cells before irradiation, in the case of BMCs, the cell cycle kinetics studied as the mitotic index, was found to increase at all the dose rates as compared to the BSO-untreated cells. In HPBLs, like the mouse BMCs, the cell cycle delay was reduced in the presence of BSO as determined from the AGT value.

The present data indicate that the depletion of endogenous GSH by BSO improves the cell cycle kinetics considerably after irradiation in mouse BMC and marginally in HPBLs. However, in each cell system, presence of BSO increased the frequency of CAs induced by radiation. Therefore, it seems that in BSO-treated mouse BMC and HPBLs the cell cycle kinetics after radiation was improved than radiation alone and not allowing the cells to repair the damages caused by radiation. It seems that these damaged cells may ultimately die apoptotically .

In this study 200mg kg^{-1} BSO alone induced CAs considerably in mouse BMCs. Revesz *et al.*, (1994) showed about the selective toxicity of BSO to melanoma cells both in vivo

and in vitro. It has also been reported earlier that extremely low concentration of BSO i.e 4mg kg^{-1} induced sister chromatid exchanges significantly in mouse BMC without inducing any CAs (Chatterjee *et al.*, 1995). Therefore, present induction of CAs by higher concentration of BSO in mouse could be attributed due to better depletion of endogenous GSH-level and this could be an indication about the important protective role of endogenous GSH in the cell against peroxides and free radicals which are formed by normal metabolic pathways (Meister, 1983). However, the failure of BSO (5mM) to induce CAs in human lymphocytes is not clear. It could be that the generation of internal free radicals might be negligible in non-cycling lymphocytes (before PHA addition) and thus depletion of cellular GSH could not increase the free radical induced spontaneous CAs frequency.

In conclusion, our results indicate that BSO-mediated GSH depletion increases radiation induced CAs at all dose-rates and this could be due to reduction of DNA shielding effect, failure in rejoining of DNA DSB free ends and apoptosis. Such BSO-treatment failed to increase the frequency of radiation induced exchange aberrations in mammalian cells. The reduction of sparing effect of LDR radiation in BSO-treated cells indicates a probable involvement of endogenous GSH in DNA dsb-joining.

Chapter II

GSH and interaction of double strand breaks

Literature Review

Extensive studies of the biological action of radiation at the cellular level have placed the DNA molecule at the top of the hierarchy of possible targets. Reproductive cell death, mutation and transformation are closely related to molecular damage that can eventually lead to molecular damage in DNA. After irradiation of the cell, there is a broad spectrum of radiation-induced damage, which can eventually lead to the different endpoints. The understanding of mechanisms of biological action of radiation requires identification of the individual molecular damage and determination of their significance for various final effects. Since ionising radiation induces a wide range of types of damages to DNA, the critical subcellular target for radiation, considerable efforts are being made to identify the biological effects of particular lesions. Among these lesions, DNA double strand breaks (dsbs) have received special attention because it is obvious that the maintenance of genetic integrity requires the accurate and efficient repair of this type of damage. Damage to both strands of DNA in a small region (with the consequent loss of complementary bases) would deprive the cellular repair system of an undamaged template for simple regeneration of the intact DNA molecule via a polymerase reaction. DNA dsb were first found to be associated with reproductive inactivation in bacterial and bacteriophage systems (e.g. Freifelder, 1965), and subsequently in the lower eukaryote *Saccharomyces cerevisiae* and it's repair deficient mutants (Frankenberg *et al.*1981).

The importance of DNA dsb in mammalian cell systems has been supported by the following observations:

1. So far, at least three of the genetic complementation groups of rodent radiation-sensitive mutants have been found to be partially defective in repair of these types of lesions. The defect in these mutants manifests itself either as a decrease in the rate of repair of dsb or as an increase in that fraction of dsb remaining unrepaired (Jeggo *et al.*1991).
2. In metazoa, just one dsb can kill a cell if it leads to the inactivation of an essential gene or, more commonly, triggers apoptosis (Rich *et al* 2000).
3. Chromosome aberrations and cell killing, similar to that caused by ionising radiation is observed in cells treated with agents that produce exclusively DNA dsb, e.g. restriction endonucleases (Obe *et al.*1992).

4. Other DNA lesions such as single strand breaks (ssb) or base damage have been shown to correlate poorly with cell killing (Radford, 1986).
5. Restriction-endonuclease-induced dsbs are capable of causing mutation in mammalian cells (Obe *et al.*, 1992)
6. Simple dsb can induce malignant transformation in mouse embryo fibroblasts (Obe *et al.*1992).
7. There is experimental evidence for a causal link between the generation of dsbs and the induction of mutations and chromosomal translocations with tumorigenic potential (Richardson and Jasin, 2000; Ferguson and Alt, 2001).

Endogenous thiols, especially the tripeptide reduced glutathione (GSH), have long been thought to affect the sensitivity of cells to radiation (Meister 1983). Ohara and Terasima (1969) and Sinclair (1969) reported that resistance to X-irradiation varies synchronously with the GSH content. The role of intracellular GSH in cellular radiosensitivity has been reported since then by a number of workers in a number of systems. In general it was found that low intracellular GSH concentrations caused sensitisation especially under anoxic and hypoxic conditions (e.g. Revesz, 1985). GSH constitutes the most important thiol intracellularly. It cannot be transported across the cell membrane from extracellular environments into the cell (Jensen and Meister, 1983; Puri and Meister, 1983; Wellner *et al.,* 1984), but it is synthesized intracellularly by γ-glutamyl cycle (Meister 1978). If the effects of an elevated intracellular GSH concentration on radiosensitivity were to be studied the GSH level would have to be raised by means other than incubation with GSH. An artificial elevation of the intracellular GSH concentration can be achieved in various ways. Russo and Mitchell (1984) described an elevation of 200-300% of controls by treatment of Chinese Hamster V79 cells with OTZ. This compound has a latent thiol group as part of a ring structure that is enzymatically opened and then serves as an intracellular cysteine delivery system, which promotes GSH synthesis (Williamson and Meister 1981; Williamson *et al* 1982). However the effect of OTZ on GSH level varies in different cell lines. Russo *et al* (1986) observed no effect in human lung fibroblast line. With cobaltous chloride ($CoCl_2$) Russo and Mitchell (1984) obtained an increase of the intracellular level of about 200% in V79 cells. The elevation of GSH content by treatment with OTZ or $CoCl_2$ provided no radioprotection of aerated cells (Russo and Mitchell, 1984). Millar and Jinks (1985) obtained after treatment with dexamethasone an almost 2 fold increase of the cellular GSH content of human glioma cell line and of some other cell lines but little or no effect in a third group of cell lines. They

suggested that the enhancement in radioresistance they observed in some cell lines could not be ascribed to an elevated GSH content in the steroid treated cells. They attributed the relatively small changes in sensitivity to other biochemical changes in the cells. A more promising way to elevate the GSH level in the cells was described by Puri and Meister (1983) who observed in liver and kidney of mice substantial increase of the GSH level after administration of monomethyl and monoethyl esters of GSH. They attributed their findings to a transport of the esters into the cells and a subsequent hydrolysis of GSH. Wellner *et al.* (1984) reported a several fold increase of GSH content in normal human fibroblast after incubation with the monoethyl ester of GSH. A similar incubation of GSH deficient fibroblast from patients with a GSH synthetase deficiency or a γ-glutamyl cysteine synthetase defeciency resulted in cellular GSH contents similar to those of normal cells incubated with GSH ester. Wellner *et al* (1984) described also an increase of GSH levels in human lymphoid cells after incubation in medium containing GSH ester and a radioprotection of such cells; however their data did not allow an estimation of the degree of protection. Preparation of GSH-monoethyl esters, uptake in tissues, and conversion to GSH after i.p. and oral administration by mice was reported by Anderson *et al.* (1985.)

In mammalian cells GSH amounts about 90% of the nonprotein bound sulphydryls NPSH. Direct evidence that sulphydryls and oxygen can compete in the ways suggested by the competition model was provided in pulse radiolysis experiments. In view of the participation of GSH in a great number of diverse metabolic functions in the cells (Kosower and Kosower 1978), it is conceivable that it will also take part in some radiation induced biochemical reactions. Many different biochemical repair reactions are likely to occur after the radical reactions, but little is known about the mechanism at a molecular level. In an attempt to clarify the possible role of GSH in one well-defined biochemical repair process, the extent of rejoining of radiation induced single stranded breaks (ssb) was determined up to 1hr after exposure. The rejoining can be error prone or error free and its relationship to survival is still a matter of debate. However, as an intermediate endpoint between the yield of ssb and survival rejoining can be considered to be of interest in it's own right. In a series if experiments $GSH^{-/-}$ cells exposed to hypoxic irradiations were found to rejoin ssb as well as $GSH^{+/+}$ (Edgren *et al,* 1981, Revesz and Edgren 1984). The rejoining was nearly complete in both cases within one hour. On the other hand when exposures were made oxically, the rejoining in $GSH^{-/-}$ cells occurred to

only about 70% within the same period, but in GSH $^{+/+}$ cells it was again nearly total. The observations can be interpreted to indicate that the repair system involved in the rejoining of oxically induced ssb differs from that involved in the rejoining of hypoxically induced ssb and is clearly dependent on GSH. Such an interpretation presupposes the difference in the nature of ssb arising in the presence and absence of oxygen. This assumption has received experimental support by chemical and enzymatic analysis of the end groups of radiation induced strand breaks (Von Sonntag *et al.*, 1981). The finding that GSH is involved in the synthesis of deoxyribonucleotide (Holmgren, 1979) may also be relevant to the participation of GSH in DNA-repair. This assumption has further strengthened after observing an increased frequency of exchange aberrations and decreased frequency of deletions in GSH/GSH-ester posttreated human PBL irradiated at 4°C. The rationale for such posttreatment to irradiated cells at 4°C is based on the premise that increased endogenous GSH level could act on radiation induced unrepaired DNA lesions (due to 4°C incubation) after shifting the temperature from 4°C to 37°C. Therefore, GSH/GSH-ester was added soon after irradiation and kept for 3h at 4°C since GSH-ester is readily transported into cells and converted to GSH increasing the level of GSH within 3-4h (Wellner *et al.*, 1984). Present observed increase and decrease in frequency of exchange aberrations and deletions respectively could be due to enhancement in rejoining (both restitution and illegitimate reunion) of radiation induced DNA DSB under the influence of increased endogenous GSH. It has been demonstrated that mammalian cells require the enzymatic machinery for joining together non-homologous DNA ends (Thode *et al* 1990). Moreover, with in vitro assay by utilizing either 'naked' agarose embedded cellular DNA (Cheong *et al* 1996) or DNA organised in chromatin as found in cell nucleus (Ganguly and Iliakis, 1995), it has been shown that efficient rejoining of radiation induced DNA DSB require activities present in cell extracts. From the present result it seems that endogenous GSH could be one of the important component of enzymatic machinery which is needed for such DNA DSB joining. This consideration finds further support in the report where inhibition of unscheduled DNA synthesis in 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 were demonstrated (Lai *et al* 1989).

DNA-dsbs are generated when the two complementary strands of the DNA double helix are broken simultaneously at sites that are sufficiently close to one another that base-pairing and chromatin structure are insufficient to keep the two DNA ends juxtaposed. As a consequence, the two DNA ends generated by a dsb are liable to become physically dissociated from one another, making ensuing repair difficult to perform and providing the opportunity for inappropriate recombination with other sites in the genome. Another barrier to rapid and error-free dsb repair is the fact that the DNA termini have often also sustained base damage, meaning that DSB ligation cannot occur until processing by DNA polymerase and/or nucleases has taken place.

There are two main pathways for DNA-DSB repair-homologous recombination (HR) and non-homologous end-joining (NHEJ). These pathways are largely distinct from one another and function in complementary ways to effect DSB repair (Cromie *et al,* 2001). During HR, the damaged chromosome enters into synapsis with, and retrieves genetic information from, an undamaged DNA molecule with which it shares extensive sequence homology. In contrast, NHEJ, which brings about the ligation of two DNA dsbs without the requirement for extensive sequence homology between the DNA ends, does not need synapsis of the broken DNA with an undamaged partner DNA molecule. Both pathways are highly conserved throughout eukaryotic evolution but their relative importance differs from one organism to another. Simple eukaryotes such as the yeasts *S.cervisiae* and *S.pombe* rely mainly on HR to repair radiation induced DNA dsbs. In contrast, in mammals the NHEJ pathway predominates in many stages of the cell cycle-particularly in Go and G_1 – although HR is also of importance, particularly during S- and G_2-phases (Johnson and Jasin, 2000).

In an attempt to further investigate the role of GSH in dsb-rejoining we have taken a different approach. We have made use of the fact 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 (Preston, 1982). Therefore, considering these possibilities and X-rays and bleomycin as the two agents chosen with similar and rapid apparent rates of repair of the DNA damage (Winters *et al* ,1992, Dar and Jorgensen, 1995) the present study has been carried out in human PBLs. It seems feasible to assume that the probability of interaction

between the DNA strand breaks induced by these 2 agents are high and therefore it will be appropriate to see the role of GSH in such interaction.

BLM was chosen because this drug has been found to induce CAs in an S-independent manner just as radiation does. BLM has been known to produce double strand breaks possessing 5'–P and 3'–phosphoglycolate (3'–PG) termini that are essentially blunt (Povirk *et al.* 1989). Ionizing radiation also produces ends similar to that produced by BLM i.e. approximately 50% of the breaks have 3'-PG termini while the rest have 3'–P ends (Henner *et al.* 1983). 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 (Winters *et al.* 1992, Dar and Jorgensen, 1995, Dar *et al.*1997, Winters *et al.*1994, Wang *et al.*1997). It has also been outlined by Preston (1982) that the damage induced by both BLM and ionising radiation is repaired rapidly showing that the repair kinetics is the same in both cases. Keeping these points in mind it was plausible to use BLM for the study of the role of GSH in dsb repair.

The bleomycins are a group of glycopeptide antibiotics isolated from the microorganism *Streptomyces verticillus.* They were discovered by Prof. H. Umezawa in 1966 (Umezawa *et al.*1966a). This compound is of significance because it has been shown to act as an antineoplastic agent. BLM at low doses has been known to cause breakage of DNA and inhibition of cell growth, which however might be unrelated to DNA damage (Vig and Lewis, 1978). BLM has been known to cause ssb as well as dsb (Suzuki *et al.* 1969, Haidle 1971), and can also release bases. Takeshita *et al.* (1978) have reported that the sequences readily recognised by BLM contain G-T or G-C. In general it has been shown that mitotically dividing cells are damaged to a greater extent than non-dividing cells by BLM with the exception of bone marrow cells (Kimura *et al.* 1972).

The mechanism of action of BLM has been reported to take place by the formation of free radicals (Sausville *et al.,* 1976), and it has been reported that BLM requires an Fe^{2+} ion as a cofactor upon binding to DNA through it's bithiazole and terminal amine moieties, for it to produce it's effects.

$$BLM + Fe^{2+} \rightarrow BLM.\ Fe^{2+}$$

In the presence of oxygen, it forms an oxygen-labile complex.

$$BLM.\ Fe^{2+} + O_2 \rightarrow BLM.\ Fe^{2+}.O_2$$

It is this oxygen labile complex that is highly unstable and is almost immediately oxidized to a more stable complex, in the process releasing a number of free radicals that produce the damages within the cell.

$$BLM.\ Fe^{2+}O_2 \rightarrow BLM.\ Fe^{3+}$$

Sometimes, in the presence of some reducing agents such as cysteine, thiols and H_2O_2, this complex may be reduced to BLM. Fe^{2+}, thereby starting the cycle of free radical production again. In this manner the damaging effects produced in the cell, in the presence of reducing agents, would be enhanced (Chatterjee *et al*, 1989). In fact it has already been reported that the depletion of the endogenous GSH, by BSO, enhances radiosensitivity, as studied by the increase of CAs upon irradiation after the administration of BSO (Chattopadhyay *et al*, 1998), increases the frequency of CAs induced by arecoline (Deb and Chatterjee, 1998) and mitomycin C (Dev-Giri and Chatterjee, 1998), depletion of endogenous GSH by BSO reduces the frequency of CAs produced by BLM in HPBLs (Chattopadhyay *et al.*1998).

The repair of a dsb requires the rejoining or interaction between two breaks. When the rejoining takes place between the dsbs produced in two different chromosomes, it gives rise to an exchange aberration. The probability of the occurrence of such misrepair or misrejoining is dependent on the number as well as the space and time of the appearance of the breaks. It has been already reported by Lobrich *et al.* (2000) that when the dose is fractionated, the misrejoining frequency decreases. Rothkamm *et al.* (2001) has also reported that MRC-5 cells irradiated at a low dose rate over 14 days showed a no misrepair as compared to the cells irradiated at a higher dose-rate (23Gy/min) which showed misrepair in 50% of the cells studied. At LDR, the breaks appear far apart and also there are pronounced separation of the breaks in space as well. As a result the probability for interactions required for misrepair is decreased. The breaks were therefore found to be efficiently repaired when LDR was given in contrast to HDR irradiation. Therefore, it is clear that the probability of efficient repair or misrepair is dependent upon the spatial proximity and also the time of appearance of the breaks.

With this background information in mind, it was decided to investigate the interaction of the BLM and radiation induced dsbs with respect to the endogenous GSH status at both HDR as well as at LDR.

Materials and Methods

Human peripheral Blood Lymphocytes (HPBLs)

For all *in vitro* experiments, heparinised peripheral blood was collected from healthy male donos in the age group of 25-30 years and used immediately after venipuncture. For the cultures, whole blood was used, but for the tGSH estimation as well as for the studies on the apoptotic cell death, lymphocytes were separated out from the heparinised whole blood on a Ficoll gradient by the method of Boyum *et al.* (1968).

I. Reagents for GSH estimation:

- DL-Buthionine-(S,R)- sulfoximine (BSO, Sigma, USA).
- Ficoll Hypaque / Histppaque (Sigma, USA) 1.077gm ml^{-1}.
- RPMI 1640 (Hyclone, USA).
- Reduced Glutathione (GSH, Sigma, USA). L-γ-glutamyl-L-cysteinyl glycine or GSH is a tripeptide, but is not derived from protein. Here a glutamic acid residue is joined in an unusual peptide linkage involving it's γ -COOH group with cysteine. This thiol has been shown to protect biological tissues by scavenging the primary radicals produced by the radiolysis of water. Different concentrations of this tripeptide were used for the standard curve.
- 5-sulfosalicylic acid (5-SSA, Merck, India)
- Ethylene-diamine-tetra aceticacid (EDTA, Merck, India)
- Potassium dihydrogen phosphate (KH_2PO_4, SD's Fine Chemicals Pvt. Ltd. India). This was prepared in distilled water. Solution A 0.1M
- Dipotassium hydrogen phosphate (K_2HPO_4, SD's Fine Chemicals Pvt. Ltd, India). This was prepared in distilled water. Solution B 0.1M

39ml of solution A was mixed with 61ml of solution B and the pH was adjusted to 7.0. EDTA was added o achieve a final concentration of 1mM EDTA phosphate buffer.

- Sodium bicarbonate ($NaHCO_3$, Merck, India) – 0.5%
- 5-5'-dithiobis-2-nitrobenzoic acid (DTNB, Roche Molecular Biochemicals, Germany). 1.5 mg of DTNB was dissolved in 0.5% of $NaHCO_3$ and was prepared fresh during use.
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- Nicotinamide adenine dinucleotide phosphate, tetrasodium salt (NADPH, SRL, India). 4mg of NADPH was dissolved in 0.5% $NaHCO_3$ and was prepared fresh at the time of use.
- Glutathione Reductase from yeast (GR, Roche Molecular Biochemicals, Germany).
- Hydrochloric acid (HCl, 10mM)

Treatments:

1. BSO was added at a concentration of 5mM to the freshly collected heparinised blood 5hrs prior to estimation.
2. At the end of that time, the blood was diluted with RPMI 1640 in a ratio of 1:1, i.e. to 1ml blood, 1ml medium was added.
3. In a centrifuge tube, this mixture was gently layered on top of 2ml of the histopaque and the tube was centrifuged at 1200rpm for 40mins.
4. The ring at the interphase of the histopaque layer and the upper plasma layer is the lymphocyte ring. This was sucked out with the help of a syringe and used for further processing.

Sample processing:

1. 50µl of the undiluted sample suspension of lymphocytes were transferred to a microcentrifuge tube containing 150µl of 10mM HCl.
   Acid treatment reduces the oxidation of GSH to GSSG and to mixed disulphides and inactivates γ-glutamyl transpspeptidase which catalyzes the following reactions that decrease the levels of both GSH and GSSG.
   GSH + aa ⇔ γ Glu-aa + CySH-Gly (Transpeptidation)
   GSH + H2O → Glu = CySH – Gly (Hydrolysis)
   GSH + GSH ⇔ γ Glu-GSH + CySH-Gly (Hydrolysis)
2. Cells were lysed by alternate freezing and thawing at least three times at 10°C and room temperature for 10 mins each respectively and centrifuged in a microfuge at 10,000rpm for 5 mins.
3. The supernatant was deproteinized using 100µl of ice-cold 5-SSA with intermittent shaking.

4. The tubes were kept in ice for 10-15 mins and the acid precipitable proteins were removed by centrifuging at 10,000rpm at 4°C fro 15mins in a Beckman J2-HS. centrifuge. The supernatant was immediately used for GSH determination by the method of Akerboom and Sies *et al.* (1981).

Advantages of the processing method:

- Acid precipitation of the samples maintains proper thiol-disulfide redox status (GSH oxidizes rapidly at pH values above 7.0) Acidification allows for the precipitation of proteins and ensures cell lysis and subsequent release of free thiols and disylfides. It also avoids GGT catalyzed degradation of GSH.
- 5-SSA was preferable for deproteinization because TCA, perchloric acid and metaphosphoric acids do not maintain true GSH:GSSG ratios and also these acids interfere with subsequent enzymatic reactions.
- Chelating agents such as EDTA were used in the sample buffer so as to prevent iron-mediated formation of peroxides in the presence of oxygen.

GSH estimation:

1. To 1ml of the buffer taken in a cuvette 50µl of the sample suspension, 50µl of NADPH, 20µl on DTNB and 20µl of GR were added. GR was added to initiate the assay.

2. The contents were mixed and the formation of TNB was followed continuously with a record for a total of 5mins. And 412nm in a Beckman DU–640 spectrophotometer.

3. The amount of GSH was calculated from the standard curve where the GSH equivalents were plotted against the rate of change of absorbancy at 412nm.

Standard curve was prepared from a stock solution of 10mM GSH in 5-SSA diluted to 100-1000nmol GSH. A sample blank lacking GSH was used to determine the background rate.

All values reported in GSH equivalents as µM/1x10^6 cells in all cases.

II. Reagents for the in vitro study:

- Bleomycin sulphate (Biochem Pharmaceuticals, India). A working solution of 75 µg/ml was prepared in RPMI 1640, and from there 20µg was added per ml blood in each culture.

- GSH monoethyl ester (Sigma, USA)-This was used at a concentration of 15mM in each culture when required.
- Buthionine sulfoximine (Sigma, USA) Used at a concentration of 5mM.
- 5-Bromo-2'-deoxyuridine (Sigma, USA) – This was dissolved in RPMI 1640 to prepare a working solution of 100 µg/ml. BrdU was added to all the cultures at a concentration of 6 µg/ml.
- Phytohaemagglutinine M (Gibco, USA)
- RPMI 1640 (Hyclone, USA) with antibiotics Penicillin and Streptomycin (Gibco, USA) and amphotericin B added to it.
- Heat inactivated Newborn Calf Serum (Hyclone, USA)
- Bis-Benzimide (Hoechst 33258)-A working solution of 50 µg/ml was prepared in double distilled water.
- Colchicine (Sigma, USA)- A working solution was prepared at a concentration of 100 µg/ml in RPMI 1640.
- Giemsa 3% (Merck, Germany)

All other chemicals used were of analytical grade.

Treatment with Bleomycin

For all the studies mentioned here, BLM was used at a concentration of 20 µg/ml blood. The stock solution of BLM was prepared at a concentration of 3 mg/ml, and from there a working solution was prepared with a concentration of 75 µg/ml. Whenever BLM was added to blood, it was given for a period of 3hrs. During this period of time, the treated blood was kept in an incubator at 37°C. After this time it was washed off from the system by the addition of pre-warmed RPMI 1640. Whenever BLM was given in combination with any of the chemicals used here or radiation, the total duration of treatment with BLM was retained at 3hrs. Whenever GSH ester was used, it was added to the cultures 30mins before the administration of BLM in all cases and the blood was incubated at 37°C. GSH-ester was given at a concentration of 15mM and just before addition to the cultures; it was weighed and dissolved in a few drops of RPMI 1640. In case of BSO-treatment BSO was added directly to the blood after weighing. BSO was added to the blood 5hrs prior to irradiation or 2hrs prior to addition of BLM. The blood was incubated at 37°C after that.

Treatment with radiation

The doses used for this particular investigation in all cases were 2 and 4Gy. The dose rates used here were 0.2, 0.68 and 6 Gy min^{-1}. The irradiations at 0.2Gy min^{-1} were given from X-ray machine (Faxitron, 43855D) and the irradiations at the dose rate of 0.68 and 6Gy min^{-1} was done from a ^{60}Co source. 1ml of the blood was taken and placed in a 25ml flat-bottomed sterilized beaker for irradiation whenever required. All cultures were set up after 1hr of the completion of irradiation. When apoptotic cell death was evaluated or total cell death was studied by the trypan blue dye exclusion test, the dose-rates used were 0.2 and 3 Gy min^{-1}, and in this case irradiation was done with X-rays. A single dose was used for these two studies, and that is 4Gy. Irradiations were always done 2hrs after the administration of BLM or 5hrs after BSO treatment or 30 mins after GSH-ester addition.

Culture Procedure for aberration study

One hour after irradiation treatment, the cultures were set by the addition of 0.2ml PHA per ml of blood, followed by RPMI 1640 supplemented with 10% heat inactivated newborn calf serum. BrdU was added to each culture at a concentration of 6μg/ml for the differential staining. All the cultures were then incubated at 37°C. In case of BLM-treatment the treated samples were washed with prewarmed RPMI 1640 and then the culture were set up. After every 12hrs, the cultures were gently shaken and they were harvested at 52hrs. 3hrs prior to harvesting, colchicine was added to each culture at a concentration of 0.01μg/ml. All experiments were repeated at least three times with the exception of one.

Preparation of metaphases and differential staining

The preparations of the metaphases and differential staining were done in the same procedure as mentioned in the chapter I.

Preparation of cells for study of apoptotic cell death:

The lymphocytes were first separated out from the whole blood in Ficoll Hypaque as mentioned in the previous chapter. BLM was given where required, and 2hrs after BLM treatment, irradiations of 4Gy at LDR (0.2Gy min^{-1}) and HDR (3Gy min^{-1}) were done and the cultures were set with RPMI 1640 containing antibiotics, supplemented with 10% serum as mentioned in Chapter I. When BSO was added, it was administered 2hrs prior to BLM treatment. Samplings were done at 24 and 48hrs and they were further processed and stained in accordance with the protocol of Vral *et al.*(1992) as outlined in Chapter I.

The slides were studied under the light microscope, and the cells with pyknotic nuclei were scored as apoptotic as against the normal cells with dispersed chromatin.

Trypan blue dye Exclusion test:

The fact that viable cells do not take up certain dyes whereas dead cells do has been used here to determine the cell death that is caused by the treatments given by BLM and radiation at LDR and HDR alone and in combination. The cell deaths were determined 24 and 48hrs after the treatments were given. The lymphocytes were first separated out in Ficoll Hypaque as mentioned above, and then BLM was given for three hours. After that time period when radiation was given in combination with BLM, it was given 2hrs after administration of BLM. Samplings were done 24 and 48 hours after the treatments were over. The dose rates used for this study were 0.2 and 3Gymin^{-1}. Only one dose was used for this study and that is 4Gy.

Scoring and statistical analysis

Slides were coded at random. 100 well spread metaphase plates were studied in most cases except in a few cases. The aberrations were all scored from metaphases in the first division cycle in accordance with the guidelines of the IAEA. For the cell cycle kinetics, metaphases were categorized into 1st, 2nd or subsequent cycles based on their differential staining pattern. The aberrations that were studied in this case were the exchanges that included the dicentrics as well as the rings, chromatid breaks, and deletions. Aberrant metaphase percentages were also determined, and cell cycle delay was studied from the percentage of M1s scored.

To determine the statistical significance of the difference in the treatments given with respect to the controls, for the aberrant metaphases the 2 x 2 χ^2–contingency test was used. For each of the types of aberrations studied, the statistical significance of the differences between the treatments given in comparison to the respective controls was tested with the help of the simple χ^2 – test. The values obtained for the M1% were tested by the 2 x 2 χ^2- contingency test.

The apoptotic cell death was scored by studying the pyknotic cells under the light microscope as mentioned in the first chapter. For the statistical testing of the data obtained from the study of the apoptotic cell death, the 2x2 χ^2-contingency test was used.

The trypan blue dye exclusion test gives an idea regarding the total cell death that takes place within the system after the treatments were given. The data obtained from

these experiments were tested statistically for the difference between the control and treated groups with the help of the χ^2-contingency test.

The statistical difference between the levels of tGSH in normal and BSO treated cells was tested using the Student's t-test.

Reagents for Western Blot analysis:

- BLM (Biochem Pharmaceuticals Pvt. Ltd, India)
- RIPA (Radioimmuno Precipitation Assay) buffer. The constituents are:
  - 1%(w/v) Nonidet P-40 (Sigma, USA)
  - 1%(w/v) Sodium deoxucholate (Sigma, USA)
  - 0.1%(w/v) SDS (Sigma, USA)
  - 0.15M NaCl (Merck, India)
  - 0.01M Sodium phosphate, pH 7.2 (Merck, India)
  - 2mM EDTA (Sigma, USA)
  - 50mM Sodium fluoride (Sigma, USA)
  - 100U/ml aprotinin (trasylol, Pentex/Miles) (Sigma, USA)
- DNAse (Stratagene, USA). The concentration of the working solution was 10 U/ml.
- Genei Protein Estimation kit by bicinchoninic acid method (BCA method) (Bangalore Genei, India)
- SDS polyacrylamide gel kit (Bangalore Genei, India)
- 10% SDS Resolving gel, ready mix for SDS-PAGE (Bangalore Genei, India)
- 5% SDS Stacking gel ready-mix for SDS-PAGE (Bangalore Genei, India)
- Ammonium presulphate for SDS PAGE (Bangalore Genei, India)
- Tris Glycine buffer, 10X (bangalore Genei, India). This was diluted to 1X before use.
- Sample buffer (5X) (Bangalore Genei, India)
- Protein Molecular Weight Marker (Fermentas, USA)
- Transfer buffer (pH 8.3). The stock solution of this constitutes of:
  - Tris base (Merck, India)-18.2 gms, Glycine (Merck, India)- 90gms and *Distilled water-500ml.*

The working solution of the transfer buffer is prepared by mixing 40ml of the stock with 200ml methanol (SD Fine Chemicals Pvt. Ltd) and making up the volume to 1litre with distilled water.

- TBST. This is prepared from the following constituents:
  - 1M Tris HCl, pH 7.4 (SRL, India)-3.152gms
  - 5M NaCl (Merck, India) –7.5972gms
  - Tween 20 (Bangalore Genei, India)-2ml

These chemicals are mixed together and the volume is made up to 100ml to make a 10X stock solution. The working solution at a dilution of 1X is prepared from this stock.

- Blocking buffer-5gms Non-Fat Dried Milk (NFDM) dissolved in 100ml 1X TBST.
- *Primary antibodies:*
  - p53-Abs (Clone DO7 + Bp53-12) (Neomarker, USA)
  - Bax Ab-5 (Clone 2C8) (Neomarker, USA)
  - Ku-70 Ab-4 (N3H10)
  - Rad-51 Ab-1 (Clone 51RAD01)
  - (Pan) Actin Ab-5 (Clone ACTN05)

All primary antibodies were used at a dilution of 1:1000 except for actin which was used at 1:5000.

- Secondary antibody: Rabbit Anti mouse IgG-ALP Conjugate (Bangalore Genei, India). This was used at a dilution of 1:2000
- Substrate for Alkaline Phosphatase BCIP/NBT (BCIP: 5-bromo-4-Chloro-3-Indolyl phosphate, NBT: Nitro Blue Tetrazolium)

Treatments:

The lymphocytes were separated out first in Ficoll Hypaque by the technique of Boyum *et al* (1968) as mentioned already, before any treatment was done. To each culture where BLM treatment was given, 20µg of BLM was added. After two hours, irradiation at LDR (0.2Gy/min) and at HDR (3Gy/min) at a dose of 4Gy when the expressions of Ku-70 and Rad51 were determined, and 5Gy when the p53 and Bax levels were evaluated. One hour after irradiation, cultures were set in the presence of RPMI 1640 supplemented with 10% serum without PHA. Irradiation was done in sterilized

culture tubes made of polypropylene (Tarson, India). Samplings were done at 6hrs when the Ku-70 and Rad51 were evaluated whereas sampling was done at 24hrs when the levels of p53 and Bax were determined.

Protein extraction for Western Blotting:

- The cells were centrifuged after the respective hours after treatment at 1500rpm for 5mins.
- The supernatant was discarded thoroughly and to the pellet, 200µl of RIPA was added. This was vortexed well and kept in ice for 10 mins.
- 5 µl of DNAse (10U/ml) was added to each of these mixtures and the samples were kept in ice for half an hour and were stored at -80°C.

Protein Estimation:

- To 10µl of each of the samples, 90µl of autoclaved distilled water was added.
- 2ml of the BCA (BCA solution: $CuSO_4$::49:1) reagent was added to each and they were incubated at 37°C in a water bath for 30mins.
- Standards were prepared for which 5-80 µg of Bovine Serum Albumin (BSA) were used. For each concentration, the volume was made up to 100 µl.
- 2ml of BCA reagent was added to each and each was incubated at 37°C in a water bath for 30mins.
- The optical density of the samples and standards were taken at 562nm and the concentrations were calculated form the standard graph.

Immunoblotting:

Equal amounts of protein (95 µg) were loaded in each lane. These were separated by SDS-PAGE using 12% polyacrylamide resolving gel and 5% stacking gel at 50V for 2hrs. The proteins were transferred in the presence onto a 0.45 µm nitrocellulose at 50V over a period of 7hrs in ice. The membranes were blocked at room temperature in blocking buffer for 2hrs followed by primary antibody treatment over a period of 2hrs. this was followed by washing in NFDM three times for 10mins each followed by the treatment with the secondary antibody. The primary as well as the secondary antibodies were given dissolved in NFDM. The primary antibody was used at a dilution of 1:1000 while the secondary antibody was diluted to 1:2000. The nitrocellulose was incubated for one and a half hours in the secondary antibody, which is conjugated to alkaline phosphatase, and

was followed by washing with TBST three times for 10mins each. The substrate BCIP/NBT was then added which reacts with the ALP and is converted to a dense blue precipitate in the area of the bands. The bands are determined by their position against the molecular weight marker. To determine the equal loading of the proteins, the equal *expression of the β-actin was also determined.*

Results

Effect of BLM and Radiation alone and in combination on CAs

Induction of CAs by radiation at two different dose rates, 0.68 and 6.0 Gy min^{-1} and bleomycin (BLM) alone and in combination in human PBLs were studied and the data are presented individually in the table 2.1 and 2.2 and in pooled form in 2.3. In the table 2.1 the data are presented from 6 donors, out of which 4Gy both HDR and LDR were treated in 3 donors. Treatment with GSH-ester alongwith radiation and BLM was also given in 3 cases. Microphotographs of some aberrations obtained by treatment with BLM alone are shown in figure 2.1. Figures 2.2 a and b show the aberrations obtained when BLM was given in combination with 4Gy irradiation at LDR.

Bleomycin and X-ray-induced CAs and the M1 in Go human PBL were studied as positive control to BLM + X-ray treated samples, and the data are presened in Table 2.1. Deletion and exchanges were the most frequent type of aberrations and increased most consistently at HDR than LDR of radiation exposure. The frequency of aberrant metaphases increased significantly at higher dose-rate of 2Gy and such increment was clear at 4Gy as well. The frequency of aberrations induced by BLM was fairly consistent and it shows similar level of aberrations induced by LDR of radiation at 2Gy. However, when BLM and X-rays (2Gy) were given in combination, a significant increase in the frequency of aberrations was observed compared with the additive effects of the 2 given separately [for deletion: BLM + X-rays (0.68 Gy min^{-1}) %, 122 ± 28; additive yields %, 21± 4]. Similar pattern of increase was also observed in the frequency of aberrations, except exchanges, while BLM was combined with the HDR exposure at 2Gy. The increase in the frequency of exchanges was not consistent with BLM and HDR at 2Gy, however, such enhancement is consistent with BLM and LDR at 2Gy. The degree of enhancement in the frequency of aberrant metaphases induced by 2Gy was more when BLM and low dose-

rate of radiation (33% to 61%) were given in combination than BLM and high dose-rate (54% to 68%) combination.

At 4 Gy of X-rays exposure the frequency of deletions and exchanges were significantly increased at HDR than at LDR. While BLM combined with low dose-rate of X-rays (4Gy) a significant increase in the frequency of deletions and exchanges were observed (Fig. 2.1) in all the individual cultures compared with the additive effects of the 2 given separately [for deletion: BLM + X-rays (0.68 Gy min^{-1})%, 134±7; additive yields %, 74±5]. Surprisingly when BLM and 4Gy at HDR were given in combination there was a significant reduction of deletions and exchanges although there was a mild increase in the frequency of aberrant metaphases. This was observed consistently in all the three donors (Table 2.1). In order to see whether this difference in induction of aberrations by BLM + 4Gy is due to different dose-rate of radiation we had exposed two more blood samples at further lower dose-rate (0.2 Gy min^{-1}) and observed further increase in the frequency of exchange aberrations when 4Gy (0.2 Gy min^{-1}) were given in combination with BLM (Table 2.2). This combination showed increased in the frequency of chromatid breaks significantly while the frequency of deletions was reduced.

The data indicate that BLM treatment did not increase the frequency of M1 cells whereas the radiation induced increase in the frequency of M1 cells are clear. Radiation at HDR showed higher frequency of M1 than LDR radiation. The combined treatment of BLM and radiation mostly showed marginal increase in the frequency of M1 cells.

These results show that BLM-induced damage can interact better with low dose-rate radiation induced damage to produce chromosome aberrations particularly exchange aberrations.

Influence of GSH-ester and BSO on BLM+X-ray induced CAs

Table 2.1 represents the data of individual experiment where GSH-ester was added in three samples (donor no. 4, 5 and 6). GSH-ester showed a positive tendency of potentiation to BLM-induced CAs particularly deletions and chromatid breaks. When GSH-ester was added before BLM + low dose-rate X-ray combined treatment the significant elevation on the frequency of aberrant metaphases, exchanges and chromatid breaks was observed in all the three samples, however, such elevation was not there when high dose-rate of X-ray was combined with BLM. In fact the frequency of aberrant metaphases and all types of CAs were reduced while high dose-rate of X-rays was combined with BLM.

Contrary to the anticipated sensitizing effect of GSH-depletion by BSO, a tendency of reduction in the frequency of aberrations and aberrant metaphases induced by BLM was observed in both the samples (Table 2.2). However, BSO-treatment before 4 Gy irradiation showed significant increase in the frequency of deletions whereas there was reduction in the frequency of exchanges. When BLM and X-rays (4 Gy; 0.68 Gy min^{-1}) were given in combination to BSO-treated samples, a highly significant increase in the frequency of deletions and drastic reduction in the frequency of exchange aberrations were observed compared to BLM + 4 Gy combined treatment to normal cells. Similar trend was observed in both the individual samples (Table 2.2). The data obtained for the exchange aberrations obtained when BLM was given in combination with radiation in the presence of normal and exogenously added GSH and also when GSH is depleted, is presented in figure 2.3.

Level of reduced GSH

Levels of reduced GSH in human PBLs with or without BSO are shown in Table 2.4. and figure 2.4 The concenration of GSH in HPBLs showed a range between 3.67 to 6.55 μmol in 10^6 cells with an average of 5.61 ± 0.56 μmol in 10^6 cells in normal blood lymphocytes. This GSH concentration was depleted to 85% of the control value after 5h treatment with BSO. The statistical difference between the mean GSH concentrations of these 2 groups was significant.

Cell death by trypan blue

The data obtained from the trypan-blue dye exclusion study are presented individually in table 2.5 and as pooled form in Table 2.6. The individual data showed that both BLM and 4Gy killing the cells which was more at 48h than at 2 and 24h after treatment. BLM alone killing slightly higher number of cells than 4Gy at LDR. However, the frequency of cell death was more with 4Gy at HDR than LDR. The combination of radiation with BLM always showed higher cell death than radiation and BLM alone and at 48h it showed more than 2 and 24h.

Apoptotic cell death:

The data obtained from the study of the apoptotic cell death are presented individually in table 2.7 and as pooled form in Table 2.8. Microphotograph of the cells with pycnotic nuclei is shown in figure 2.2. The individual data showed that both BLM and 4Gy at LDR induced almost similar level of apoptotic cells both at 24 and 48h, however, pooled

data showed that 4Gy induced marginally more apoptotic cells than BLM. The induction of apoptotic cell death was significantly higher when samples were irradiated with 4Gy at HDR. The combination of radiation with BLM always showed higher induction of apoptosis than radiation and BLM alone and at 48h it showed more than 24h. Interestingly, such combined treatment when given to BSO-treated cells there was an appreciable reduction in the apoptotic cells. Moreover, treatment of BLM to BSO-treated cells also showed less frequency of apoptosis than BLM alone.

Western blot for Ku-70, Rad 51, p53, and Bax protein expression:

The results obtained regarding the level of p53 and bax protein are presented in fig. 2.5 and for the level of Ku-70 and Rad 51 are presented in Fig 2.6.

It shows higher expression of Bax after 24h in only irradiated sample whereas in BLM-treated sample the level was lower than untreated control. The similar pattern shows in both the samples. However, in combined treatment the level of Bax is more than untreated control but lower than only irradiated sample. The level of p53 after 24h of treatment shows similar to untreated control level although the level shows further lower in only BLM-treated sample.

Regarding the level of Ku-70 both the samples show higher level of Ku-70 protein in BLM+LDR radiation combined treated lane than radiation alone and BLM-alone treated samples. However, for Rad 51 the level was very low in all the protein samples.

Fig 2.1 A & B: Microphotographs of aberrant metaphase plate in HPBLS showing deletions (dl) dicentric(d) and tricentric(t) induced by the combined treatment of BLM and LDR irradiation.

d
t
a

d
t
d
b

Fig 2.2: Microphotograph of apoptotic cell death in HPBLs (→) in response to BLM and radiation.

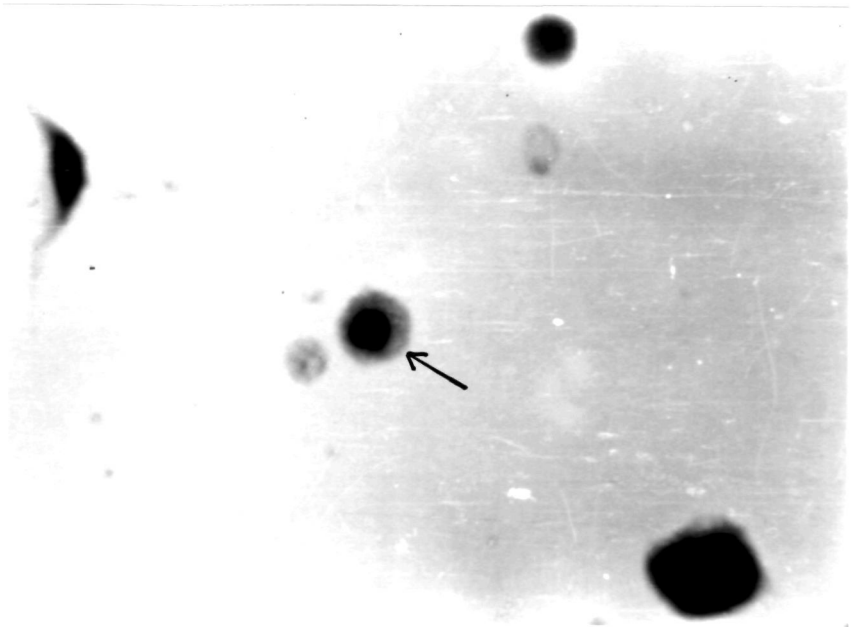


Table 2.1: Induction of CAs by BLM or radiation (with different dose-rate) alone or in combination in HPBLs

Donor No.	Exptl. Condition	TM_1	M1%	TM_2	Aberrant M %	Exchanges ±F %	Chtd. bk %	Deletion %
1	Untreated	088	86	074	2	0	1	1
	BLM	152	88	132	39	4	8	25
	2Gy (0.68)	133	91	115	32	10	5	28
	BLM+2Gy	117	96	108	59*	14	7	92#
	2Gy (6)	133	97	121	54	20	11	21
	BLM+2Gy	109	99	103	67*	16	13	76#
2	Untreated	181	87	124	3	0	2	1
	BLM	136	89	122	38	9	11	12
	2Gy (0.68)	120	92	108	31	14	3	20
	BLM+2Gy	135	94	124	63*	17	10	140#
	2Gy (6)	129	97	115	58	22	8	21
	BLM+2Gy	137	99	126	70	22	18	131#
3	Untreated	134	80	123	3	0	2	1
	BLM	127	82	113	37	6	13	9
	2Gy (0.68)	142	83	133	33	14	9	14
	BLM+2Gy	147	88	133	60*	20	16	146#
	2Gy (6)	122	86	112	54	17	11	18
	BLM+2Gy	139	91	129	72$	24	21	172#
	4Gy (0.68)	141	87	126	87	36	7	59
	BLM+4Gy	132	100	109	82	178#	18	170
	4Gy (6)	149	90	130	88	60	11	95
	BLM+4Gy	130	95	119	97	29#	6	55@
4	Untreated	210	79	185	3	0	1	2
	BLM	153	77	130	21	7	14	7
	2Gy (0.68)	161	81	127	37	12	9	10
	E+BLM+2Gy	139	88	118	87*	72#	32	97#
	2Gy (6)	132	89	109	52	15	18	18
	E+BLM+2Gy	163	94	126	50	21	4	27
	4Gy (0.68)	172	87	143	81	39	5	77
	BLM+4Gy	133	92	121	75	68#	0	112
	4Gy (6)	097	93	087	89	65	19	85
	BLM+4Gy	124	72	104	88	36#	6	76#
5	Untreated	127	76	112	2	0	2	0
	BLM	110	76	087	33	6	12	14
	E+BLM	122	81	091	36	4	27	19
	2Gy (0.68)	109	80	079	32	10	6	12
	BLM+2Gy	111	81	088	53$	22	13	82#
	E+ BLM+2Gy	125	83	101	81*	60#	29	94
	2Gy (6)	097	84	073	49	12	15	22
	BLM+2Gy	117	88	082	68$	23	23	158
	E+BLM+2Gy	126	89	096	43	15	3	28
6	Untreated	228	84	199	2	0	2	1
	BLM	213	84	183	33	6	10	12
	E+BLM	173	87	151	18	3	22	16
	2Gy(0.68)	169	87	147	35	12	8	12
	E+BLM+ 2Gy	143	94	121	89*	62#	25	60#
	2Gy (6)	162	93	133	54	14	13	22
	E+BLM+2Gy	151	96	137	48	21	3	25

	4Gy (0.68)	227	94	201	84	35	7	57
	BLM+4Gy	150	100	129	81	124#	7	120#
	4Gy (6)	147	97	122	88	67	15	78
	BLM+4Gy	161	99	139	94	36#	9	52#

TM_1 = Total metaphases scored for M_1%; M_1% = First division cycle; TM_2 = Total metaphases scored for aberration study; Aberrant M = Aberrant metaphases; F = Fragments; Chtd. Bk =Chromatid breaks.
* Significant at $p<0.01$ 2x2 χ^2-contingency test; $ Borderline of $p<0.05$ 2x2 χ^2-contingency test.
# significant at $p<0.01$ simple χ^2- test; @ borderline of $p<0.01$ 01 simple χ^2- test.

Table 2.2. Effect of BSO on the combined treatment of BLM and radiation at different dose-rates on HPBLs.

Donor	Exptal.	TM	M1%	TM_1	Abt. M%	Aberrations(%)		
No.	System					Exchs.	Chtd.Bk	Deln.
1	Untreated	276	74	199	02	0	2	1
	BLM	262	74	183	36	5	5	13
	4Gy (0.2)	105	87	081	67	31	19	100
	BLM+4Gy	136	94	106	78	222#	56	60#
	4Gy (0.68)	226	100	222	84	39	8	65
	BLM+4Gy	159	94	139	79	134#	7	106
	BSO+ 4Gy (0.68)	124	98	104	92	32	18	100
	BSO+BLM	177	76	147	10	3	4	10
	BSO+BLM+4Gy	097	100*	090	100*	29#	26	220#
2	Untreated	277	74	199	2	2	3	1
	BLM	238	72	152	34	6	5	8
	4Gy (0.2)	133	98	105	72	32	21	94
	BLM+4Gy	142	99	125	84	221#	51	82#
	4Gy (0.68)	147	96	117	81	37	9	67
	BLM+4Gy	153	93	132	82	131#	5	85#
	BSO+ 4Gy (0.68)	138	94	111	77	23	13	107
	BSO+BLM	177	74	128	8	4	3	8
	BSO+BLM+4Gy	111	100*	103	100*	32#	30	203#

TM_1 = Total metaphases scored for M_1%; M_1% = First division cycle; TM_2 = Total metaphases scored for aberration study; Aberrant M = Aberrant metaphases; F = Fragments; Chtd. Bk =Chromatid breaks; exch = Exchanges; deln = Deletions.
* Significant at $p<0.01$ 2x2 χ^2-contingency test; $ Borderline of $p<0.05$ 2x2 χ^2-contingency test.
# significant at $p<0.01$ simple χ^2- test; @ borderline of $p<0.01$ 01 simple χ^2- test.

Table 2.3. Pooled data for combined effect of BLM and radiation dose-rate variation in HPBLs.

Exptal. Condtns.	TM	AbM%	Exch(%)	CB(%)	Del(%)
Untreated	1215	2.38±0.18	0±0	01.75±0.16	1.00±0.19
BLM	1102	33.88±2.00	06.13±0.52	09.50±1.37	10.25±1.99
2Gy(0.2)	304	45.50±0.5	22.50±0.50	11.50±0.50	26.00±4.00
BLM+2Gy(0.2)	276	51.00±0.00	27.50±3.48	16.00±2.00	23.00±1.00
2Gy(0.68)	709	33.30±0.91	12.00±0.73	06.67±0.99	10.67±2.89
BLM+2Gy(0.68)	365	60.67±1.20*	19.30±2.38	11.00±2.65	115.00±17.24
E+B+2Gy(0.68)	340	85.67±2.40*	65.00±1.51#	28.67±2.03#	84.0±12.45#
2Gy(6)	206	53.5.0±1.20	18.83±2.40	12.67±1.43	20.17±1.11
BLM+2Gy(6)	358	68.3.0±2.73*	23.67±2.00	17.33±2.33	134.0±28.57#
E+BLM+2Gy(6)	359	47.00±2.08*	19.00±0.58	03.33±0.33#	27.00±3.46
4Gy(0.2)	186	69.5.0±2.5	31.50±0.50	20.00±1.00	97.00±3.00
BLM+4Gy(0.2)	231	81.00±3.00*	224.5±0.50#	53.50±2.50#	46.00±4.00#
4Gy(0.68)	809	83.4.0±1.12	38.80±26.43	07.20±0.66	64.00±4.06
BLM+4Gy(0.68)	623	80.20±1.53	145.8±26.4#	16.60±9.07#	134.00±6.55#
4Gy(6)	339	88.3±0.33	67.30±2.19	15.00±2.31	86.00±9.59
BLM+4Gy(6)	362	93.00±2.64	37.67±4.33#	07.00±1.00	61.00±7.42#
BSO+BLM	275	9.00±1.00	05.50±0.5	03.50±0.50	02.50±2.50
BSO+4Gy(0.68)	215	84.50±7.5	37.00±12.0	15.50±2.50	88.00±19.00
BSO+BLM+4Gy(0.68)	193	100±0.00*	30.50±1.5	28.00±2.00#	211.5±8.50#

The numbers in parenthesis indicate the dose rate in Gy min^{-1}. TM_1 = Total metaphases scored; Abt M = Aberrant metaphases; CB =Chromatid breaks; Exch = Exchanges; Del = Deletions.

* Significant at $p<0.01$ 2x2 χ^2-contingency test; # significant at $p<0.01$ simple χ^2- test;.

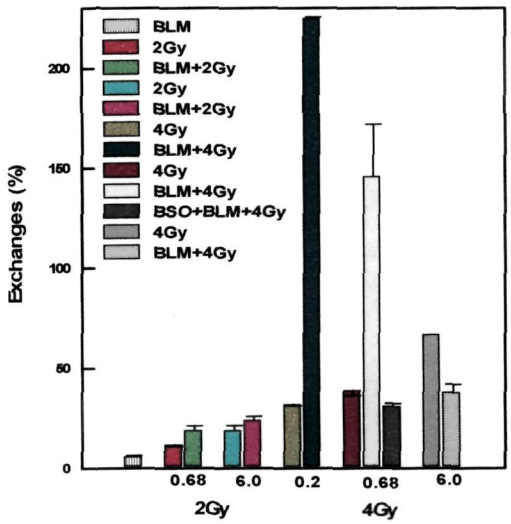


Fig. 2.3 **Induction of exchanges induced by BLM and radiation at high and low dose-rates and the influence of GSH in HPBLs.**

Ls 5h after a single treatment of BSO

		Total GSH (μM 10^{-6} cells	Mean ± SEM (increment%)
		6.55	
		3.67	
		6.44	5.61 ± 0.56
		5.57	
5.	0	5.04	
6.	0	6.41	
7.	5	0.85	
8.	5	0.78	0.86 ± 0.05*
9.	5	0.98	(- 85%)
10.	5	0.79	
11.	5	0.92	

* $p<0.05$ a student's t-test

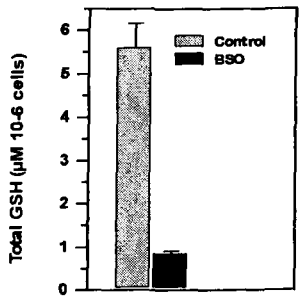


Fig. 2.4 Level of tGSH in normal and BSO (5mM) treated HPBLs.

Table 2.5 Individual data on trypan blue test in HPBLs after treatment with BLM(20µg/ml) and 4Gy irradiation at different dose-rates.

Treatment	%cells dead 24H	%cells dead 48H
C	2.03	5.85
	3.22	6.93
BLM	6.195	9.16
	7.83	10.98
4(0.2)	4.22	6.18
	5.14	7.77
BLM+4(0.2)	9.8	15.3
	11	17.6
4(3)	10.94	10.86
	11.59	11.86
BLM+4(3)	12.34	22.89
	13.66	22.62

Table2.6 Pooled data for percentage of dead cells after treatment with BLM(20µg/ml) and 4Gy irradiation at different dose-rates.

Treatment	Dead cells (%)	
	24hrs	48hrs
C	3 ± 0.6	6 ± 0.5
BLM	7 ± 0.8	10 ± 0.9
4Gy(0.2)	5 ± 0.5	7 ± 0.8
BLM+4Gy(0.2)	10 ± 0.6	$17 \pm 1.2^{*}$
4Gy(3)	$11 \pm 0.3^{*}$	11 ± 0.5
BLM+4Gy(3)	$13 \pm 0.7^{*}$	$23 \pm 0.1^{*}$

*Significant at $p<0.05$ χ^2-contingency test as compared to control.

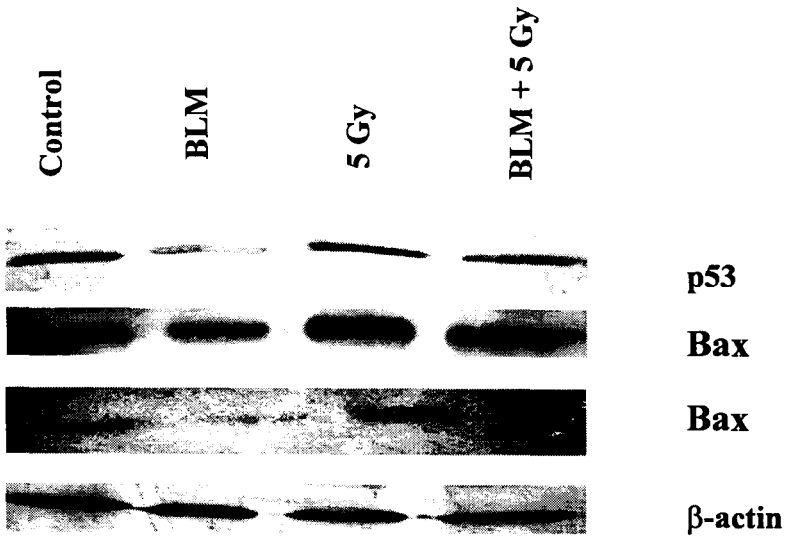
Table 2.7. Individual data on apoptotic cell death in HPBLs in response to BLM treatment at different dose rates of irradiation.

Treatment (dose-rate)	Apoptotic cell death (%) (cells scored)	
	24H	48H
Untreated	18.5(1121)	22.3 (1015)
	17.6(1353)	21.3(1234)
BLM	30.7(1006)	40.1(1212)
	33(1115)	41.6(1368)
4 Gy (0.2)	32.2(1264)	43.6(1341)
	34.6(1124)	46.7(1017)
BLM+4Gy (0.2)	42.7(1234)	49.2(1435)
	44.3(1323)	51.1(1257)
4Gy (3)	51.3(1281)	63.8(1213)
	53.8(1245)	65(1267)
BLM+4Gy (3)	60.2(1359)	72.5(1324)
	62.8(1219)	75.4(1222)
BSO+BLM	22.6(1520)	23.3(1420)
	17.7(1301)	24.5(1139)
BSO+BLM+4Gy (0.2)	37.8(1256)	48.7(1328)
	41.6(1127)	51.1(1148)
BSO+BLM+4Gy (3)	55.4(1344)	67.6(1152)
	58.2(1146)	68.2(1257)

Table 2.8. Apoptotic cell death in response to BLM treatment and irradiation at different dose-rates in HPBLs.

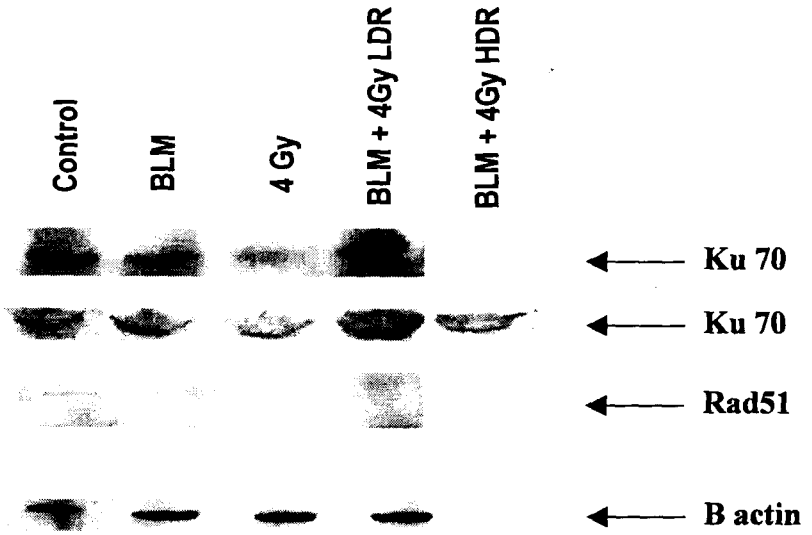
Treatment	Apoptotic cell death (%)	
	24hrs	48hrs
Untreated	18.1 ± 0.5	21.8 ± 0.5
BLM	31.9 ± 1.2*	40.9 ± 0.8*
4(0.2)	33.4 ±1.2	45.2 ± 1.6
BLM+4(0.2)	43.5 ± 0.8*	50.2 ± 0.9
4(3)	52.6 ± 1.3	64.4 ± 0.6
BLM+4(3)	61.5 ± 1.3*	73.9 ± 1.5*
BSO+BLM	20.2 ± 2.5	23.9 ± 0.6
BSO+BLM+4(0.2)	39.7 ± 1.9*	49.9 ± 1.2*
BSO+BLM+4(3)	56.8 ± 1.4*	67.9 ± 0.3*

The numbers in parenthesis indicate the dose-rate in Gy min^{-1}
*Significant at $p<0.05$ χ^2-contingency test.



Level of p53 and Bax in human PBL (24h)

Fig. 2.5



The level of Ku 70 and Rad 51 in HPBLs after treatment with either radiation or BLM or in combination (6H)

Fig. 2.6

Discussion

In this study BSO was used to evaluate the effect of GSH depletion on DNA DSB ends joining in HPBLs after exposing the cells to BLM and X-rays. An exchange aberration is thought to arise as a consequence of illegitimate reunion ('misrejoining') of free ends from different DNA DSB (Cornforth and Bedford, 1993). The incidence of dicentric chromosome aberrations in HPBLs has been proven to be a sensitive indicator of radiation damage because of its high frequency relative to other types of radiation induced aberrations and its low natural incidence (Lloyd *et al* 1986). Therefore, in the present study we have scored different CAs including exchanges which gave an indication of the phenomenon of DSB-misrejoining. In the previous chapter, our results indicate that the presence of BSO increased the cellular radiosensitivity in human PBL and it could be due to depletion of endogenous GSH as it was shown earlier (Chattopadhyay *et al* 1999). The reduced-GSH estimation in this study indicate that 5h incubation with BSO (5 mM) could deplete GSH level significantly with respect to control.. The freshly drawn blood was incubated with BSO for 5h since in cultured cells more than 75% depletion was achieved within 4-5h duration by 500μm to 10mM of BSO (Shrieve *et al* 1985, Edgren and Revesz 1987). In this study the concentration of BSO used was 5mM since significant sensitization by BSO was observed at this higher concentration with respect to radiation-induced CAs. In accordance with the data reported by different authors (Virsik and Harder, 1980, Fabry, 1986) it was also found that LDR of radiation induced less CAs than HDR of radiation pointing of repair of sub-lethal damage during prolonged radiation exposure time at LDR. Although the range of dose-rate used in the present study (0.2 Gy/min to 6 Gy/min) was extremely narrower than the range used in other studies (0.0005 Gy / min to 0.4 Gy / min), nevertheless, higher induction of different aberrations by the higher dose-rate of radiation is clear and the sparing effect of LDR is evident in the previous chapter. It has been shown that fractionating a given X-ray dose significantly decreases the DSB misrejoining frequency and increases the probability of joining correctly (Lobrich *et al* ,2000). In another study (Rothkamm *et al,* 2001) it was shown that continuous LDR irradiation results in very efficient overall rejoining in MRC-5 cells. Therefore, it seems that in the present study correct joining of DNA DSBs took place during LDR-exposure and as a result of such DNA repair mechanism the number of exchange aberrations as well as deletions were reduced. However, this is not the case when radiation was given to BSO-treated cells. It was shown earlier that the sparing effect of LDR in BSO-treated cells was reduced at both 2 and 4Gy. This indicates that BSO might interfere the DNA-repair mechanism and therefore overall

DNA DSB-ends joining was affected. It was shown that there is sparing effect of LDR in normal cells and is absent in AT cells and has been interpreted as a repair-defect (Cox, 1982).

In order to see the role of endogenous GSH on joining of DNA DSBs we allow to interact DNA lesions induced by X-ray and BLM since the chemistry of the end of DNA strand breaks are similar (Henner *et al,* 1982, Bases *et al,* 1990). It has also been shown that BLM-induced DNA dsbs are rejoined in vitro with efficiency similar to that measured for radiation-induced DNA DSB (Cheong and Iliakis, 1997). In the present study it shows that the frequency of exchanges was increased substantially when BLM combined with LDR radiation. Similar significant increase in the frequency of exchanges and deletions was observed by Preston (1982) in HPBLs while BLM 15 μg/ml was combined with 1.5Gy X-ray at the dose-rate of 1Gy / min. Since the 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. However, such interactive effect between the lesions induced by BLM and HDR radiation was not seen. In the LDR experiments, only very few breaks are induced at any given time and such breaks which appeared very close to BLM-induced breaks could interact and form exchanges. In case of HDR radiation exposure multiple DSBs will appear at the same time and therefore the probability of misrejoing between the DSBs induced by X-ray and BLM should be enhanced. On the contrary in this study DSBs induced by HDR radiation exposure shows poor interaction to BLM-induced lesions. It is true that radiation induces a number of DNA lesions, like base damage and crosslinks in addition to DNA dsb and it is likely that a cell may encounter multiple lesions at any given time by HDR than LDR radiation exposure and therefore, the presence of multiple lesions of different types may affect directly or indirectly the chromatin structure which may subsequently affect the enzymatic activities involved in dsb rejoining. If this is the case then the frequency of deletion and chromatid break could have increased significantly but the frequency of both the aberrations was reduced in the present study. Therefore, it is difficult to understand why BLM in combination to HDR radiation induces less aberrations. However, it could be possible that a portion of free radicals out of large number of free radicals generated by HDR radiation could combine with the radicals generated due to presence of BLM and thus less number of free radicals interact with the target molecule DNA and induced less damage. Another possibility is that radiation exposure at HDR induces higher number of DNA strand-breaks at a given time and such treatment to BLM-pretreated cells could lead more intense toxic insult to the cell and thus

could elicit necrosis (Dypbukt *et al,* 1994, Bonfoco *et al,* 1995). It is known that in high doses of ionizing radiation and complex DNA damage as produced by high-LET radiation, an increased fraction of these cells will die by necrosis (Harms-Ringdahl *et al,* 1996). Moreover, BLM+HDR radiation treatment might also induces severe cell-membrane or mitochondrial damage which may lead to necrotic death (Szumiel, 1994). Therefore, such necrotic death of heavily damaged cells could eliminate from the cell population and showed less frequency of CA induction. This speculation also gets support from the present trypan-blue dye exclusion tests where it was shown that BLM in combination with radiation at HDR induced more cell death than BLM and LDR-radiation exposure. However, besides necrotic death the damage loaded cells might also die apoptotically. This is also evident from the present study since BLM + HDR-radiation exposure showed significantly higher apoptotic cell death at any given time than BLM+LDR radiation treatment.

It is likely that the cells loaded with high frequency of aberrations after BLM+LDR radiation treatment could have followed apoptotic death but such apoptotic response has been counteracted by the addition of mitogen (Holmberg *et al,* 1996) in the culture. However, this is also true for BLM+HDR radiation treated samples but it shows fewer aberrations than BLM+LDR radiation exposed cells. From the present trypan-blue dye exclusion study and the apoptotic cell death study it seems that at any given point more cell death occurred at BLM+HDR radiation treated samples and thus it shows less frequency of aberrations.

The interesting observation in the present study is the failure of the frequency of exchange aberrations by BLM and LDR radiation treatment in BSO-pretreated cells to increase in spite of significant enhancement of chromatid and deletion type of aberrations. It has been demonstrated that depletion of endogenous GSH by BSO reduced the clastogenic action of BLM (Chattopadhyay *et al,* 1997) and enhanced the clastogenic action of radiation (Chattopadhyay *et al,* 1999). The present data show that combination of BLM and LDR radiation induced higher frequency of aberrant metaphases and deletions in BSO-treated cells than BSO-untreated cells whereas in the later the frequency of exchanges was increased substantially. Therefore, BSO-mediated GSH depletion does not allow the interaction of DNA lesions induced by BLM and LDR radiation exposure. In an attempt to clarify the possible role of GSH in biochemical repair processes, the extent of rejoining of radiation induced single strand breaks (Ssbs) in oxic condition was determined upto 1h

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 hypoxically induced Ssds. The assumption that the presence of GSH has a role to play in interaction of DNA dsbs to form exchanges was strengthened by the observation of an increased frequency of exchange aberrations and decreased frequency of deletions in GSH/GSH-ester post-treated human PBL irradiated at 4°C (Chattopadhyay *et al* 1999). The present study has made another attempt by different approach to show the role of endogenous GSH in the interaction of dsbs induced by two different agents. The data indicate an efficient interaction between BLM-induced lesions and LDR radiation induced lesions as determined by the increased frequencies of exchanges obtained when the cells were treated with GSH-ester before BLM treatment and irradiation. 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 *et al* 1989, Chattopadhyay *et al,* 1998). Therefore, it seems that LDR radiation induced lesions interact efficiently with the increased BLM-induced lesions under the influence of increased endogenous GSH level.

The present observed consistent increase in the frequency of exchange aberrations in cells treated with BLM in combination with LDR irradiation could be due to the enhancement of the interaction between the BLM induced and radiation-induced dsbs. From the present data it is clear that a slower rate of irradiation is better for the misrejoining/misrepair to take place thereby giving rise to exchange aberrations. This process of repair of lesions within the cells may take place in a number of ways, but in quiescent mammalian cells, it has been reported to take place prevalently by the non homologous end joining process (NHEJ) (Roth and Wilson, 1986, Karran, 2000) NHEJ is regarded as the dominant mechanism for dsb repair in vertebrates, especially in Go and G1 phases of the cell cycle ((Khanna and Jackson 2001). This NHEJ involves DNA end-binding heterodimer Ku70 / Ku 80, the catalytic subunit of the DNA-PK, the XRCC4 gene product and DNA ligase IV (Karran 2000). The present result shows higher level of Ku 70 protein and extremely low level of Rad-51 in BLM+LDR-irradiated sample justifying its more involvement in NHEJ of DNA dsbs induced by BLM and LDR.

If the present DNA dsbs, induced by BLM and LDR-radiation combination, are the candidates for the NHEJ-repair pathway then how such extremely high frequency of

exchanges are formed would be one of the questions. In fact whether the NHEJ pathway is inherently error-prone under varying circumstances is unclear. Studies with mouse cells null for components of the NHEJ pathway suggest the importance of these proteins for protecting against genetic instability and tumorigenesis (Pierce and Jasin, 2001). On the other hand, 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* ,2000). In addition, studies of NHEJ activity in some of the inherited chromosomal instability syndromes that predispose to leukemia suggest that error-prone NHEJ may be induced by different routes (Lundberg *et al,* 2001,Gaymes *et al,* 2002). Therefore, in the present study it could be that the LDR radiation induced DNA dsbs that appeared slowly and very close to BLM-induced dsbs interact under the influence of NHEJ-repair machinery and formed chromosomal exchange aberrations.

Apoptosis is a term used to describe the terminal morphological and biochemical events seen in Programmed cell death (Wyllie, 1992). Apoptosis has recently been of great interest because it has been shown clearly to mediate cell death not only during the process of development, but also in neoplasia in response to chemo or radiotherapy (Hickman, 1992; Eastman, 1990). Increased interest in apoptosis is also because of the involvement of a large number of genes that can regulate the apoptotic process. In response to the induction of DNA damage by irradiation or by the treatments with chemotherapeutic drugs, one of the first proteins that get activated is the p53 (Levine, 1997). p53 is involved in several aspects of the cell cycle, apoptosis, control of genome integrity and DNA repair. The protein p53 influences cell proliferation by acting on both the G1 as well as the G2 phases of the cell cycle. p53 functions are mediated by direct interactions with other proteins or with the DNA (Kastan, 1991; Kuerbitz, 1992). Exposure to radiation leads to an increase in the levels of the proteins that derives from an alteration in its half life as a result of post translational modifications (Levine 1997; Ko and Prives, 1996). Similar observation was also obtained with exposure to BLM and other chemotherapeutic drugs (Lu and Lane 1993). When the expression of p53 was evaluated at 24hrs, it was seen that when BLM treatment was given the intensity of the band decreased. This could probably indicate that p53 is not being induced much. This result in fact can be correlated with the results obtained in this study that have clearly shown that BLM is a poor inducer of cell cycle delay in comparison to radiation. When 5Gy of

radiation was given, p53 is induced to an extent much more than BLM treated samples. When a combination of the drug and radiation is given, an intermediate intensity between the expressions of BLM alone or radiation alone is obtained.

One of two pathways of induction of apoptosis involves the mitochondrial pathway that is controlled by the Bcl-2 family of proteins. There are at least 15 members of this protein family, and these may either promote survival viz. Bcl-2, Bcl-X_L, or promote apoptosis viz. Bax, Bak. The pro and anti apoptotic family members are able to form heterodimers with each other and this has lead to the idea that their relative abundance may act as a rheostat controlling the mitochondrial initiation pathway. When the expression of Bax was studied, a pattern similar to that obtained for p53 was seen. When radiation was given alone, the expression of Bax was found to be more as compared to when BLM was given alone. The combined treatment showed an expression intermediate between radiation and BLM treated samples. Therefore, it seems that BLM induces apoptotic cell death through p53-independent pathway. It has been shown that the lung cells and small intestinal cells respond to the bleomycin treatment in different ways in terms of the induction of apoptosis and that p53 carries out an essential role in the early response to and repair of DNA damage by a non-apoptotic mechanism which appears to be crucial in the noncycling lung cells and enterocytes. The p53-p21 pathway and apoptosis are unlikely to be essential for bleomycin-induced tissue injury in the lung (Okudela *et al.*, 1999). Therefore, the present results suggest that in human PBLs BLM failed to induce delay in cell cycle and was unable to raise the level of p53 protein and bax and therefore the apoptotic cell death could be through p53-independent pathway.

Chapter III

Antitumor activity of the combination of BLM and radiation at different dose-rates

Literature Review

The reasons for the development of cancer might be many. The different processes involved in the development of cancer, however, are not a mystery any longer. During the past two decades, investigators have made astonishing progress in the deepest bases of the process, i.e. those at the molecular level. Translation of new understanding into clinical practice is however complicated, slow and expensive. The term 'cancer' refers to more than 100 forms of the disease. Almost every tissue in the body can spawn malignancies, and some have even been found to yield several different types. The interesting observation however is that each cancer has unique features. The 30 trillion cells in the body of a normal, healthy, human live in a complex, interdependent condominium, regulating one another's proliferation. In fact normal cells reproduce only once when instructed to do so by cells in their vicinity. Such unceasing collaboration ensures that each tissue maintains a size and architecture appropriate to the body's needs. Cancer cells, in stark contrast, violate this scheme, so that the usual controls on proliferation do not work on them. Instead they follow their own internal agenda for reproduction. They also possess as even more insidious property – the ability to migrate from the site where they began, invading nearby tissues as well as distant sites within the body. Tumours composed of such malignant cells become more and more aggressive over time and they become lethal when they disrupt the tissues and organs needed for the survival of the organism as a whole.

Over the past 25 yrs, scientists have uncovered a set of basic principles that govern the development of cancer. It is now known that cells in a tumour descend from a common ancestral cell that at one point initiated a program of inappropriate reproduction. Further the malignant transformation of a cell comes through the accumulation of mutations in specific classes of the genes within it. Therefore the studies of these genes provide the key to understanding the processes at the root of human cancer. Two classes of genes, which together constitute only a small portion of the full genetic set, play major roles in triggering cancer. In their normal configuration they are involved in the regulation of the sequence of events that takes place during the life cycle of the cell. Proto-oncogenes belong to one of those classes of genes and these are required for growth of cells whereas tumour suppressor genes inhibit such growth. The collective effects of both these two classes of genes account for much of the uncontrolled cell proliferation seen in human cancers.

The treatment modalities that are in use for the management of cancer are radiotherapy, chemotherapy and surgery. Radiotherapy is one of the two most effective treatments for cancer. Surgery, which of course has a longer history, is in the majority of cases, the primary form of treatment and it leads to good therapeutic results in the range of early non-metastatic tumours. Radiotherapy has replaced surgery for the long-term control of many tumours of the head and neck, cervix. bladder, prostrate, and skin, in which it often achieves a reasonable probability of tumour control with good cosmetic results. Wide field irradiations are also successful in Hodgkin's disease and other lymphomas where surgery would not be possible.

In radiotherapy, the rate of delivery of the dose is an important factor to consider. As dose-rate is lowered, the time taken for the delivery of a particular dose increases. Therefore, it is possible that during that time of irradiation, a large number of biological processes take place and modify the radiation response. Following the studies of Hall and Bedford (1996), it is known that a decrease in the dose-rate generally leads to a decrease in the lethal effect for a given dose of ionizing radiation. Keeping this in mind, different dose-rates of radiation have been delivered to patients for the treatments of various types of cancer. HDR radiotherapy has been used to treat cervical cancer advanced esophageal cancer and prostrate cancer. In fact, the use of higher dose-rates results in improved tumor control, especially in the cases of small tumors (Mayer et al 2003). It is however believed that continuous low dose-rates of exposure are the most efficient way of achieving full recovery from radiation damage in the shortest overall time. It minimizes the effects of cell proliferation, which is an advantage in terms of damage to the tumour cells, but a disadvantage for the tolerance of acutely responding normal tissues. High continuous dose-rates are biologically equivalent to the use of large dose per fraction in fractionated radiotherapy; greater late damage to normal tissues for a given level of tumour response is therefore to be expected.

Chemotherapy is the third most important treatment modality at the present time. Following the early use of nitrogen mustard during the 1920s, it has emerged to the point where upwards of 30 drugs are available for the, management of cancer. A large proportion of patients receive chemotherapy at some point in their management and useful symptom relief and disease arrest are often obtained.

Reliable information about the relative roles of surgery, radiotherapy and chemotherapy is difficult to obtain. Often quoted in this regard is the analysis by De Vita *et*

al. (1979) of the probable outcomes of treatment in 700,000 cases in USA in 1977. De Vita estimated that local treatment, which includes surgery and/or radiotherapy could be expected to be successful in approximately 40% of these cases. In 15% of the total, radiotherapy would be the principal form of treatment. In contrast to this, De Vita estimated that only 2% of the cases where chemotherapy was the main line treatment were likely to achieve long-term survival. This is generally because of the resulting toxicity of the chemicals that are used for the treatments of the cancers. While there is a high cure rate with chemotherapy in patients with this disease, some long-term complications from chemotherapy have now been recognised, including secondary leukaemia, therapy-related solid tumours, nephrotoxicity, neurotoxicity, pulmonary toxicity, vascular toxicity and infertility (Chaudhury and Haldas, 2003). However, in spite of their toxic effects within the system, chemotherapy does have to be used for the treatment of a number of chemo sensitive diseases.

In the present day context, complex multimodality treatments play an important role in modern oncology (Dunst, 2002). The objectives of these strategies are not only to increase local control and survival, but also to preserve the tumor-affected organ. A major disadvantage of the complex and combined treatment schedules is the risk of higher rate of side effects and complications. The knowledge about the nature of the side effects with such combined regimes and information on how to deal with them is essential for the oncologist to avoid harm to the patients and to achieve the desired treatment goals. Radio chemotherapy is generally used when only one of the treatment modalities is unable to achieve complete remission underlining the insufficiency of treatment with a single antineoplastic agent. Therefore, in light of the observations made in the HPBLs when the drug BLM was given in combination with LDR and HDR radiation in Chapter II, it would seem pertinent to investigate the prospects of it's applications in the field of therapy as well.

Bleomycin (BLM) is one of the drugs being used for chemotherapy alone or in combination with other drugs, as well as for radiotherapy+chemotherapy. BLM is a toxic chemical, but its use is often mandatory in view of its efficacy. BLM has been successfully used for the treatments of head and neck cancer, skin cancer, cervical carcinoma, choriocarcinoma, treatocarcinoma, Hodgkin's lymphoma, reticulum cell carcinoma and lymphosarcoma.

While the delivery of chemotherapy and radiotherapy, a number of factors have to be taken into account. The limiting factor for any therapeutic strategy against cancer has always been its toxic effects on normal tissues; indeed combinations of chemotherapy and radiotherapy have produced severe and even fatal complications. These have been mainly observed in the tissues treated within the irradiated volume. If radiotherapy and chemotherapy have to be given sequentially and in full doses, the consequences of this long protraction can be high-level repopulation (Tubiana *et al*, 1985, Tubiana *et al*, 1988). Withers *et al.* (1988) pointed out that if chemotherapy is given prior to radiotherapy, increased repopulation between dose fractions could then lessen the effect of the subsequent radiotherapy. When radiotherapy is delivered first, this delays the employment of chemotherapy and thus permits the further growth of occult metastates located outside the irradiated volume. This is why chemotherapy is generally given first; however, radiotherapy cannot be delayed too long after that because chemotherapy often has only a limited efficacy, and can even elicit the development of radioresistance. Therefore, radiotherapy is usually carried out after two to four cycles of chemotherapy. This is the reason why in the present investigation, the drug BLM had been injected prior to irradiation.

Materials and Methods

Dalton's Lymphoma cells: The tumor Dalton's lymphoma was originated in the thymus gland of a DBA/2 mouse at the National Cancer Institute, Bethesda, USA in 1947. Subsequently an ascitic form was developed by repeated intraperitoneal transplantation of the tumor by Chakrabarti *et al.* in 1984. This ascetic form was used for the antitumor tests.

Dalton's lymphoma cells were extracted from the peritoneal cavity of the transplanted mouse and washed with 0.85% NH_4Cl by centrifuging at 1200rpm to lyse out the RBCs. Folowing this, the cells were washed with PBS (pH 7.2), and then 1 x 10^6 cells were injected into the peritoneal cavity of each mouse. For this purpose, inbred Swiss albino mice, each weighing 20-25 gms, were used.

Treatments: The survivality of mice were studied after the following treatments were given to the mice bearing the Dalton's Lymphoma (DL) cells.

Bleomycin: When BLM was given, it was administered i.p. at a dose of 10 or 20μg per mouse where required. When BLM was given alone, it was given on the 5^{th} and the 9^{th}

day. In the experiments where BLM had been combined with radiation, BLM was given on the 5th day only, 2hrs before irradiation.

Irradiation: Irradiations were done with the help of an X-ray machine (Faxitron, 43855D). Two dose-rates were used for this purpose. The dose-rates used were 0.3Gy min^{-1} for LDR and 2.3Gy min^{-1} for HDR. The doses used in this particular study were 1 and 2Gy. Irradiation was done on the 5th day in all cases where radiation was given. When radiation was combined with BLM treatment, irradiation was done 2hrs after BLM was administered. When irradiations were done, the mice bearing the DL cells were etherized and put into a lead chamber that shields the torso exposing only the peritoneal region and the hind limbs to the X-rays. After completion of irradiation, the mice were revived.

Reagents:

- NH_4Cl (Merck, India)- 0.85% in distilled water.
- PBS pH 7.2 – This is a mixture of a number of salts in the following proportions:
    - NaCl (Merck, India) - 8.00gms
    - KCl (Merck, India) – 0.20gms
    - Na_2HPO_4 (Merck, India) – 1.14gms
    - KH_2PO_4 (Merck, India) – 0.20gms

The above salts were dissolved in 1lit of double distilled water.

Anti-tumor activity:

The anti tumor activity test of the treatments given were assessed in accordance with the U.S. National Cancer Institute standard protocols for primary screening. The evaluation of this activity was done by monitoring the survival period of the mice that had been treated with BLM and/or radiation at different doses and dose rates. After the survival times were observed, the T/C values were computed for each of the treated groups of animals. T is the median survival time of the treated group while C is that of the control group. The T/C ratio is given as a percentage. A compound is termed active if it has a T/C percentage ≥ 120% (U.S. National Cancer Institute Instruction, 1978)

Results

The results of the present investigation are presented in Table 3.1. It was observed that the administration of the higher concentration of BLM i.e. 20µg yielded a better T/C value. The treatment of 10µg BLM too showed a T/C of 124, but then 20µg BLM showed a T/C of 154. When BLM was given in combination prior to irradiation, there was no increase in the survivalities as compared to the controls. When BLM 20µg was

combined with 2Gy of X-rays, the survivality of the treated mice at LDR was similar to the survivality
of the mice when they were irradiated at HDR (2Gy) in the presence of BLM (20 μg). T/C values of the BLM(20)+LDR(2Gy) was 82 while BLM(20)+HDR(2Gy) showed a T/C of 77. Even when the dose of both the drug as well as radiation was reduced to 10 μg and 1Gy respectively, the T/C values were not improved much. The resulting T/C values therefore were all below 120% with the combination of BLM.

When only radiation was given at HDR and LDR, and a dose of 2Gy was given, the survivality was seen to increase by a large number of days in LDR irradiated mice as compared to the HDR treated ones. The T/C value for LDR in one case was 206 and in another was 182, while for HDR it was 118 and 135. When the HDR irradiation was split also, the T/C was found to be 147 which was well above the figure given by the NCI.

Table 3.1: Effect of radiation and bleomycin on survivality of in mice transplanted with DL cells.

Exptal. Condtn.	No. of animals	Bleomycin (days)	Irradiation (days)	Median Survival Time(days)	Range of Survival (days)	T/C %
Untreated	3	0	0	19	18-20	-
BLM(10)	3	5^{th},9^{th}	0	21	21-22	124
BLM(20)	3	5^{th},9^{th}	0	26	25-27	153
Untreated	3	0	0	16	15-17	-
LDR(2)	3	0	5^{th}	35	29-39	206
HDR(2)	3	0	5^{th}	23	18-26	135
BLM(20)+LDR(2)	3	5^{th}	5^{th}	14	07-27	82
BLM(20)+HDR(2)	3	5^{th}	5^{th}	13	07-26	77
Untreated	3	0	0	16	15-16	-
LDR(1)	3	0	5^{th}	16	13-20	94
HDR(1)	3	0	5^{th}	18	16-20	106
BLM(10)+LDR(1)	3	5^{th}	5^{th}	20	19-20	118
BLM(10)+HDR(1)	3	5^{th}	5^{th}	16	11-20	94
LDR(2)	2	0	5^{th}	25	21-29	147
HDR(2)	2	0	5^{th}	20	17-22	118
HDR(1+1)	2	0	5^{th},9^{th}	31	25-37	182

T/C = (Median days of survival of treated group/ Median days of survival of control group) x 100

Discussion

The association of RT and CT is clearly beneficial in tumors which are both chemo and radiosensitive (Tubiana *et al*, 1987). However, contrary to the expectations, in most solid tumors this association did not lead to significant progress in long-term survival. In head and neck cancers e.g. despite large numbers of controlled clinical trials, there is not yet a clear-cut demonstration in survival (De Vita *et al.* 1986, Tannock and Browman, 1986, Taylor, 1987, Tubiana *et al.*, 1985). These disappointing results are probably due to two factors; I) the insufficient effectiveness of the available drug on most solid tumors, 2) the cumulative toxic effects of radiation and drugs ion the normal tissues which limits the dose of both modalities in combined treatments. Combinations of RT and CT is a promising method of management of cancer, however, this regimen is of value only in those types of tumors which are both chemosensitive and radiosensitive and in which there is a significant repopulation rate (Tubiana *et al*, 1985, 1987) and/or a marked propensity towards early development of resistance to treatment modalities. The combination of RT and CT in fact, involves the complexity of both modalities plus the interactions between them. A drug, which is optimal in a treatment by CT alone, might not be optimal when CT is combined to RT, if the cumulative toxicity on normal tissues is too high (Tubiana *et al.* 1989).

In the present observation, BLM at 20μg showed a better T/C value than BLM at 10 μg. Therefore, it was obvious that the drug at a higher concentration was better from the therapeutic point of view. When this dose of 20 μg was combined with radiation of 2Gy, the survivality at neither of the two dose rates used were enhanced. The same trend was observed when BLM 10 μg was given in combination with 1Gy of X-rays. In a study conducted by Collis (1981) and Steel *et al.* (1979), it had been observed that when BLM was administered 7-28 days before radiation there was a moderate enhancement in the lung damage in mice. Therefore even in the present case where BLM had been given 2hrs prior to irradiation a similar event may have taken place, as a result of which none of the mice that had been given the combined treatment were found to show better survivality.

It is known that HDR in the case of sparsely ionizing radiation always shows better cell killing than LDR as reported by Evans *et al.* (1985) in the case of mouse lymphoma cell lines. He reported that there was better survival of the x-ray sensitive LY-S at low

dose-rate whereas there was more cell killing in comparison in the LY-R cells. Therefore, in this case too, it was expected that HDR would show a better survivality than LDR. However, it was surprising to note that when radiation was given alone at high and low dose-rates in the present study, the mice irradiated at LDR showed a better survivality as observed from the better T/C percentage, than the mice given HDR irradiation. The mice that had been given the split dose of 2Gy at HDR also were found to show a better survivality than continuous HDR irradiation but had a lesser T/C values than the LDR irradiated mice. This indicates that damage to the normal tissues was involved when HDR irradiation was given causing an increased mortality. When radiation was given, the mice were shielded by a lead shielding exposing the ventral peritoneal region and the hind limbs. Therefore, the tissues in that region received irradiations as well as the ascites. In fact, it is known that the small intestine has a high radiation and drug sensitivity and commonly represents one of the major limiting normal tissues in cancer therapy (Potten *et al.*1994). Therefore it is possible that damage to the small intestine could be one of the factors upon BLM treatment followed by irradiation that was the causal agent in determining the survivality of the mice. Indeed, acute toxicities in clinical treatments when BLM was given in combination with other drugs and irradiation have been reported by Aviles and Delgado (1998). BLM alone has been reported to cause a number of toxicities such as acute lung injuries (Betsuyaku *et al,* 2003) and pulmonary fibrosis (Azoulay *et al,* 2003). So, even though, in this case BLM was administered alone, in the absence of any other drugs, preceding irradiation, toxicities were still present which could have been enhanced by irradiation, causing mortality such treated groups.

Summary

The endogenous aminothiol which has far-out the highest intracellular concentration is glutathione (GSH) Its concentration in most animal tissues is in the range of 0.5 – 10 mM and is thus substantially higher than many other intracellular metabolites. It has already been proved that endogenous thiols of which GSH is the most prevalent one are important in cellular radiosensitivity and in general it was found that low intracellular GSH concentration cause sensitization especially under anoxic or hypoxic condition . The role of glutathione in protecting the cells against radiation damage has been studied extensively by a number of workers. Most workers have used BSO for such studies. BSO inhibits GSH synthesis and very low levels of GSH can be obtained over a relatively short time periods without appreciable toxicity. When GSH levels are depleted very rapidly, cells are sensitized to radiation. However, it has been suggested that the compartmentalization of GSH within the cells take place and the GSH within the nucleus and in particular close to the DNA is important in determining cellular radiosensitivity. GSH has also been hypothesized to act as a repair agent. Mitchell *et al.* have reported the role of GSH as a chemical repair agent in mammalian cells. That GSH also plays a role as a cofactor in the enzymatic repair processes in the cell has also been outlined, and the participation of GSH in the repair of single strand breaks in oxic conditions has been reported. GSH cannot be transported across the cell membrane from extracellular environments into the cell but it is synthesized intracellularly by γ-glutamyl cycle. If the effects of an elevated intracellular GSH concentration on radiosensitivity were to be studied the GSH level would have to be raised by means other than incubation with GSH. A several fold increase of GSH content in normal human fibroblast after incubation with the monoethyl ester of GSH was obtained many workers.

The effects produced by ionizing radiation depends on the energy absorbed by the system, and among other factors, largely, this depends on the rate at which this energy is deposited. This dose-rate effects covers significant areas in radiobiology applied to radiation therapy as well as radiation protection. Dose rate effects have been widely studied and numerous examples quote the effect of variation of dose-rates of radiation on different endpoints within the biological system. It is known that when radiation is given over a long period of time, a number of biological processes take place within the cell modifying it's response to irradiation. It is possible for the cell to repair the sub-lethal damages during that time. Therefore,

keeping this in mind, we decided to study the role of GSH at different dose-rates of radiation.

Deoxyribonucleic acid is a critical cellular target for the cytotoxic, mutagenic and carcinogenic effects of ionizing radiation. Radiation induced chemical modifications of DNA involve the formation of ionic, radical and excited intermediates as a result of the deposition of energy within the biopolymers and indirect process involving water radiolysis species . Despite the diversity of damages in DNA quoted by ionizing radiation as outlined above, there is evidence that the double strand breaks (dsb) is the lesion which if unrepaired or misrepaired is most likely to lead to cell death.

There is an interest in the development of assays that might predict response to radiotherapy and it is based upon observations that there is a range of radiation responses of cultured tumour and normal cells from different individuals. So far there is no simple method has been devised that can rapidly and accurately predict patient sensitivity to ionizing radiation. Because measurements of rejoining of DNA strand breaks can be performed rapidly, they are appealing for use in a predictive assay. While a correlation between radiosensitivity and dsb repair capacity for tumour cells has been observed by some workers, others have failed to find one.

The role of GSH in ssb repair had been outlined way back, but that GSH might have a role to play in dsb repair was recently outlined in a recent study when increased exchanges and decreased deletions had been reported at 4°C by GSH/GSHester posttreatment. Therefore, in the present study, we have made an attempt to look at the role of GSH in dsb repair from another aspect. We have made use of the fact 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, BLM, a radiomimectic drug, and radiation were given in combination. It has been reported that when radiation is fractionated or given over a prolonged time, i.e. at a low dose rate, the probability of misrejoining decreases. As a result the probability for interaction of the breaks are reduced. Keeping this in mind, we therefore decided to investigate the interaction of BLM and radiation induced dsb interaction at high and low dose-rates, with respect to the role of GSH.

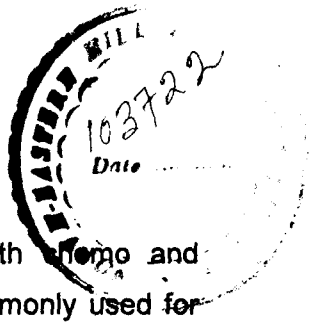
Rejoining of strand breaks can take place either by HR or NHEJ predominantly in mammalian cells. The process of rejoining in quiescent cells takes place mainly by the NHEJ process. This involves the interaction of a number of proteins, of which the Ku heterodimers are the first to recognize and bind to the breaks. Thus the levels of expression of the proteins involved in break rejoining would give us an idea regarding the pathway of repair induced within the cells. Therefore, the levels of Ku-70 and Rad51 were evaluated by western blot.

A number of factors may be mediating the differences in radiosensitivity found amongst both normal and tumour cell lines. The first one is the efficiency with which the external beam low-LET ionizing radiation induced DNA dsb, thought to be the critical lesion leading to cell death, may differ between cell types. The second factor that may contribute to differences in radiosensitivity is mode of cell death after irradiation.

Apoptosis and necrosis are two distinct forms of cell death, which are triggered among other insults by ionizing radiation and chemicals. These two forms of cell death have different morphological and molecular features and implications for the surrounding tissue. Apoptosis is an active process of cellular destruction characterized by cell shrinkage, chromatin aggregation with extensive genomic fragmentation and nuclear pyknosis. It eliminates cells that have accrued genetic mutations and may thereby become cancerous. Indeed induction of apoptosis is the mode of action of many anti tumor therapeutic agents that are currently in use. Thus, the necessity to investigate necrotic as well as apoptotic cell death in this investigation arose.

A number of genes are involved in triggering the process of apoptosis. One of the important classes of proteins involved in apoptosis is p53 has numerous roles to play within the cell including determining the cell cycle delay/arrest. It is also involved in apoptosis and induced a number of genes in a cascade manner leading up to chromatin fragmentation at the final step in apoptotic cell death. The process of apoptosis is however regulated by the pro and anti apoptotic proteins within the cells. One pro apoptotic protein is Bax involved in this process. The expression of this protein would give an idea regarding apoptotic cell death as well. Therefore the expressions of p53 and Bax were evaluated.

In the current context, for the treatment of cancer, radiochemotherapy is one of commonly used modalities. The use of the combined treatment is required when



the treatment by one is insufficient or when the disease is both chemo and radiosensitive. Even though bleomycin is a toxic chemical, it is commonly used for the treatment of many types of cancers because of its high efficacy. The use of radiation too is common for the therapy for cancer, and it is known that even though HDR irradiation induces more cell death, in the long run, LDR is better for the achievement of complete remission. This is because LDR causes less severe damages to the normal tissues. Therefore, keeping these facts in mind, an attempt was made to investigate the antitumor activity of the combined treatment of BLM and radiation at different dose-rates.

The major observations from the present investigation are outlined below:

⇒ With increasing dose-rate, aberrant metaphases and all types of CAs were increased. The decrease in the exchange aberration formation at LDR as compared to HDR, indicated that efficient rejoining was taking place when the dose was delivered over longer periods of time. Data indicate that BSO-mediated GSH depletion increased radiation induced CAs, apart from exchange aberrations. In the presence of BSO the sparing effect for deletions in HPBLs as well in isochromatid and chromatid breaks in the case of BMCs showed a decreasing trend indicating the role of BSO in the process of repair.

⇒ Interaction of DNA-lesions induced by BLM and radiation was more when samples were exposed to LDR of radiation. Significantly high frequency of exchange aberrations was induced in this combined treatment and such DNA double-strand breaks misrejoining could be due to NHEJ-pathway for repairing DNA dsbs, as shown by the western blot analysis of the expression of the Ku-70 and Rad51 proteins.

⇒ Combined treatment of BLM and LDR radiation to BSO-treated cells significantly reduced the exchange aberrations formation and simultaneously increased the frequency of deletions indicating further that presence of GSH is required for end joining of the dsbs.

⇒ Presence of BSO improved the cell kinetics after radiation in mouse BMCs and did not alter in HPBLs. BLM did not induce any delay in cell proliferation. Cells treated with BLM were found therefore not to express a higher intensity of p53 on evaluating the western blot results. This is correlated with the fact that BLM did not induce cell cycle delay from the study of the first cycle metaphases.

⇒ Apoptotic cell death with increasing dose-rate was increasing. In combination with BLM and radiation apoptosis as well as total cell death was more at HDR than LDR by light microscopy. The expression of the pro-apoptotic protein bax correlated with the expression pattern of p53 by western blot analysis, indicating that BLM did not induce apoptotic cell death in a p53 dependant manner.

⇒ Combination of radiation and BLM did not improve the survivality of mice bearing Dalton's Lymphoma cells. LDR irradiated mice showed better survivality than mice irradiated at HDR. It is most likely that the combination of the drug with radiation was toxic for the normal tissues.

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