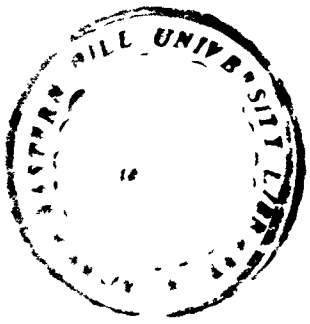
RELATIONSHIP BETWEEN CELLULAR RADIO AND CHEMOSENSITIVITY AND ENDOGENOUS GLUTATHIONE IN MAMMALIAN CELLS WITH RESPECT TO CYTOGENETICAL END POINTS



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

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

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CONTENTS

ABBREVIATIONS

Ab M.	Aberrant metaphase
AGT	Average generation time
AET	β-aminoethyl isothiouronium
BLM	Bleomycin
BMC	Bone marrow cells
BSO	Buthionine Sulfoximine
BUdR	5-Bromo 2-deoxyurudine
CAs	Chromosome aberrations
Chd. bk.	Chromatid breaks
DEM	Diethyl malate
Dicen.	Dicentric
DTNB	5-5' dithiobis 2 nitro benjoic acid
DTT	Dithio threitol
EDTA	Ethyl diamine tetraacetic acid
Exch	Exchange
FPG	Fluroscence plus Giemsa
GSH	Reduced-Glutathione
GSH-ester	Glutathione ethyl ester
Gy	Gray
HCT	Homocysteine thiolacetone
HPBL	Human peripheral blood lymphocytes
Isochd. bk.	Iso-chromatid break
KCl	Potassium Chloride
M1	Metaphase 1
M2	Metaphase 2
MISO	Misonidazole
MPA	Metaphosphoric acid
MPG	Monopropionyl glicine
NaCl	Sodium Chloride
Na_2HPO_4	di-Sodium Hydrogen Phosphate
Na_2CO_3	Sodium Carbonate
$NaHCO_3$	Sodium Bicarbonate

OER	Oxygen enhancement ratio
SCE	Sister Chromatid exchange
SCU	Sister Chromatid Union
SSb	Single strand break
TCA	Trichloro Acetic acid
TM	Total metaphase
WR-1065	Aminopropyl amino ethanethiol
WR-2721	Amino-propylamino ethylphodphorothioic acid

INTRODUCTION

The journey of the discovery of chemical radioprotectors in living systems started almost about 50 years ago. In early 1940's, the role of sulphur compounds in protection against radiation damage in complex chemical systems was recognised. Dale in 1942, first reported that colloidal sulphuur and thiourea can protect some enzymes against inactivation by X-rays. In 1948, Laterjit and Epharti reported that bacteriophages were effectively protected against radiation damage by thioglycolic acid, glutathione, cysteine and cysteine. In 1949, Barron et al. provided evidence that radiation induced inactivation of some SH-containing enzymes could be inhibited by glutathione(GSH). However, the first in vivo study of radioprotection was reported by Patt et al. (1949), that cysteine, a naturally occurring amino acid can increase the survivality of mice.

From the earliest days of research in this area, it was also proposed that radiotherapeutic procedures could be improved by the use of radioprotectors to protect normal tiissues, but not tumors, from radiation damage. As research developed, it was clear that studies with radioprotectors could also provide important information in the mechanisms of interaction of radiation with biomolecules. Ideas on the use of protective agents in combination with chemotherapeutic agents as well as in combination with radiotherapy, were merging because of the possibilities of common mechanisms of damage by the two trearment modalities. In addition to this, studies on endogenous protective systems that are important in protection against radiation and chemical induced damages were increasing dramatically.

Glutathione (γ- Glutamyl-cystenyl-glycine, GSH), is present almost universally in animal cells, most plant cells and bacteria. It is the dominant low molecular weight nonprotein thiol (NPSH) in mammalian cells and its level is inversely correlated to the cellular radiosensitivity (Revesz et al. 1963). It has been demonstrated that GSH plays an important role in cellular detoxification processes (Revesz and Modig 1965, see Revesz et al. 1984), regulates various enzymatic pathways by acting as a cofactor (see Meister and Anderson 1983) and it is involved in cell growth and replication process (Mazia 1961; Holmgreen 1979). Several reports suggest that increased radiation sensitivity is associated with defective GSH-metabolism. Various diseases like cataract of lens (Beutler and Srivastava 1974), 5- oxoprolinuria (Larson 1081), leukemia and anaemia (Sabine 1964; Macdougall 1968) have been reported to be associated with defective GSH metabolism

Data available on the protective effect of GSH on radiation induced chromosome aberrations (CAs) are limited to deletion in *Tradescantia* root tips (Mikaelsen1952), anaphase bridges in grasshopper (Chaudhuri 1968) and *Hordeum* root tips (Reddy 1971) and sex linked lethals in *Drosophila* (Jacob and Roychoudhuri 1973). Only one study was made using Fluorescence plus Giemsa (FPG) staining to restrict scoring in the first cycle metaphases (Chatterjee and Jacob-Raman 1986). They reported that reduced GSH minimized X-ray induced CAs and cell cycle delay in muntjac lymphocytes.

In contrast to radiation, very few informations are available on the role of endogenous GSH, on chemosensitivity. Studies have shown an increased level of GSH and its related enzymes in tumour tissues, indicating a correlation to drug resistance (Carmichael et al. 1988; Cook et al. 1991; Di Ilio et al. 1988; Volm et al. 1991). GSH was also found to provide resistance to several anticancer drugs. These include alkylating agents (nitrogen mustards, nitrosourea-s), redox-cycling agents (adriamycin) and also to vinca alkaloids and epipodophilotoxins (Black and Wolf 1991). Mytomicin C (MMC) toxicity was found to be inversely proportional to cellular GSH-level (Kennedy et al. 1985).

Therefore, it will be interesting to see the influence of endogenous GSH-level on cellular damages induced by radiomimetic chemical bleomycin (BLM), an important anticancer drug.

Accumulated evidences clearly indicate that endogenous GSH is involved in many radiation induced chemical processes in cells and thus represents an important factor in determining internal cellular radiosensitivity. The role of endogenous GSH on radiosensitivity have been observed with both GSH-deficient and proficient cell lines, however, its potential contribution to the *in vivo* response has not been quantified,

So far, the yield of DNA single strand breaks (SSbs), and clonogenic survivality have been used as a criteria for the radiation response and cytogenetical parameters have not yet been considered as end points. Survivality may not be an appropriate criteria due to probable interference of biochemical processes and the damages on DNA analyzed by SSbs also do not reflect the true picture at chromosomal level (Hittelman and Pollard 1982). Therefore, in the present investigation, analysis of CAs, Sister chromatid exchanges (SCEs) and cell cycle kinetics are considered as the end points to asses the radio and chemosensitivity in normal and GSH-depleted cells.

The bone marrow cells of mouse (BMCs) and human peripheral blood lymphocytes (HPBLs) is considered to be an well established *in vivo* and *in vitro* experimental system respectively. This two systems were selected in the present investigation with the following objectives:

- Evaluation of the influence of endogenous GSH on CAs induced by γ-irradiation in both in vivo and in vitro systems
- Analysis of the involvement of GSH in repair/misrepair type of phenomenon after induction of damages on DNA by radiation.
- Analysis of the influence of endogenous GSH on radiomimetic chemical induced CAs, with a view to draw a comparison between the mechanism of action of the two clastogens and the influence of endogenous GSH on them.
- Determination of the role of GSH alone on cell cycle delay and SCEs.

The present investigation is presented on the basis of each objective. Therefore, total five separate chapters are formed. Each chapter comprises more background literature survey along with materials and methods, results and discussion. However, the present study is mainly inclined to establish a relationship between cellular radio and chemosensitivity and endogenous GSH-level. Therefore, the first chapter is mainly concerned about the measurement of endogenous GSH both in mouse bone marrow cells and human peripheral blood lymphocytes.

CHAPTER I

Measurement of cellular glutathione level

LITERATURE REVIEW

Thiols have long been thought to affect the sensitivity of cells to irradiation (Revesz et al. 1963; Meister 1983) and there is increasing evidence that glutathione (GSH), the most prevalent intracellular thiol, is a major determinant of cellular radiosensitivity (Meister and Anderson 1983). The radioprotective function that has been ascribed to GSH include destruction of radiation induced radicals, donation of hydrogen to radiation damaged molecules and mediation of DNA repair, an effect, which may reflect, at least in part, its role as a cofactor in deoxyribonucleotide synthesis (Kossower and Kossower 1976; Holmgren 1979). In the effort to elucidate the function of GSH in radioprotection and in other biological processes, attempts have been made to deplete cellular GSH. The experimental procedures used for such depletion include application of compounds that oxidize GSH to GSH-disulfide (GSSG) and compounds that react with GSH to form GSH-adducts (Kosower 1978; Plummer et al 1981). Such methods have major disadvantages, including those associated with nonspecificity, rapid reversibility and the production of a high concentration of GSSG. Depletion of GSH by inhibition of GSH-synthetase would be expected on the basis of studies of patient's with a deficiency of this enzyme to be complicated with marked metabolic acidosis (Meister 1982). In contrast, inhibition of Υ-glutamyl cysteine synthetase offers a satisfactory approach to the production of experimental GSH deficiency.

Buthionine Sulfoximine (BSO) was developed in Meister's laboratory (Griffith and Meister 1979; Griffith et al 1979) as a potent and selective inhibitor of Υ-glutamyl cysteine synthetase. This and similar agents are used in both in vitro (Griffith et al. 1979; Griffith and Meister 1979) and in vivo (Griffith et al. 1979; Meister 1983).

Mice treated with BSO do not have convulsions. They exhibit a rapid decline in GSH in kidney, liver, plasma, pancreas and muscle and after prolonged treatment also show lowered concentrations of GSH in other tissues (Griffith and Meister 1979). Concentration of GSH in various cells grown in tissue culture (Dethmers and Meister 1981) and in erythrocytes in suspension (Griffith 1982) also decline rapidly in the presence of BSO. In vitro studies, using human tumour cell lines have shown that depletion of cellular GSH by BSO can indeed increase the cytotoxicity of a variety of anticancer drugs (Green et al 1984; Lee et al. 1986).

However, it is not yet clear whether a therapeutic benefit can be obtained by selectively depleating tumour GSH contents while partly or wholly sparing the critical normal tissues Consequently additional information on the in vivo use of BSO would greatly

assist the planning of such clinical trials. In the present investigation, attempt has been made to establish a relationship between endogenous GSH level and cellular radio and chemosensitivity both in vivo and in vitro in mammalian cells. In order to deplete the endogenous GSH level BSO was used and the level was measured by the method proposed by Beutler et al (1963).

MATERIALS AND METHODS

Materials

1. L- Buthionine S-R Sulfoximine (BSO; Sigma, USA): Potent inhibitor of GSH-synthesis. This was synthesized in the course of efforts to prepare selective inhibitors of ϒ- glutamyl cysteine synthetase.

2. 5,5'-dithiobis (2-nitro) benjoic acid (Ellman reagent, DTNB ;Sigma, USA): In order to increase the sensitivity of the spectrophotometric procedure, DTNB was used. DTNB after reacting with GSH produces a chromophoric product, viz.,2-nitro, 5-thio benjoic acid, possesing a molar absorption at 412 nm approximately twice that of reduced di-triphosphopyridine nucleotide at 340 nm (Tietze 1969).

3. Other materials, which were used are mentioned below:

Trichloro acetic acid (TCA) (Merck), Metaphosphoric acid (MPA) (Merck), Sodium Chloride (NaCl) (Merck), Ethyl diamine tetraacetic acid (EDTA) (Merck), Di-Sodium hydrogen phosphate (Na_2HPO_4) (Merck), Sodium Citrate (Merck).

Experimental Systems

This investigation was carried out in the in vivo mouse bone marrow cells and in vitro human peripheral blood lymphocytes.

Inbred male BALB/C mice of 2-3 months of age, weighing about 25-30g were used (2n =40; all acrocentric). Mice were maintained in laboratory community cages, with sterile bed, under controlled temperature (20°C ± 2) and lighting condition (12h light and 12h dark). Standard animal feed (Goldmuhar; Bilkak traders, New Delhi) and water was provided *ad libitum*. Bone marrow cells (**BMCs**) were isolated from femur bones.

In vitro experiments were carried out using human peripheral blood lymphocytes (**HPBLs**), collected from healthy young (25 - 30 yrs of age) donors, mostly males. The method of collection of blood and further teatments are described later. This system is being used widely and well known.

Methods

Experimental procedure

a. Treatment of BSO

The concentrations used in in vivo experiments were 1, 4 and 200 mg kg^{-1} and for in vitro experiments, 5 mM. For each experiment , the working solution of different concentrations of BSO (100 µg ml^{-1} to 10 mg ml^{-1}) were prepared in phosphate buffer solution. For in vitro experiments they were dissolved in triple distilled water. In this case 5mM of BSO was added into the 1ml aliquot of whole blood for 3h prior to estimation.

b. Measurement of GSH in mouse BMCs

The GSH level of cells was measured according to the protocol (Beutler et al 1963) described below:

Solutions:

1. NaCl - EDTA: 4.2g of NaCl + 0.3684g EDTA were dissolved in 500ml of distilled water.

2. 0.3 M Phosphate buffer: 4.2588g Na_2HPO_4 was dissolved in 100ml of distilled water.

3. 20% TCA : 20g of TCA was dissolved in 100 ml of distilled water.

4. DTNB reagent: 4mg DTNB was dissolved in 10 ml of 1% Na-Citrate solution (Stored at 4^0C in dark).

Procedure

BMCs were flushed into ice-cold NaCl-EDTA solution and the volume was made to 2ml. Cells were counted in haemocytometer. 1ml of cell suspension was added to 1ml of 20% TCA, vortexed and kept at 4^0C for 20 min. It was centrifuged for 15 min at 5000 rpm at 4^0C. 1ml of supernatant was added to 6ml of 0.3M phosphate buffer and then 1ml of DTNB was added and vortexed. The optical density of the samples were measured at 412 nm by UV- visible spectrophotometer (Hitachi model 2005) relative to a blank containing DTNB, buffer and TCA.

The standard curve was plotted using different concentrations of GSH solution (100 µM to 400 µM).

The concentration of GSH was calculated using the formula:

$$\text{GSH concentration } (\mu\text{mol} / 10^6 \text{ cells}) = \frac{\text{Spectophotometric concentration}}{\text{Number of cells } (\times 10^6)} \times 2(\text{dilution-factor})$$

c.Measurement of GSH in whole blood of human

Solutions:

1. Precipitating solution:

Glacial MPA - 1.67g ; EDTA - 0.2g; NaCl - 30g. Dissolved in 100ml of distilled water.

2. 0.3M Phosphate solution:

4.2588g Na_2HPO_4 was dissolved in 100ml of distilled water.

3. DTNB reagent : 4mg of DTNB was added in 10ml of 1% Na-citrate solution (Stored at 4^0C in dark).

Procedure

0.2ml of blood was mixed with 1.8ml of distilled water. 3ml of precipitating solution was added and allowed to stand for 15 min It was then filtered, using Whatman (No. 1) filter paper.

1ml of filtrate was mixed with 6ml of phosphate solution and 1ml of DTNB. A blank sample was made using 6 ml of phosphate solution and 1 ml of diluted precipitating solution (3 precipitating solution : 2 distilled water) and 1ml of DTNB was added to it.

All the solutions were vortexed and the optical density was measured at 412 nm.

The standard curve was plotted using different concentrations of GSH solution (100 μM - 400 μM). The concentration of GSH was calculated by multiplying the spectrophotometric concentration with 10.

The concentration was expressed as μmol ml^{-1} of blood.

Statistical clulation

Data are presented as Mean ± SD. The results were analysed with the Student's t-test.

RESULTS

Data of GSH concentrations from individual BSO - treated and untreated mouse BMCs are shown in table 1.1. Five or more mice were used per point in this experiment. The GSH concentrations showed a rather marked range varying from 1 to 3 μmol 10^{-6} with

an average of 1.693 ± 0.62 μmol 10^6 cells in BSO untreated group. It was found that significant GSH depletion in normal mouse BMCs to a level of about 1.17 ± 0.32 and 0.768 ± 0.54 μmol 10^6 cells (69% and 45% of the BSO untreated mice) was achieved after treating the mouse with 4 and 200 mg kg^{-1} BSO for 10h. The data clearly indicate that depletion of endogenous GSH is BSO dose dependent. However, the total depletion of GSH may differ from mouse to mouse and it depends on the GSH content of normal cells before BSO administration.

Estimation of blood GSH concentration of BSO treated and untreated HPBLs are presented in table 1.2. The blood GSH concentrations showed a range between 720 to 813 μmol ml^{-1} with an average of 759.4 ± 33.93 μmol ml^{-1} in the BSO untreated human blood. When BSO (5mM) was present in the blood for 3h, the GSH concentration was depleted to 70% of the control value. The statistical difference between the mean GSH concentration of these two groups was significant.

Table 1.1 GSH concentration in BSO treated and untreated mouse bone marrow cells

BSO (mg kg^{-1})	# Cells ($x10^6$)	Spectrophotometric Absorb.	Spectrophotometric Conc.	GSH (μmol 10^{-6} cells)	GSH X ± S.D.
00	32	0.024	28.77	1.80	**1.693 ± 0.62[a]**
	31	0.029	18.19	1.17	
	19	0.020	10.02	1.05	
	36	0.024	43.90	2.40	
	18	0.015	27.50	3.00	
	32	0.016	31.00	1.90	
	32	0.020	16.00	1.00	
	25	0.016	19.83	1.58	
	35	0.023	28.12	1.60	
	35	0.030	39.86	2.27	
	38	0.021	29.16	1.53	
	36	0.034	18.37	1.02	
01	35	0.021	25.70	1.47	**1.533 ± 0.41**
	33	0.029	33.73	2.04	
	25	0.027	16.47	1.31	**(90 %)**
	35	0.036	24.21	1.38	
	30	0.032	20.99	1.39	
	30	0.026	32.12	2.14	
	38	0.015	18.97	1.00	
04	35	0.021	25.70	1.47	**1.17 ± 0.32[b]**
	34	0.020	24.52	1.44	
	33	0.006	18.00	1.09	**(69 %)**
	33	0.004	28.00	1.20	
	51	0.120	27.00	1.06	
	39	0.025	30.69	1.57	
	29	0.018	21.83	1.50	
	32	0.021	11.31	0.71	
	29	0.024	13.68	0.95	
	35	0.022	12.60	0.72	
200	43	0.021	30.03	1.39	**0.768 ± 0.54[c]**
	54	0.025	34.66	1.32	
	46	0.024	09.48	0.41	**(45 %)**
	52	0.023	08.45	0.32	
	48	0.024	09.69	0.40	

GSH concentration as percent of untreated control is given in parentheses.
b-a $p < 0.05$; c-a $p < 0.01$ Student's t-test.

Table 1.2 Estimation of GSH-level in BSO treated and untreated human peripheral blood .

BSO (mM)	Spectrophotometric Absorb	Conc.	GSH (μmol ml^{-1})	GSH X $\pm$ S.D.
00	0 058	81.32	813	**759.4 $\pm$ 33.93[a]**
	0 054	76 23	762	
	0 055	71 96	720	
	0.057	75 53	755	
	0.049	74.65	747	
05	0.031	45.11	451	**531.4 $\pm$ 57.59[b]**
	0.042	60.35	603	
	0 041	51 31	513	**(70 %)**
	0.042	52.39	523	
	0.037	56.77	567	

GSH concentration as percent of untreated control is given in parentheses.
b-a $p < 0.001$ Student's t-test.

DISCUSSION

A major aim of the present study was to establish a relationship between endogenous GSH content and cellular radio and chemosensitivity with respect to cytogenetical parameters. Since mouse bone marrow cells were used in vivo and human peripheral blood lymphocytes was used in vitro studies, the measurement of endogenous GSH have been observed in these two tissues. However, instead of lymphocyte, we have measured the GSH of whole blood, since isolation of lymphocyte by Ficoll-gradient may cause leaking of endogenous thiols from cells.

The wide spread distribution of GSH and its apparent involvement in various biological functions (Meister 1983) have generated a continual interest in methods of analysis of this cellular component ever since its discovery and isolation in early thirties. Although this tripeptide can exist in both reduced (sulphydryl) and oxidised (disulfide) form, it is maintained in vivo predominantly in the former state, through the action of the equally ubiquitous enzyme glutathione reductase (Knox 1960). Since the reduced form comprises in most instances, the bulk of cellular non protein thiol groups, measurement of acid soluble thiol has been commonly employed for the estimation of GSH levels of tissue extracts. In order to increase the sensitivity of the spectrophotometric procedure the sulphydryl reagent DTNB was used. The chromophoric product resulting from reaction of the reagent with GSH viz., 2-nitro-5-thiobenzoic acid, possesses a molar absorbtion at 412 nm.

For this reason and in conjunction with the use of double beam spectrophotometry, it has proved possible to extend the useful lower limits of the present assay to optical density changes corresponding to GSH contents of 1-10 ng ml^{-1} of assay mixture, a level of sensitivity which is at least an order of magnitude greater than that obtainable with the previous methods of analysis that have been commonly employed.

The present results demonstrate considerable depletion of cellular GSH level in both BMCs and HPBLs with the specific GSH depleting agent BSO. The concentrations of BSO used for in vivo study was 1, 4 and 200 mg kg^{-1} and for in vitro study was 5mM. The concentrations used in in vivo study was in the lower side, particula-rly 1 and 4 mg kg^{-1} was extremely low concentrations. Nevertheless, we have used such extremely low concentration since 4 mg kg^{-1} of BSO treatment induced significant sister chromatid exchanges in the mouse BMCs (data shown in Chapter V). The present data

showed that the GSH content of the BMCs following BSO treatment of 4 and 200 mg kg^{-1} came down to 69% and 45% of its initial level. 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). Intermediate rates of depletion were seen in the bone marrow with nadirs at 8 -12h. They have observed that the GSH content of the BMCs following depletion was to be 17% of the initial level. In the present data, the level of depletion was 45% of its initial level, which could be attributed due to lower concentration of BSO used in the present experiment. The incubation period of BSO treatment was kept for 10h in all studies according to Lee et al. (1987). The doses of exposure time of BSO administered in the present studies showed no toxicity on inhibition of cellular growth. BSO may be toxic at higher doses or longer exposure time. This has been demonstrated in V79 cells by Midander and Revesz (1984) In separate studies, BSO was treated in mice for as long as 14 days, without any apparent toxicity (Meister 1983). Therefore, considering all these facts, the concentration of BSO was preferrred towards the lower side in the present study. The concentration of BSO, used in in vitro study was 5mM during GSH measurement, and showed 70% of the initial GSH level following depletion. In this investigation, the radio and chemosensitivity was assessed in peripheral blood lymphocytes, however, for the measurement of endogenous GSH level, the whole blood was considered. It has been demonstrated that BSO was least effective in depleting GSH of RBCs (Lee et al 1987) and therefore, in the present study, the observed less depletion of endogenous GSH in the whole blood could be due to least depletion occurred in RBCs. If this is the case, then perhaps, the level of depletion in lymphocytes was much higher.

It is a matter of debate as to whether it is a) the absolute GSH concentration following depletion or b) the percent depletion of the initial GSH content, which is the important parameter governing the chemo and radiosensitization effects of GSH-depletion. Since different tissues have vastly different steady state of GSH levels, the effects of GSH depletion as indicated by these two parameters can differ dramatically. For example, the GSH content of the liver, following depletion to 25% of its initial level is still greater than that of the untreated lungs (Lee et al. 1987). Therefore, the percent depletion parameter is more appropriate and was used in the following sections.

In the course of our subsequent studies, the data are explained on the basis of endogenous GSH level, which was observed in this section.

CHAPTER II

Endogenous glutathione level and its effect on cellular radiosensitivity

LITERATURE REVIEW

The ability of GSH to protect cells against damages induced by ionizing radiation was reported almost 50 years ago (Laterjit et al. 1948). Revesz et al. in 1963 and then Ohara and Terashima (1970) reported that reduced GSH is an inherent radioprotector. The increased endogenous 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 reduced radiosensitivity. Ohara and Terashima (1970) established the cyclic variation of cellular NPSH content during the cell cycle of HeLa S3 cells and its correlation to the cyclic variation of X-ray sensitivity. Meanwhile, several studies have been carried out, where, reduced GSH was added exogenously. It was found that, exogenous addition of GSH, could protect cells against ionizing radiation, moderately in vivo (Cronkite et al. 1951; Alexender et al. 1955) or in cellular experiments in vitro (Vergrossen et al. 1964). Jacob and Roy-Chaudhuri (1973) reported about the significant protection shown by reduced GSH against sex linked recessive lethals, when injected in all broods of *Drosophila*. Chatterjee and Jacob-Raman (1986) studied the modifying effect of reduced GSH on X-ray induced chromosome aberrations (CAs) and cell cycle delay in muntjac lymphocytes in vitro, employing differential staining technique. They used three concentrations of GSH; 10, 15 and 25 mM and treated them either 30 min prior to X-irradiation (2, 3 and 4 Gy) or just after the irradiation. GSH pretreatment reduced the frequency of deletions against all doses of X-irradiation. Exchange type of aberations were also protected by 25 mM of GSH, when cells were irradiated with 3 and 4Gy. They also demonstrated significant reduction of X-ray induced cell cycle delay by GSH-pretreatments.

The observation of an association of radioresistance with increased cellular thiol levels were subsequently confirmed in other biological materials, like in mouse lymphoma cell lines (Alexander et al. 1965), bacterial strains (Bruce and Malcham 1965) and yeast cells (Brunborg 1977). Workers felt the requirement of experimental investigations into the role of cellular thiols in the post-irradiation processes. In order to have this information, an approach was taken to manipulate intracellular thiol concentrations by treating with several chemical compounds.

The radiosensitivity of cells increased when treated with thiol reactive substances like misonidazole (Varnes et al. 1980) and dimethyl malate (DEM) (Mitchell et al. 1983) or by

chemically blocking GSH biosynthesis using BSO (Bump et al. 1982; Griffith and Meister 1979). It has also been shown that the application of DEM and BSO enhanced the radiosensitizing effect of misonidazole (MISO) in mammalian cells in vitro (Bump et al. 1984; Hodgkiss and Middleton 1983; Clark et al. 1984). However, a similar gain in the radiosensitizing efficiency of MISO in vivo was not observed with either BSO or DEM (Rojas et al. 1984; Minchinton 1986).

Although in most, if not in all cases, the artificial change of the thiol level by treatment with such compounds can also be associated with some non-specific side effects and the results can not be interpreted unequivocally. Type of cells with GSH deficiency, due to a specific genetic defect in the activity of a particular enzyme GSH-synthetase (Larson 1981) is now used. These cells are derived from blood samples or subcutaneous biopsis from patients with 5-oxoprolinuria or from their close relatives with no chemical symptoms of the disease. Experiments were performed, with different cell strains, homozygous or heterozygous, with regard to this defect. The homozygous cells had a GSH level of about 6% of the GSH proficient cells, whereas, the level in heterozygous cells was almost 50% of the controls. In comparison to controls the total NPSH concentration was decreased in both cases about 50 and 70% respectively (Edgren et al. 1981).

The enhancement of radiosensitivity by oxygen is probably determined by competition between oxygen and cellular thiols for radiation induced radicals in key targets (Howard-Flanders 1960). GSH-deficient (GSH^-) cells are a useful biological material for experimental tests of this competition hypothesis. Several tests have been performed using the yield of single strand breaks (SSbs) of DNA, micronucleus frequency and clonogenic survivality as end points (Edgren et el. 1980; Deschavanne et al. 1981; Midander 1983). A clear correlation appears to exist between oxygen enhancement ratio (OER) and GSH-content. The enhancement of sensitivity being critically dependent on the GSH-availability. They also demonstrated that GSH is specific for determining the enhancement of radiosensitivity by oxygen and the role of other cellular aminothiols may be insignificant.

Attempts were also made to change the sensitivity of GSH^- cells after treating the cells with different radioprotective thiols (Edgren et al. 1983). The OER of the treated cells was determined after exposure to radiation under hypoxic and oxic condition. It was found that the OER for the thiol substituted GSH^- cells do not reach the values calculated for GSH^+

cells treated in a similar manner. This suggests that, none of the added exogenous thiols can fully replace endogenous GSH in the competition process.

Therefore, reported evidences are clearly indicating that thiols define radiosensitization of hypoxic cells by compairing the response of GSH-proficient and deficient bacterial cells (Fuchs and Warnes 1975; Morse and Dale 1983). The radioprotective effect of endogenous thiols can also be studied in cells, where GSH is depleted by thiol reactive agents such as MISO, DEM and BSO. All these studies considered, DNA SSbs or micronucleus frequency or cell survival as end points. The effect of cellular GSH on the radiation induced DNA double strand breaks (dsbs) was also investigated using GSH deficient yeast cells (Frankenberg et al. 1987). They demonstrated that, induction of dsbs is increased by a factor of 1.5 under oxic and 1.8 under anoxic irradiation condition.

Experimental data are accumulating which suggest that, DNA dsbs may be the primary lesions leading to CAs (Natarajan and Obe 1978, 1984). Therefore, in the present investigation we have studied the effect of endogenous GSH on the frequency of CAs induced by γ - irradiation.

MATERIALS AND METHODS

Materials

1. Buthionine Sulfoximine (BSO; Sigma, USA): This is used as a potent inhibitor of GSH-synthesis. Details about it has already been described in Chapter I.

2. 5-bromo-2- deoxyuridine tablet/powder (BUdR- tablet; Boehringer-Manheim, Germany): In in vivo experiments, paraffin coated (50mg) tablet was implanted subcutaneously in each mouse. In in vitro experiments, BUdR powder (Sigma, USA) was used after dissolving in sterilized double distilled water and added to each vial with a concentration of 6 $\mu g\ ml^{-1}$.

3. Radiation: Gamma irradiation was performed, with a ^{60}Co source, with dose rates 18.6 Gy min^{-1} and 14.4 Gy min^{-1}. The calibration was done with the method of Fricke dosimeter.

Experimental Systems

In in vivo studies, mouse BMCs and in in vitro studies, HPBLs were used. Details of the mice keeping procedure and the collection of human blood are available in the same section of the preceeding chapter (Chapter I).

Methods

Experimental procedure

a) Treatment with BSO

BSO with different concentrations (1-300 mg kg^{-1} body weight) were prepared in phosphate buffer solution (pH 7.4) and injected intraperitoneally (i.p.), 10h prior to irradiation. In one case mice were kept for 15 and 18h after BSO treatment.

For in vitro experiments, 1 and 5 mM of BSO was added into 1ml aliquot of blood for 5h prior to irradiation.

b) Irradiation

Mouse was kept inside thin glass bottle and irradiated whole body in the ^{60}Co Gamma chamber. The radiation doses used were, 0.5, 1.5, 2.0 and 3.0 Gy. 13h after irradiation the BMCs were fixed.

In case of HPBLs, 1ml aliquot of whole blood for each ϒ-ray dose was taken in a sterilized small flat bottom 25 ml glass beaker. The lymphocytes were exposed to 1.0, 1.5, 2.0 and 2.5 Gy of ϒ- irradiation at room temperature. After irradiation, blood samples were kept at 37^0C in an incubator for an hour before setting up the cultures. A diagram representing the experimental protocol is presented in Fig. 2p.

c) Culture procedure

Cultures were set up in medium containing 10ml of RPMI 1640 (Gibco, USA) with antibiotics (200 IU of penicillin and 100 μg of streptomycin per ml) and supplemented with 1ml of heat inactivated Foetal-calf serum (Biological Industry Ltd., Israel). In each culture, the amount of blood was 1ml. In order to stimulate the lymphocytes for entering in cell division, 0.3 ml of phytohemagglutinin M (PHA) (Gibco, USA) was added in each culture. BUdR (6 μg ml^{-1}) (Sigma, USA) was added in each culture during initiation of cultures. All cultures were incubated at 37^0C. After about every 12h they were shaken gently. Cultures were harvested at 48h. All experimrnts were repeated a minimum of two times.

d) Chromosome preparation

The animals were sacrificed 13h after irradiation by cervical dislocation with 2h colchicine treatment prior to it. The femur bones were dissected and the BMCs were obtained by injecting 2ml of 0.075M KCl (hypotonic solution) into one end with a 26 gauge needle. A single cell suspension was made in hypotonic solution and incubated for 15min at 37^0C.

They were centrifuged at 1200 rpm for 5min and fixed in two changes in fixative (methanol : acetic acid 3:1) for 30 min and 10 min respectively, resuspended in 0.5 ml of fixative and dropped onto chilled slides and flame dried.

In whole blood cultures, colcemid was added at a concentration of 0.01 $\mu g\ ml^{-1}$, 3h before harvesting. Hypotonic treatment was done for 18 min and cells were fixed and slides were prepared according to the flame drying method, same as for the BMCs.

e) Staining

Slides prepared from BMCs were stained with 5% Giemsa (BDH Chemicals Ltd. UK) for 3-4 min. They were rinsed in distilled water, air dried and mounted in DPX. During treatment, when BUdR (tablet/powder) was added (for in vivo study BUdR-tablet was used only in unirradiated mice in order to know the cell cycle kinetics) the metaphase slides were treated for differential staining technique (Goto et al. 1975) as follws:

Slides were treated for 10min with Hoechst 33258 (50 $\mu g\ ml^{-1}$; Sigma, USA) at room temperature in dark, rinsed in distilled water, mounted in 2 X SSC (NaCl-Na-citrate, pH 6.8) and kept in sunlight for 30-40min, depending upon the intensity of sunlight. After rinsing in distilled water, slides were stained in 2% Giemsa (BDH Chemicals Ltd, UK) for 3-4min, air dried and mounted in DPX.

Scoring

Slides were coded at random. At least 100 well spread metaphase plates were selected for study. For scoring cell cycle kinetics, metaphases were categorised into 1st, 2nd or subsequent division cycles based on their differential staining pattern.

For bone marrow cells, chromosomes were classified as Exchanges (exch.), Chromatid breaks (Chd. bks.), Iso-chromatid breaks (Iso-chd. bks.) and Sister chromatid union (SCU). In case of HPBLs, the categories of aberrations scored were dicentric and rings (with or without fragments), terminal and interstitial deletions and chromatid breaks. Number of normal and aberrant metaphase cells were also recorded.

Photomicrographs

Photomicrographs were taken in Leitz (Ortholux) (Germany) photomicroscope with 100X magnification using slow speed (8 ASA, Agfa Copex), black and white films. Fine grain film developer (Agfa) was used and photographs were printed in hard printing paper (Agfa,

Brovira). Graphs were plotted from the pooled data using Microcal Origin (version 3.5) program implemented on a Toshiba T2100 computer.

Calculation and Statistics

a. To verify the significance of the protective and synergistic effect of the chemical measured in terms of reduction and enhancement in aberrant metaphases, 2 X 2 contingency Chisquare (χ^2) formula was applied on the experimental and positive control data.

$$\chi^2 = \frac{n \{ (ad - bc) - n/2 \}^2}{(a + b)(c + d)(a + c)(b + d)}$$

Where, "a" and "c" are the number of normal metaphases and "b" and "d" are the number of aberrant metaphases tested in experimental and control lots.

N = a + b + c + d ; degree of freedom (df) = 1

b. To verify the significance of the protective and synergistic effect of the chemical measured in terms of reduction and enhancement in different types of aberrations, simple Chisquare (χ^2) test has been used.

RESULTS

The influence of endogenous GSH on CAs Induced by ϒ- irradiation in mouse BMCs

Initially few experiments were performed to see the cell cycle pattern of unirradiated BMCs by fixing them at 13, 17 and 20h after BUdR-tablet implantation. The data (table 2.1) showed that the frequency of cells in first cycle metaphase (M1) is very high (96.5%) at 13h fixation time. Since the M1 cells should be considered for chromosome aberration study, all the subsequent in vivo experiments were carried out by fixing the cells at 13h, considering the delay induced by ϒ-irradiation in the cell cycle. Microphotographs, representing different cell cycle metaphases are presented in Fig. 2.1

Gamma ray induced CAs were studied in mouse BMCs as positive control to BSO treated mice in each experiment and their results are represented individually in table 2.2a, 2.2b and 2.4. The pooled data are presented in table 2.3 and 2.5. The negative control value is presented from 3 mice Mice were treated with 0, 0.5, 1.5, 2 and 3 Gy of ϒ- irradiation. In

BSO treated mice, different concentrations of BSO (1, 3, 4, 200 and 300 mg kg^{-1}) were selected. A minimum of 3 mice were used in each point.

The whole experiment was conducted in 2 sets and therefore two different dose rate had to use. In the first set of experiments, the dose rate was 18.6 Gy min^{-1} and in the second set 14.4 Gy min^{-1} was used. Mean percentage of aberrant metaphases and mean frequencies of all types of aberrations per cell with standard error of the mean were calculated and there values are plotted (Fig 2.3 and 2.4). Details of the results are presentd below.

Aberrations were scored in four categories - I) Exchanges (exch): All inter changes involving two or more different chromosomes. ii) Sister chromatid union (SCU): Kind of intra arm interchanges, taking place between lesions within a chromosome iii) Iso-chromatid break (Iso-chd. bks.) and iv) Simple Chromatid breaks (Chd. bks.), Translocations were not scored. Microphotographs representing these aberrations are presented in Fig 2.2.

The frequencies of Iso-chd. and Chd. bks. were the most frequent type of aberrations and showed increase in frequencies with increasing radiation doses. However, there was no difference in frequency of Chd. bk. between 2 and 3 Gy. The individual data showed that at 3 Gy, the frequency of Chd. bk. was in the range of 2.55 to 3.69 per cell, which was higher than the range of 2.22 to 3.28 per cell induced by 2Gy.

The frequency of exchanges showed sharp increase from 0.5 to 3Gy. It is also clear from table 2.3 that the frequency of SCUs showed a significant increase from 1.5 Gy to 3 Gy of radiation doses.

The frequency of aberrant metaphases was increased from 0.5 to 2Gy, however, difference was not observed between 2 and 3Gy of Υ-irradiation in the first set of experiment (table 2.3). In the second set of experiment, where the dose rate was 14.4Gy min^{-1}, the frequency of aberrant metaphase was higher in 3 Gy than 2 Gy (table 2.5). All other aberrations showed similar trends, as it was in the first set of experiments. The only difference was that the frequency of each category of aberrations was lesser in the second set than in the first one. Dose dependent increase in the frequency of aberrations was clear from the graphs plotted from the data (Fig 2.3, 2.4, 2.5 and 2.6).

Increased sensitivity was observed in BSO treated mice, although the extent of sensitivity varried according to the concentrations of BSO and the dose of radiation. Extremely low concentration of BSO (1 and 3 mg kg^{-1}) sensitized mice against 0.5 Gy, however, this was

not found with higher doses of radiation. In the first set of experiments, BSO treatment with 4 mg kg^{-1} sensitized the mice at all radiation doses, however, with 3Gy, it shows mild sensitization. The maximum sensitization in the frequency of aberrant metaphases was observed when mice were treated with 200 mg kg^{-1} of BSO (table 2.5)

The frequency of Chromatid breaks was the class of aberration, which showed consistently significant enhancement in mice, when sensitized by BSO treatment (Fig 2.4 and 2.6). BSO 4 mg kg^{-1} could sensitize the frequency of Chd. bks induced by all radiation doses, except with 3 Gy in the second set of experiment. However, using 200 mg kg^{-1} of BSO a considerable increase in the frequency of aberrations was observed. The frequency of Iso-chd bks also increased both by 4 and 200 mg kg^{-1} of BSO in all the experiments.

The frequency of exchange type of aberrations (Exch. and SCUs), on the other hand, was not enhanced at all in all the BSO treated mice. In several cases, there frequency was similar in BSO treated and untreated mice, whereas in others the frequency was reduced after BSO treatment. Graphs plotted from the pooled data showed that the frequency of exchanges reduced in BSO treated sample than untreated one (Fig 2.4 and 2.6).

It is worth noting, that mice treated with extremely low concentrations of BSO (1 and 3 mg kg^{-1}) showed reduction in the frequency of all types of aberrations, induced by 1.5 and 2Gy (Fig 2.3 and 2.4).

The effect of BSO itself on the formation of spontaneous aberrations was studied on unirradiated mice. With 1 and 4 mg kg^{-1} of BSO treatment, no effect was found on the frequency of any type of aberrations, however, a significant increase in the frequency of aberrant metaphases and Chd. bks were observed after 200 mg kg^{-1} BSO treatment (Chapter V).

Therefore, as a whole, mice treated in vivo with BSO (4 and 200 mg kg^{-1}) showed higher sensitivity to Υ-irradiation than BSO untreated mice. This increased sensitivity of mice to irradiation was reflected by significant enhancement of the frequency of Chd. bk., Iso-chd. bk. and aberrant metaphases but not in any type of exchanges.

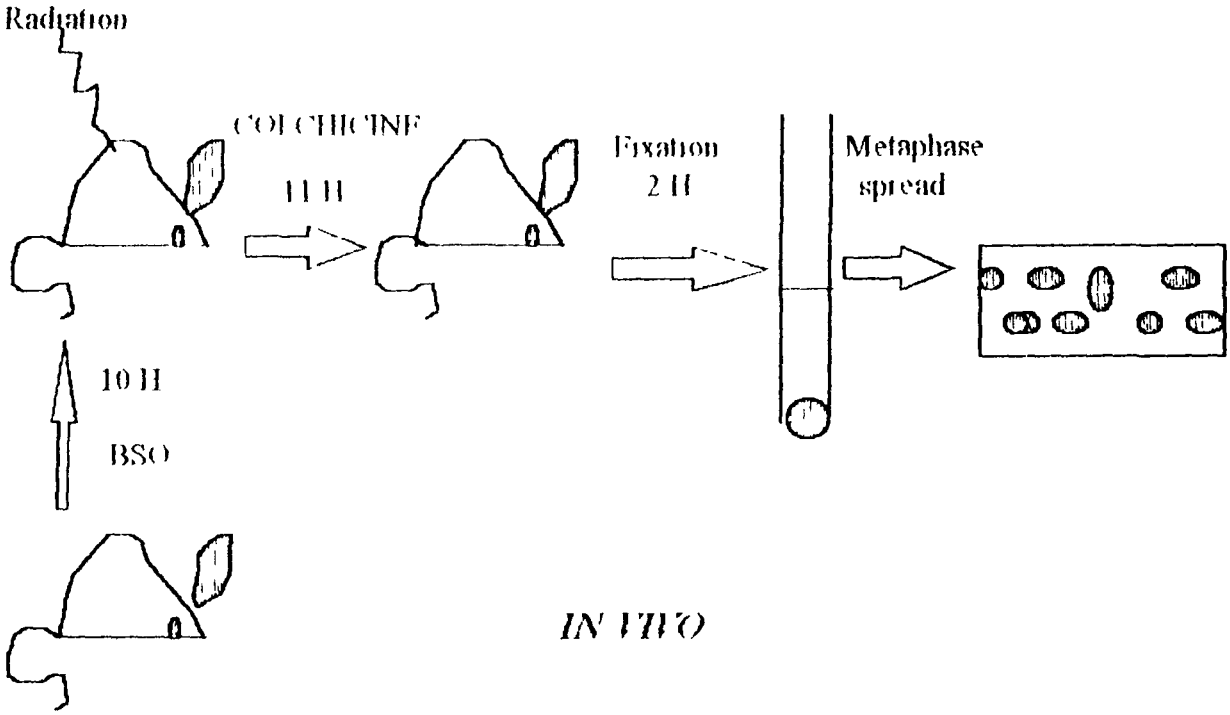
Table 2.6 shows the data of CA induction by 1.5Gy of Υ -irradiation in mice treated with BSO (5mg kg^{-1}) for 10, 15 and 18h. The frequency of aberrant metaphases as well as Chd. bks and Iso-chd. bks were not showing any appreciable difference between the mice treated with BSO for different time periods.

The influence of endogenous GSH level on CAs induced by ϒ-irradiation in HPBLs

Gamma ray induced CAs have been studied in HPBLs as positive control to BSO treated cultures and their results are presented both individually and in pooled form (table 2.7 and 2.8).

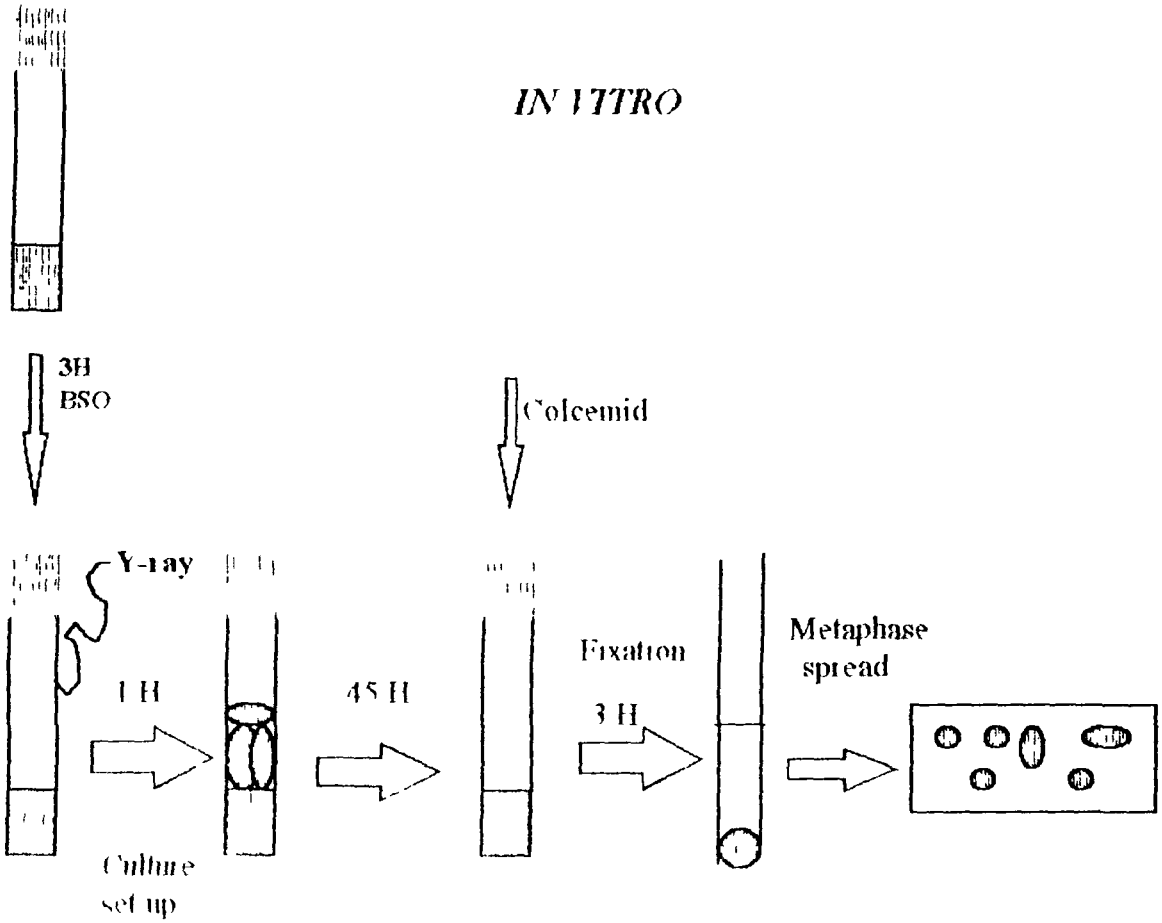
The negative control values have been calculated from two different blood samples. Blood were treated with 0, 1.0, 1.5 and 2.0Gy of ϒ -irradiation. In one case 2.5 Gy was also used. BSO 1 and 5 mM were used. Aberrations were scored from M1 (Fig 2.7). The scored aberrations were: i) Exchanges including dicentrics and rings (both with or without fragments) ii) deletions (including both terminal and interstitial) and iii) Chromatid breaks (Chd. bks). Translocations were not scored. Microphotographs representing aberrations are presented in Fig 2.8a. The frequency of total rearrangements (dicentric+rings), deletions and aberrant metaphases induced by ϒ-irradiation (0 - 2Gy), clearly showed an increase in frequency with increasing radiation doses. However, the frequency of Chd. bks was very low at all the radiation doses. Significant increment in the frequency of deletions and aberrant metaphases were observed in all BSO treated samples. Frequency of Chd. bks did not increase after BSO treatment (table 2.7 and 2.8). The frequency of radiation induced rearrangements did not increase at all in BSO treated samples, on the contrary a tendency of reduction was observed.

The effect of BSO (5mM) itself on the spontaneous CAs was also studied. No significant effect was observed (table 2.7 and 2.8). Therefore, as a whole, the blood sample, treated in vitro with BSO (1 and 5 mM) showed increased sensitivity to radiation than BSO untreated samples. Like in vivo, the frequency of rearrangements were not increased, whereas, the frequency of both aberrant metaphases and deletions increased significantly.



IN VIVO

EXPERIMENTAL PROTOCOL



IN VITRO

Fig. : 2p

Table 2.1 The frequency of first cycle metaphases (M1) in mouse bone marrow cells fixed at different hours

Fixation at (H)	Total Metaphases	M1 (%)	$\bar{X} \pm$ S.D. M1
13	092	97	
	194	96	96.5 ± 0.71
17	302	49	
	160	34	44 0 ± 10.60
20	178	08	
	156	24	16.0 ± 11 31

Table 2.2a Effect of BSO treatment on chromosome aberrations induced by Gamma irradiation in mouse bone marrow cells

Radiation dose(Gy)	BSO mg kg^{-1}	Abt. M (%)	TM	Aberrations/cell			
				Exch.	Isochd. bk.	Chd. bk.	SCU
Dose rate: 18.6 Gy/min.							
0	0	05	112	0	0 02	0 03	0
		01	120	0	0 03	0 01	0
		03	110	0	0 00	0 02	0
	1	02	174	0	0 00	0 04	0
		03	110	0	0 02	0 02	0
	4	04	150	0	0 01	0 03	0
		02	106	0	0.00	0.01	0
		06	110	0	0 02	0 02	0
0 5	0	49	194	0 04	0 19	1 19	0.03
		49	114	0 03	0.11	0.75	0 00
		63	114	0 07	0 26	1.72	0.04
		54	119	0 01	0 13	1 22	0.03
		50	121	0 03	0 14	1 17	0.03
	1	61	197	0 05	0 25	1.50	0.05
		59	152	0.09	0 17	1.06	0.00
		61	121	0 16	0 23	1 48	0.06
	3	75	114	0 09	0 23	1 88	0.05
		77	123	0 04	0 57	3.22	0.03
		70	114	0 10	0 12	1 50	0 08
		67	119	0 08	0 09	1.70	0.10
	4	75	118	0 10	0 94	2 75	0 00
		79	100	0 07	0 34	2 49	0.06
		67	095	0 09	0 20	2.30	0.13
		67	123	0 08	0 22	2 09	0.06
1 5	0	80	123	0 26	0 80	1 88	0 04
		70	108	0 08	0 64	2 07	0.02
		69	196	0 10	0 83	2 32	0.06
		80	113	0 24	0 72	2.65	0.05
		71	121	0 12	0 32	2 03	0 10
	1	79	129	0 22	0 95	2 34	0 18
		63	165	0 13	0 25	1 66	0.06
		67	116	0 10	0 40	1 39	0 00
		74	104	0 12	0 58	2 56	0.13
	3	60	170	0 09	0 94	1 94	0 05
		79	147	0 12	1 10	3 44	0 05
		83	110	0 05	0 94	3 88	0 06
		80	090	0 22	0 42	2 06	0 06
		87	113	0 29	0 46	2 18	0 19
	4	80	138	0 21	1 99	4 54	0 03
		84	070	0 07	2 53	5 50	0 03
		96	077	0 18	1 41	5 75	0.05

Table 2.2b Effect of BSO treatment on chromosome aberrations induced by Gamma irradiation in mouse bone marrow cells

Radiation dose(Gy)	BSO mg kg^{-1}	Abt M (%)	TM	Aberrations/cell			
				Exch	Isochd. bk.	Chd. bk.	SCU
Dose rate: 18.6 Gy/min.							
2.0	0	88	101	0.23	0.53	3.49	0.02
		90	100	0.13	0.48	3.16	0.16
		95	102	0.30	0.66	3.28	0.08
		90	088	0.28	0.56	2.22	0.28
		89	118	0.28	0.58	2.22	0.28
	1	88	100	0.21	0.36	3.21	0.11
		78	100	0.21	0.28	2.92	0.12
		72	112	0.11	0.78	2.22	0.07
		84	120	0.30	0.56	2.59	0.23
	3	84	101	0.19	0.17	2.77	0.13
		74	109	0.12	0.50	2.22	0.06
		83	115	0.17	0.64	3.20	0.20
	4	97	086	0.03	0.60	4.55	0.00
		92	102	0.05	0.55	3.97	0.00
		88	101	0.04	0.85	3.94	0.00
		80	134	0.10	0.83	2.34	0.06
		91	122	0.29	0.91	3.58	0.18
		77	083	0.07	1.05	4.36	0.02
3.0	0	83	087	0.18	0.80	2.55	0.21
		93	080	0.25	0.69	3.69	0.33
		86	095	0.54	0.75	2.60	0.29
	1	90	105	0.40	0.81	3.81	0.20
		98	080	0.25	0.69	3.69	0.33
		87	120	0.20	1.05	3.10	0.28

Table 2.3 Effect of BSO tretament on chromosome aberrations induced by Gamma irradiation in mouse bone marrow cells in vivo

Radiat. dose	BSO mg kg^{-1}	Abt.M.(%) ± SEM	TM (n)	Aberrations/cell ± SEM Exch	IsoChd. bk.	Chd. bk.	SCU
Dose Rate: 18.6 Gy / min							
0 Gy	0	03 ± 0.01	342 (3)	0.00	0.01 ± 0.006	0.02 ± 0.004	0.00
	1	03 ± 0.01	184 (2)	0.00	0.01 ± 0.005	0.02 ± 0.003	0.00
	4	04 ± 0.02	366 (3)	0.00	0.02 ± 0.007	0.03 ± 0.005	0.00
0.5 Gy	0	53 + 2.66	662 (5)	0.04 ± 0.01	0.17 ± 0.03	1.21 ± 0.15	0.03 ± 0.01
	1	60 ± 0.58	470 (3)	0.10 ± 0 03	0.22 + 0.02	1.35 + 0.14	0.04 ± 0.02
	3	72 ± 2.29^{+}	470 (4)	0 08 ± 0.02	0.25 ± 0.11	2.07 ± 0.39**	0.07 ± 0.01
	4	72 ± 3 00^{+}	436 (4)	0 09 ± 0 01	0 43 + 0.17**	2.40 + 0.14**	0.06 + 0.03
1.5 Gy	0	74 ± 2.47	661 (5)	0.16 ± 0.04	0.66 ± 0.09	2.19 ± 0.13	0.05 ± 0.01
	1	71 ± 3 56	514 (4)	0.14 ± 0 03	0.55 ± 0.15*	1.98 ± 0.27*	0.09 ± 0.04
	3	78 ± 7.1	640 (5)	0.15 ± 0 02	0 77 ± 0.14*	2.70 + 0.04**	0.08 + 0.03
	4	87 ± 4 8^{+}	285 (3)	0 15 ± 0 04	1.94 ± 0 35**	5.26 + 0.37**	0.04 + 0.01
2.0 Gy	0	90 ± 1.21	509 (5)	0.24 ± 0 03	0.56 ± 0.03	2.87 ± 0.27	0.14 ± 0.05
	1	81 ± 3.50^{+}	432 (4)	0.18 ± 0.04	0.50 ± 0.11	2.74 ± 0.21	0.13 ± 0.03
	3	80 ± 3 18^{+}	325 (3)	0.16 ± 0.02*	0.44 + 0.14*	2.73 ± 0.28	0.13 ± 0.04
	4	88 ± 3.10	628 (6)	0.10 ± 0.04*	0.80 ± 0.08**	3.79 ± 0.32**	0.04 ± 0.03*
3.0 Gy	0	87 ± 2.96	262 (3)	0.37 ± 0.10	0.87 ± 0.10	2.79 ± 0.21	0.22 ± 0.04
	4	92 ± 3.28	312 (3)	0 28 ± 0.06*	0.85 ± 0.10	3.53 ± 0.22**	0.27 ±0.04

n - number of animals; SCU- sister chromatid union; TM- total metaphases
+ $p<0.001$ 2 x 2 contingency X^2-test ; * $p<0.05$, ** $p<0.001$ X^2-test at df=2

Table 2.4 Effect of BSO treatment on chromosome aberrations induced by Gamma irradiation in mouse bone marrow cells.

Radiation dose(Gy)	BSO mg kg^{-1}	Abt M (%)	TM	Aberrations/cell			
				Exch.	Isochd. bk.	Chd. bk.	SCU
Dose rate: 14.4 Gy/min.							
2.0	0	60	127	0.12	0.30	2.08	0.08
		65	115	0.18	0.18	1.90	0.04
		57	105	0.20	0.23	1.73	0.05
	4	67	113	0.08	0.34	2.23	0.03
		64	119	0.07	0.30	2.36	0.03
	200	78	093	0.07	0.52	2.94	0.05
		59	107	0.04	0.30	2.06	0.00
		90	087	0.05	0.33	3.88	0.01
		81	101	0.09	0.36	3.93	0.01
	300	75	077	0.04	0.49	2.47	0.12
		64	090	0.03	0.32	1.92	0.04
		67	116	0 07	0.40	2.84	0.01
3.0	0	73	111	0.28	0.82	3.11	0.18
		71	108	0.24	0.43	2.86	0.16
		83	121	0.39	0 56	3.46	0.14
		79	111	0 42	0 36	3.29	0.09
	1	90	105	0 40	0 81	3.81	0.20
		81	067	0 27	0.54	2.28	0.15
		87	120	0.20	1 05	3.10	0.28
	200	90	114	0.32	0.69	5.21	0.11
		92	126	0.43	0.98	5.38	0.08
		83	091	0 26	0.91	4.81	0.06

Table 2.5 Effect of BSO treatment on chromosome aberrations induced by Gamma irradiation in mouse bone marrow cells in vivo

Radn dose	BSO mg kg^{-1}	Abt M (%) ± SEM	TM (n)	Aberrations/cell ± SEM Exch.	Isochd. bk	Chd.bk	SCU
Dose Rate: 14.4 Gy / min							
0 Gy	0	03 ± 1.00	342 (3)	0	0.01 ± 0.005	0.02 ± 0.007	0
	4	04 ± 0.40	366 (3)	0	0.01 ± 0.007	0.02 ± 0.006	0
	200	04 ± 1.03	356 (3)	0	0.01 ± 0.003	0.02 ± 0.006	0
2 Gy	0	61 ± 2.33	347 (3)	0.16 ± 0.02	0.24 ± 0.03	1.90 ± 0.10	0.05 ± 0.01
	4	65 ± 1.50	232 (2)	0.07 ± 0.01*	0.32 ± 0.02*	2.29 ± 0.06*	0.03 ± 0.01
	200	77 ± 6.52^{+}	388 (4)	0.06 ± 0.01*	0.37 ± 0.05*	3.20 ± 0.44**	0.02 ± 0.01
	300	69 ± 3.28	283 (3)	0.05 ± 0.01*	0.40 ± 0.05*	2.41 ± 0.27*	0.06 ± 0.03
3 Gy	0	76 ± 2.75	451 (4)	0.33 ± 0.04	0.54 ± 0.10	3.18 ± 0.12	0.14 ± 0.02
	4	86 ± 2.64^{+}	292 (3)	0.29 ± 0.06	0.80 ± 0.10*	3.06 ± 0.40	0.21 ± 0.04
	200	88 ± 2.72^{+}	331 (3)	0.33 ± 0.05	0.86 ± 0.09**	5.13 ± 0.17**	0.08 ± 0.01

n - number of animals; SCU- sister chromatid union; TM- total metaphases
+ $p<0.001$ 2 x 2 contingency X^2-test ; * $p<0.05$, ** $p<0.001$ X^2-test at df=2

Table 2.6 Induction of chromosome aberrations by Gamma irradiation in BSO-treated (5 mg kg^{-1}) mouse bone marrow cells for different time periods.

BSO-treat. period (h)	Radiat. dose	TM	Abt. M (%)	Aberrations/cell			
				Exch	Isochd. bk.	Chd. bk.	SCU
Radiation Dose Rate: 18.6 Gy/min							
0	1.5	123	80	0.26	0.80	1.88	0.04
		108	70	0.08	0.64	2.07	0.02
		196	69	0.10	0.83	2.32	0.06
		113	80	0.24	0.72	2.65	0.05
		121	71	0 12	0.32	2.03	0.10
10	1.5	129	84	0.25	1.14	3.70	0.08
		131	83	0.10	1.70	4.60	0.03
		097	90	0.07	0 78	4.04	0.02
15	1.5	118	82	0.08	0.90	3.40	0.02
		109	88	0.08	1.70	4.50	0.01
18	1.5	106	95	0.16	1.08	3.96	0.01
		111	80	0 10	1.06	3.50	0.01

Table 2.7 Effect of BSO treatment on chromosome aberrations induced by Gamma irradiation in human peripheral blood lymphocytes in vitro.

Dose (Gy)	BSO (mM)	Abt. M (%)	TM	Aberrations/cell			
				Dicen.	Deln.	Chd. bk.	Ring
Radiation Dose Rate: 12.39 Gy/min							
0	0	02	101	0	0.005	0.01	0
		02	102	0	0.005	0.01	0
	5	03	113	0	0 010	0.02	0
		02	110	0	0.010	0.02	0
1.0	0	37	096	0.08	0.450	0.01	0.02
		41	103	0.09	0.390	0.02	0.01
	5	61	102	0.08	0.740	0.04	0.01
		68	111	0.06	0.690	0.06	0.00
1.5	0	51	108	0 10	0.480	0.04	0.01
		53	101	0.13	0.540	0.04	0.01
	1	61	121	0.09	0 670	0.04	0.02
		69	107	0 07	0 700	0.04	0.00
	5	69	113	0.08	0.760	0.06	0.01
		73	116	0 05	0.810	0.07	0.01
2 0	0	77	109	0 15	0.740	0.05	0.05
		69	091	0 13	0.700	0.05	0.02
		77	093	0.16	0.780	0.03	0.03
	1	81	114	0 10	1 300	0.07	0.04
		85	089	0 13	1.390	0.09	0.02
	5	91	106	0 15	1 980	0 20	0.03
		92	093	0 13	1 790	0 16	0.02
2.5	0	53	108	0 45	0 670	0.05	0.06
	1	76	075	0 40	1.04	0.29	0.03
	5	78	105	0 25	1 14	0 09	0 07

Table 2.8 Effect of BSO treatment on chromosome aberrations induced by Gamma irradiation in human peripheral blood lymphocytes in vitro.

Radiat. dose	BSO mM	Abt M % ± SEM	TM (n)	D + R	Aberrations/cell + SEM Deletion	Chd.bk.
Dose Rate: 12.39 Gy / min						
0 Gy	0	2.0 ± 0	203 (2)	0	0.005 ± 0.00	0.01 + 0.00
0 Gy	5	2.5 ± 0.5	223 (2)	0	0.01 ± 0.00	0.02 ± 0.00
1.0 Gy	0	39 ± 2	199 (2)	0.10 ± 0.00	0.42 ± 0.005	0.01 ± 0.00
	5	64 ± 3.5^{+}	213 (2)	0.07 ± 0.01	0.71 ± 0.01**	0.05 ± 0.01
1.5 Gy	0	52 ± 1	209 (2)	0.12 ± 0.01	0.51 ± 0.00	0.04 + 0.00
	1	65 ± 4^{+}	228 (2)	0.09 ± 0.02	0.68 ± 0.00*	0.04 + 0.00
	5	71 ± 2	229 (2)	0.07 ± 0.01	0.78 ± 0.005**	0.06 + 0.00
2.0 Gy	0	74 ± 3	293 (3)	0.18 + 0.01	0.74 ± 0.006	0.04 + 0.01
	1	83 ± 2	203 (2)	0.14 ± 0.00	1.34 ± 0.01**	0.08 + 0.01
	5	91 ± 0.5^{+}	199 (2)	0.16 ± 0.01	1.80 ± 0.02**	0.18 ± 0.02
2.5 Gy	0	53	108 (1)	0.51	0.67	0.05
	1	76	075 (1)	0.43	1.04**	0.29
	5	78	105 (1)	0.32*	1.14**	0.09

+ $p<0.001$ 2 x 2 contingency X^2-test ; * $p<0.05$, ** $p<0.001$ X^2-test at df=2
TM Total Metaphase; n number of sample; D+R Dicentric + Ring

Fig 2.1: Hoechst- Sunlight- Giemsa staining pattern of chromosomes of mouse bone marrow cells grown in presence of BUdR.

a, one division cell cycle; b,c,two division cycles;
SCE, Sister Chromatid Exchanges.

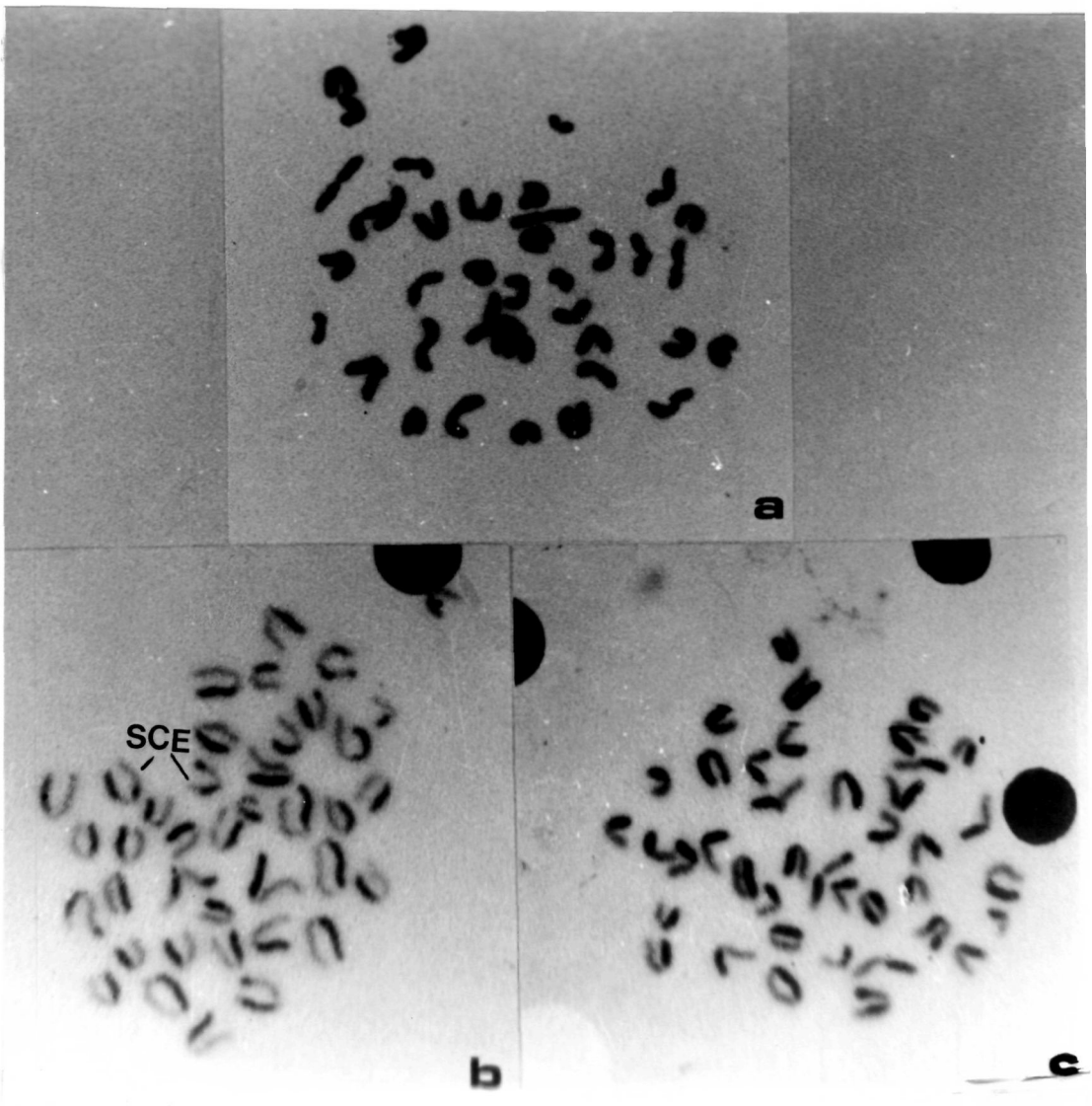
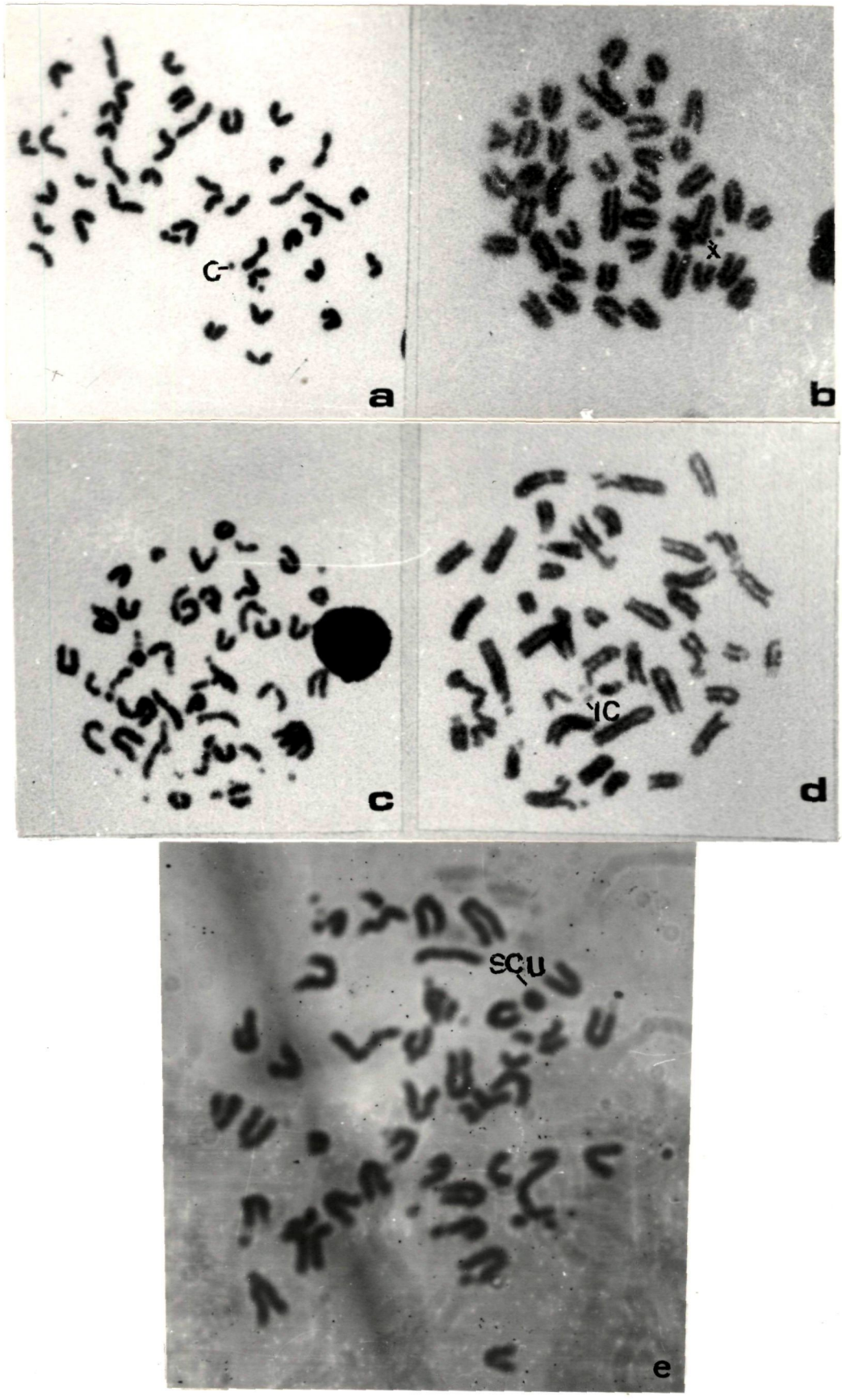
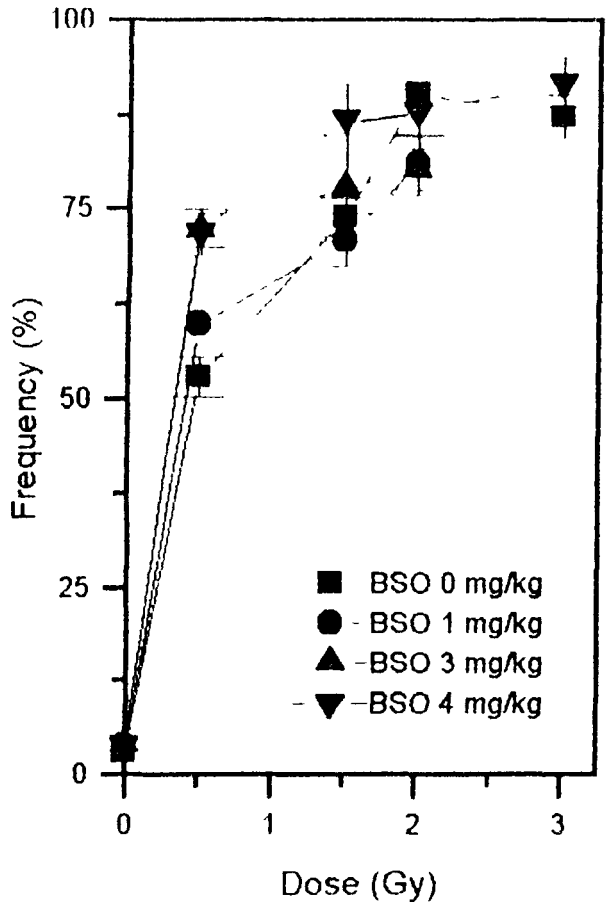

a
SCE
b
c

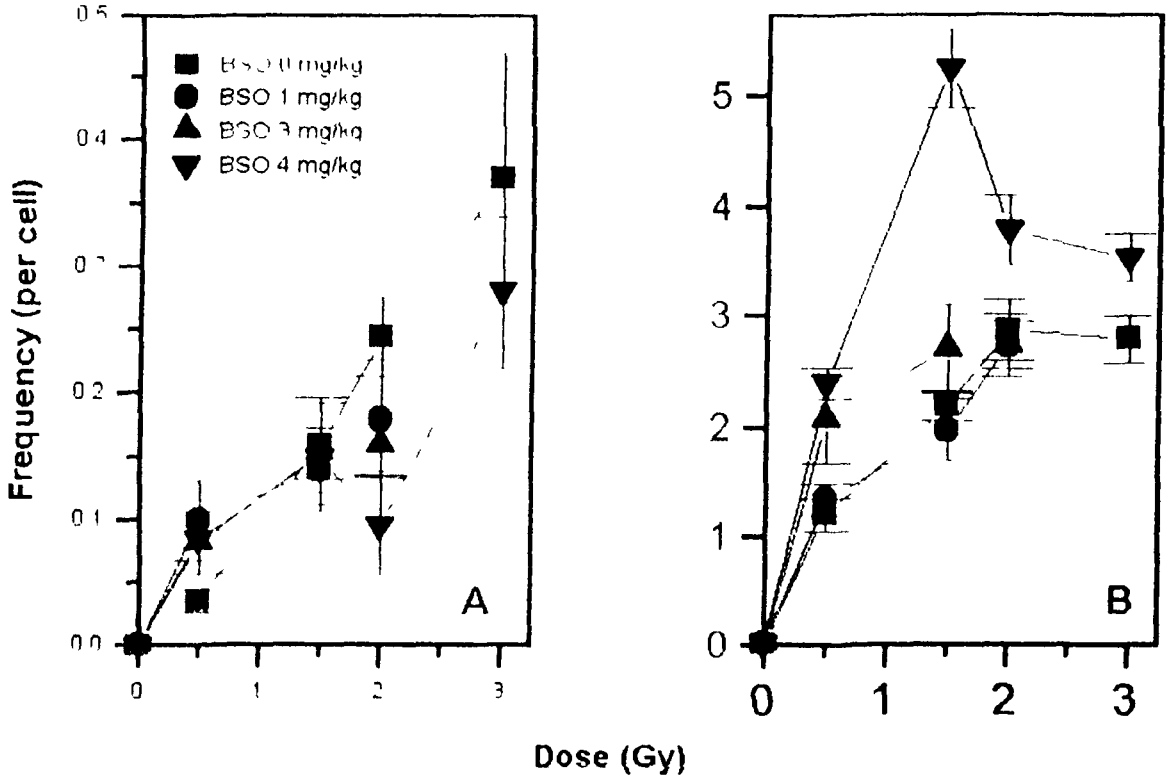
Fig 2.2: Microphotographs showing Gamma irradiation induced chromosome aberrations in first cycle metaphases of mouse bone marrow cells. C, Chromatid break; IC, Isochromatid break; X, Exchange; SCU, Sister Chromatid Union.


C
a
X
b
c
IC
d
SCu
e



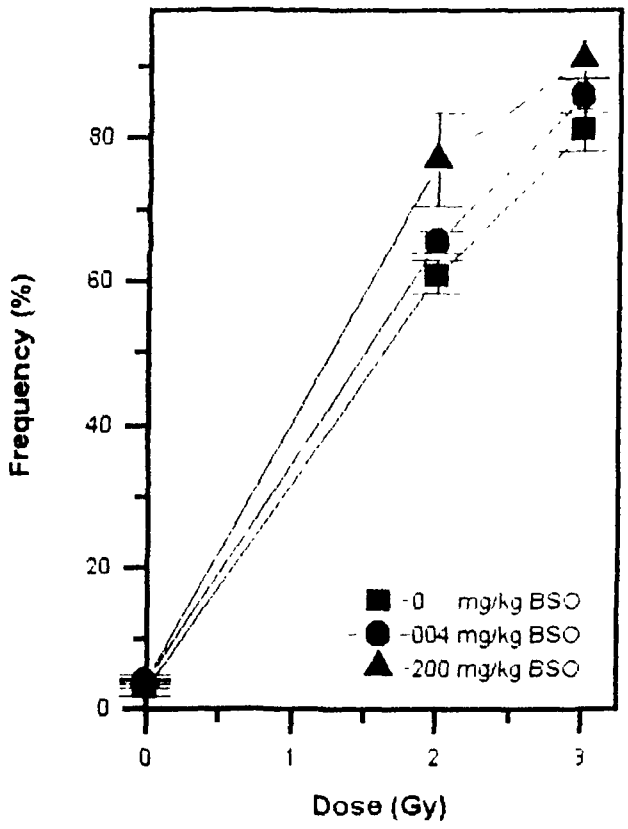
Frequency of radiation induced aberrant metaphases with or without BSO in vivo

Fig. 2.3



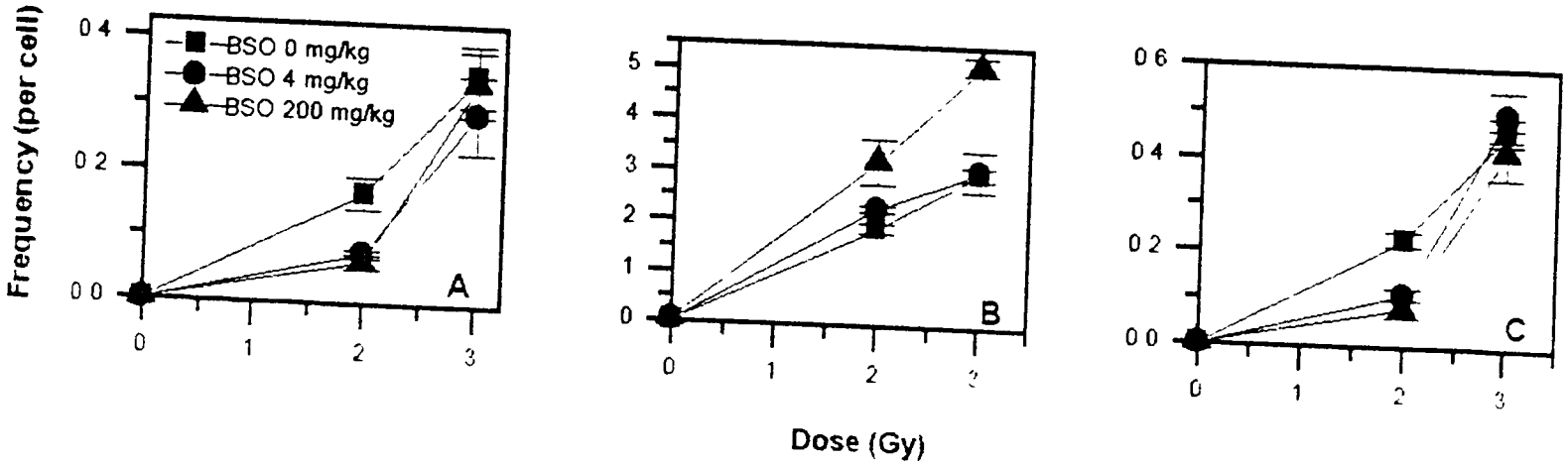
Frequency of exchanges (A) and chromatid breaks (B) induced by Gamma irradiation with or without BSO in vivo

Fig. 2.4



Frequency of radiation induced aberrant metaphases with or without BSO in vivo

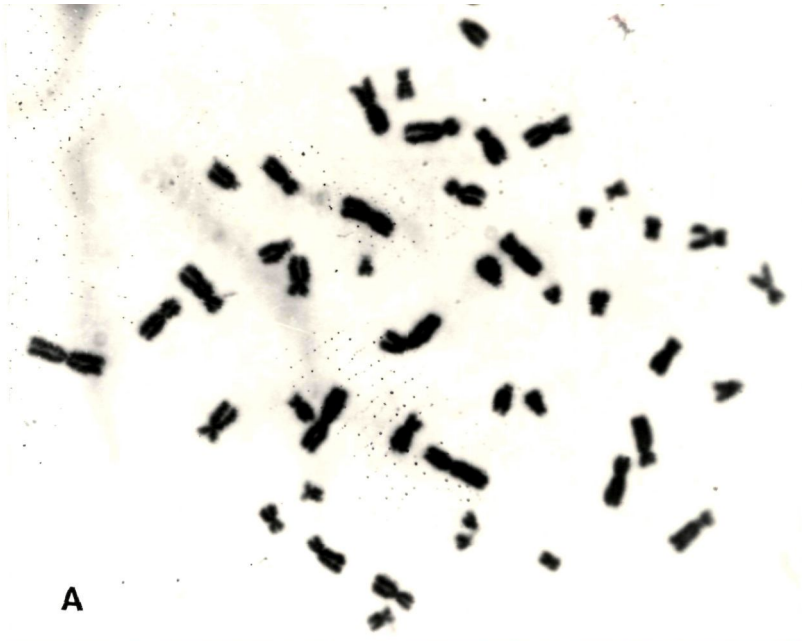
Fig. 2.5

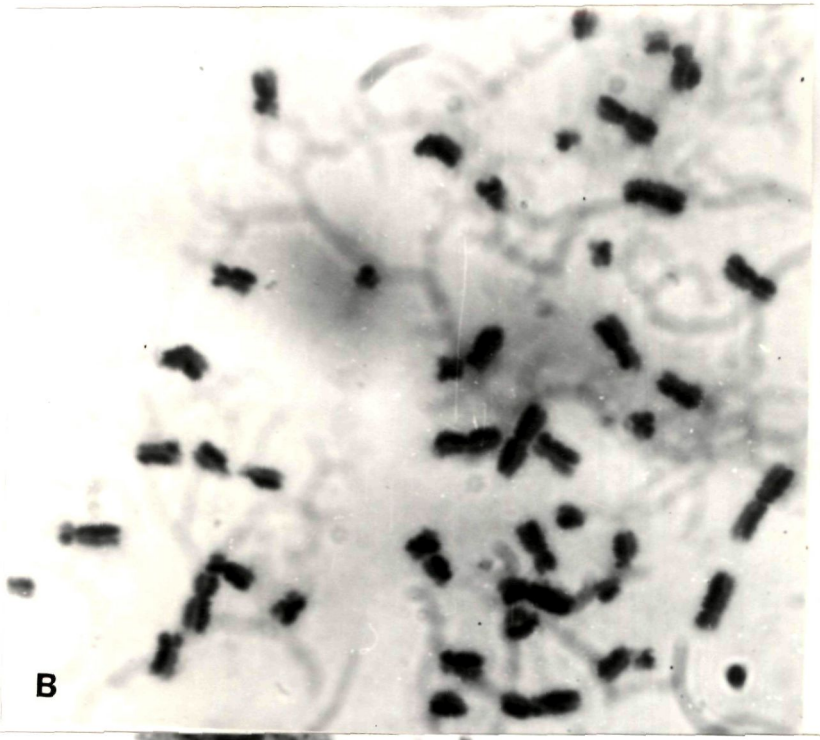


Frequency of radiation induced excnanges (A). Chromatid breaks (B) and total rearrangements (C) with or without BSO in vivo

Fig. 2.6

Fig 2.7: Hoechst- Sunlight- Giemsa staning pattern of chromosomes of human peripheral blood lymphocytes grown in presence of BUdR. A, one division cycle; B and C, two division cycles.


A


B

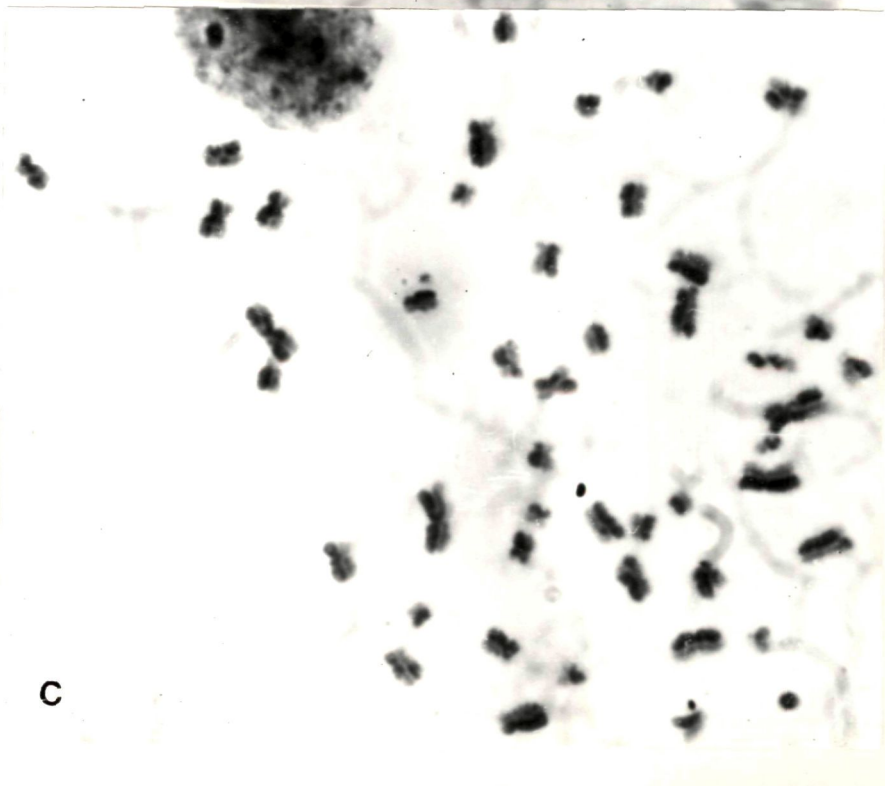
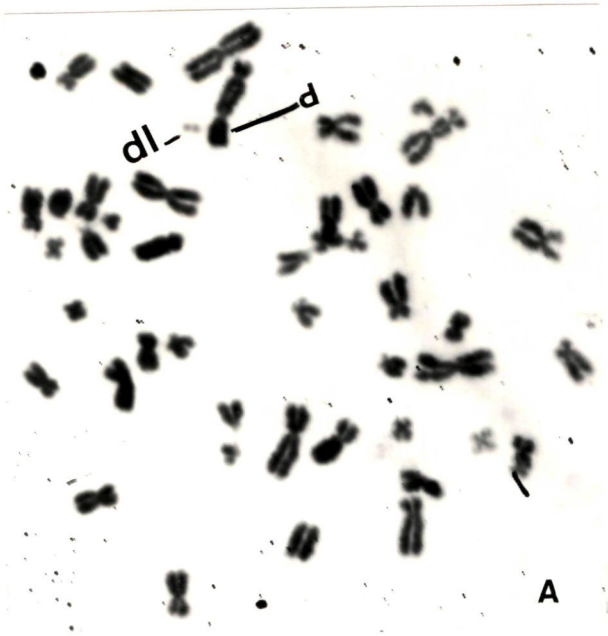
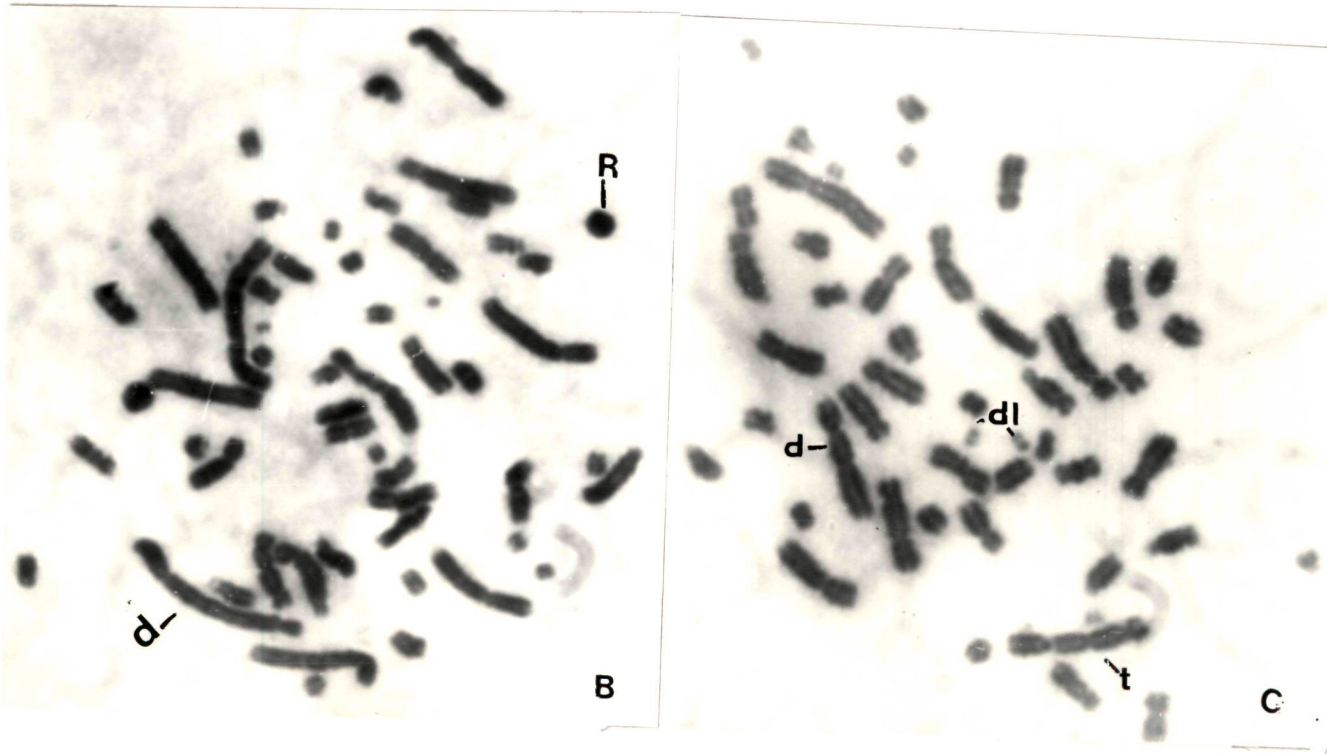

C

Fig 2.8: Microphotographs showing A, Gamma irradiation induced; B and C, Bleomycin induced chromosome aberrations in first cycle metaphases of human peripheral blood lymphocytes. d, dicentric; dl, deletion; t, tricentric; R, ring.


dl
d
A


R
d
B
d
dl
t
C

DISCUSSION

Historically, studies on the radiosensitizing effect of oxygen directed attention towards cellular thiols as a major determinant of the radiation response of the cells. The well known competition theory was introduced in 1955 by Alexander and Charlesby, the major feature of which was the assumption of competition between damage restituting and damage fixing reactants for radiation induced radicals in key target molecules (Alper 1979; Edgren et al. 1985). The restituting species is identified as cellular thiols, which repair the damaged target by hydrogen donation.

Many experimental observations have been made, which are consistent with the competition model. In many of the tests, cellular survival was used as the end points (Alper 1979) The competition model concerns the initial physico-chemical processes. On the other hand, survival, determined after several hours or days of radiation exposure involves several biochemical reactions, which may modify the effect of the earlier radical processes, eg. by repairing the proportion of damages. Therefore, cell survival may not be an appropriate end point for the study of the effect of radical reactions. Another question concerns, the role of the cellular thiols. In mammalian cells, GSH amounts to about 90% of the non protein thiols Direct evidence that, sulphydryl and oxygen can compete in the way suggested by the competition model was provided in pulse radiolysis experiments (Adam et al. 1968) In view of the participation of GSH in a great number of diverse metabolic functions in the cell (Kosower and Kosower 1978), it is conceivable that it will also take part in some radiation induced biochemical reactions.

Over the past few years, advantage has been taken of the GSH deficient cell strain in extensive studies to clarify the role of GSH in radiation response of the cells. In view of the possible participation of GSH in both physicochemical (restorative) and biochemical (repair) radiation - reactions, particul-ar attention was given to the choice of end points in an attempt to investigate the effect of the two proceeses separately.

In this study CAs is the end point considered, which is generated from the DNA-dsbs (Natarajan et al 1980; Bryant 1984). DNA SSbs and DNA base damage have also been suggested as DNA - lesions, giving rise to indirectly induced DNA dsbs with subsequent formation of CAs (Preston 1980, 1982). BSO was used to study the effect of GSH depletion on radiosensitization of BMCs in vivo and HPBLs in vitro. The rationale for BSO treatment is based on the premise that GSH serves as a major endogenous cellular

radioprotector (Meister 1983) and that GSH depletion itself may lead to significant radiosensitization.

In order to score CAs, it is advisable to use the BUdR differential method to determine the most suitable harvesting time to have an optimum number of cells in M1 (Bianchi et al. 1982). Therefore, initially one experiment was carried out to select suitable fixation time for mouse BMCs Maximum number (95%) of M1 cells were observed at 13h fixation time and since ionizing radiation induces delay in cell cycle kinetics (Lloyd et al. 1977), therefore, in all in vivo experiments, cells were fixed at 13h For HPBLs, BUdR powder was added in cultures and only M1 cells were considered for CAs studies.

Initially, an attempt was taken to delineate the treatment timing of BSO before exposure of whole mouse to Y-irradiation. The observed data clearly indicate that BSO-incubation period for 10, 15 and 18h increased the radiosensitivity to a same degree. It was reported that intermediate rates of depletion were seen in BMCs of mouse with nadirs at 8-12h (Lee et al 1987). The present results indicate, that maximum level of GSH depletion could be achieved at 10h of incubation following single dose of BSO and therefore, in all in vivo studies, the BSO treatment period was 10h.

Present results indicate, that the presence of BSO increased the cellular radiosensitivity, when CAs is considered as end point This increased radiosensitivity is dependent to BSO concentrations. In this study (in vivo), extremely low (1,4 mg kg^{-1}) and moderately high concentration (200, 300 mg kg^{-1}) of BSO was used. Out of three extremely low concentrations used, 4 mg kg^{-1} is the one, which is effective to all the radiation doses, except 3Gy, where the degree of increment was very less. Both 1 and 3 mg kg^{-1} is also effective at 0.5 and 1.5 Gy of Y-irradiation but not at higher doses. It is clear that BSO 200 mg kg^{-1} is more efficient to increase the cellular radiosensitivity than 4 mg kg^{-1}. This could be due to better depletion of endogenous GSH by 200 mg kg^{-1} than 4mg kg^{-1} (Chapter 1). Incidentally, 1 and 3 mg kg^{-1} of BSO has provided significant reduction in the frequency of CAs induced by 1 5 and 2Gy However, the reason is not quite clear. Very recently there is a report regarding a protective effect of BSO in micronuclei induction by radiation in mouse BMCs (Sarma et al 1996).

In this study, of all the aberrations scored in mouse BMCs, Chd bks. are the most frequent one and showed consistent sensitization in BSO treated cells. The second major group is the Iso-chd bks which also showed similar sensitization after BSO treatment. However,

the other important group, exchanges and rearrangements did not increase at all. This shows that Chd. bks and Iso-chd bks, which are open type of breaks were increased readily by BSO treatment but endogenous GSH depletion failed to enhance exchange type of aberrations. To the best of our knowledge, no data are available on the effect of BSO on radiation induced mammalian CAs. However, our present observation regarding the increased frequency of Chd bks and Iso-chd bks. is not in agreement with the reported failure in increase in radiosensitivity with respect to DNA SSbs in GSH depleted cells by BSO under aerobic conditions (Edgren et al. 1985). Others have also found, little or no sensitization of aerated cells by depleting cultures of GSH (Bump et al. 1982; Clark et al. 1984). However, equal sensitization of aerated and hypoxic cells using similar in vitro systems has also been reported (Koch et al. 1984; Mitchell et al. 1983). Depletion of intracellular GSH by BSO, sensitized cells to X-irradiation at all oxygen tensions in V79 - 379A cells, has also been reported by Shrieve et al. (1985). Therefore, reports are available, both in favour and against the view that BSO treatment increased the radiosensitivity of aerated cells.

The radiosensitivity of GSH deficient and proficient cells were also studied by measuring DNA SSbs (Edgren et al 1981) with the unwinding technique in weak alkali. The SSb in $GSH^{-/-}$ cells remained unchanged, whether irradiation was performed in air or hypoxia. However, it has been observed, that the SSbs induced in GSH deficient cells under oxic condition was repaired much slower and remained incomplete after 2h.

In all studies, DNA SSbs were chosen as the end point, determined shortly after exposure of the cells, because the interest was directed to see the function of GSH in the initial radiation induced radical processes. Therefore, this end point, in contrast to clonogenic survival, can be assumed to reflect the effect of the initial radical reactions without any major interference of the biochemical processes which follow irradiation and some of which may themselves be GSH dependent (Edgren et al 1981; Revesz and Edgren 1984).

In the present study, CAs is the end point which has been assessed by conventional method of studying cells in first metaphase stage Since, it does not allow direct measurement of chromosome damage immediately after irradiation, therefore, interference of biochemical processes following irradiation could occur. It is wellknown that, primary lesions induced in DNA are subjected to cellular repair. Unrepaired or misrepaired lesions

give rise to CAs (Natarajan and Zwanenburg 1982) Therefore, in this investigation, the increased radiosensitivity of the BSO treated cells with respect to Chd bks and Iso-chd bks or deletions, could be a failure of efficient repair in GSH depleted cells. However, the possibility of more radical induced DNA lesions and strand breaks in GSH depleted cells can not be ruled out for the observed higher radiosensitivity.

It is conceivable, that GSH only in close proximity to the critical DNA target can function in the radical competition processes In this study, the GSH measurement has been performed in whole cells, which may not reflect the change in the amount of GSH at locations where it could be of importance in treatment. Edgren (1987), reported observations that depletion of GSH by BSO occurs to different extents in the nucleus and the cytoplasm and therefore the OER for cells depleted of GSH by treatment with BSO to a comparable degree of GSH defiency as in the genetic mutants, did not show a similar decrease in OER (Edgren et al 1986) Edgren (1987) demonstrated that the total amount of free, oxidized and reduced GSH in the cells of $GSH^{+/+}$ strain was 13.5 $\pm$ 0.7 nmol per mg protein About 10% of this amount was located in the nuclei. After BSO treatment, the GSH content in the whole human fibroblast cells of the $GSH^{+/+}$ strain was decreased to about 5%, whereas, in the nuclei it was about 55% of the normal Therefore, it could be, in the present study, that BSO treatment both in vivo and in vitro, deplete cytoplasmic content more eficiently than nuclear GSH content, and if it is so, then the initial number of free radical induced DNA lesions may not vary with great degree between BSO treated and untreated cells While extranuclear GSH, may not play any major role to save DNA from radical reactions, are involved in the numerous post-irradiation biochemical repair processes The data presented here, support this interpretation because exchange and rearrangement type of aberrations failed to increase, which needs association and rejoining of radiation induced DNA strand breaks in BSO treated cells. It implies that, presence of GSH may be important for rejoining of strand breaks induced by radiation. More increased radiosensitivity by 200 mg kg^{-1} than 4 mg kg^{-1} BSO in cells could be attributed due to better degree of GSH-depletion in both the compartment of cell by the former concentration of BSO Therefore, incresed radical induced DNA lesions in addition to low efficient repair could be the reason for significant increase in the frequency of both Chd bks and Iso chd bks in BSO treated cells

CHAPTER III

DOES GLUTATHIONE INVOLVE IN DNA REPAIR?

LITERATURE REVIEW

Endogenous thiols, especially the tripeptide GSH, play an important role in the protection of cells against the damaging effect of ionizing radiations. Modification of the radiation induced damages of cells by GSH may occur at different levels of the process leading from the primary lesions to the final cell damages, eg

1 GSH may interfere at the level of induction of lesions in the target by scavenging radicals of the radiolysis of water and respectively, by transfer of hydrogen atoms to target radicals (Howard-Flanders et al 1963; Revesz et al. 1963; Held et al. 1982).

2. GSH may be involved in enzymatic repair processes of radiation induced DNA damage (Edgren et al 1981; Edgren and Revesz 1985)

Using GSH-deficient human fibroblast and lymphoblastoid cells, the role of GSH, in the initiation of damage leading to DNA breakage has been demonstrated (Revesz et al. 1979; Edgren et al 1980). Radiation induced DNA breaks were shown to be repaired rapidly, error free or error prone, by different mechanisms (Painter et al 1971;Hanawalt et al 1979). As yet little is known about the importance of GSH in these processes. The role of GSH in regard to the post irradiation repair of DNA breaks has also been evaluated in GSH^- cells (Edgren et al 1981). When GSH^- cells were exposed to irradiation under hypoxic condition, practically all the induced single strand breaks (SSbs) were gradually rejoined within about 1h of aerobic incubation, independently of the dose of irradiation. Similar observations were made with a great number of different GSH^+ cells (Lohman 1968, Ormerod and Stevens 1970). In contrast, when irradiation was performed under aerobic conditions, GSH^- cells failed to repair a considerable part of the induced SSbs during an identical incubation period The failure of GSH^- cells, but not to GSH^+ cells to repair SSbs induced by aerobic irradiation was confirmed in experiments, in which, the cells were exposed to a radiation dose, split into two equal fractions and given with an interval of either 30 or 60 min (Nishidai et al , see Revesz et al. 1984). They suggested that this failure of rejoining of a majority of aerobically induced SSbs in GSH^- cells indicates that the activity of some of the enzymes involved in the repair of this particular radiation damage is dependent on GSH function, probably as a cofactor Therefore, they interpreted these observations as an involvement of repair system in the rejoining of oxically induced SSbs, different from that involved in the rejoining of hypoxically induced SSbs. The former is

clearly dependent on GSH In thiol substituted (GSH, MPG or DTT) GSH^- cells had the capacity to rejoin radiation induced SSbs to a fairly normal extent has also been demonstrated (Edgren et al 1981) Further support for the concept that the presence of GSH is essential for the rejoining of the particular aerobically induced SSb was obtained in experiments in which the irradiated GSH^- cells admixed with unirradiated GSH^- or GSH^+ cells and incubated together for some times (Edgren 1982). In the former case, the SSb rejoining capacity of the irradiated cells were not appreciably improved. In contrast, admixed GSH^+ cells enhanced the rejoining capacity to a normal level. This enhancement was inhibited, however, if GSH^+ cells were pretreated with misonidazole (MISO), which decreased GSH content to about 30% before admixture (Edgren, unpublished: in Revesz et al. 1984).

Few reports are also available regarding the importance of thiols in the repair mechanisms of DNA containing AP (apurinic or apyrimidinic) sites. All the cells seem to possess an AP-endonuclease activity that hydrolyses the phosphodiester bond 5' to AP sites in a DNA leaving 3'-OH and 5'-PO_4 ends. But the excision of an AP-site requires another clevage 3'- to the damage. AP-endonuclease I breaks the phosphodiester bond 3' to AP sites leaving 5' PO_4 bonds (Mosbaugh and Linn 1980), are not endonuclease but seems to be β- elimination catalysts (Bailly and Verly 1984). β -elimination acting as an AP site in DNA leaves a 3' terminal α , β- unsaturated aldehyde derived from the base free deoxyribose. Monoharan et al.1988 (see Bailly and Verly 1988) have found that thiols react with this unsaturated aldehyde. Later on it has been observed that addition of thiols to the double bond of the 3'-terminal sugar resulting from β-elimination prevents a subsequent δ-elimination (Bailly and Verly 1988).

An exchange aberration is thought to arise after lateral association between partially denatured DNA regions in two different chromosomes and such region might occur as a consequence of both DNA SSbs as well as DNA double strand breaks. An aberration then arises as a consequence of misrepair of this complex. A prerequisite for the formation of a chromosome exchange complex is also that the DNA lesions which give rise to the exchange aberration are in close proximity (Natarajan and Ahnstrom 1968; kihlman 1977).

Secondly, exchange complex formation also depends on the DNA-repair of the lesions; i.e. no exchange complexes are formed if the time for DNA-repair is shorter than the association time. Thus, short lived DNA breaks give rise to exchange complexes only for short interaction distances, whereas for longer interaction distances the exchange complexes are formed for long-lived DNA-breaks (Holmberg and Gumausker 1986).

It has been demonstrated that short lived DNA breaks can, in the absence of arabinoside-C (ara-C; repair inhibitor), be repaired within that time but in the presence of ara-C more such breaks are able to interact and form exchange complexes. In contrast, the yield of exchange aberrations formed from interactions between long-lived DNA breaks is not affected by ara-C or UV-C and the reasons could be : **a**. the DNA repair of long lived DNA breaks is not affected by UV-C or ara-C; **b**. the long lived DNA breaks are DNA double strand breaks, which are repaired by some kind of recombinational repair and this process requires an exchange complex (Holmberg and Gumausker 1986).

In the present study, we have made an attempt to resolve the role of GSH in exchange aberration formation (due to misrepair). Since DNA dsbs are responsible for CAs formations (Natarajan and Obe 1978; Natarajan et al. 1980), therefore in this study, data mainly indicate the influence of GSH on repair / misrepair of DNA dsbs induced by ϒ-irradiation.

MATERIALS AND METHODS

Materials

1. Reduced Glutathione (GSH; Sigma,USA): GSH is the dominant low molecular weight non protein thiol compound, present in all animal cells. It is a tripeptide, though not derived from protein. Glutamic acid, cysteine and glycine are bound together to form GSH (L-ϒ-glutamyl-l csteinyl- glycine). The glutamic acid residue joined unusually with its ϒ-COOH, rather than the α-COOH group, with cysteine. It is a thiol compound and has been shown to protect biological tissues by scavenging the primary radicals produced by radiolysis of water. Concentration of GSH was used 400 mg kg^{-1} in mice for in vivo studies and for in

vitro studies 20 mM was used in HPBLs. The solution was prepared freshly in sterile distilled water and was used.

2. Glutathione ethyl ester (γ-glutamyl-cysteinyl-glycyl ethyl ester; Sigma, USA):

This is a derivative of GSH. It can enter cells more easily than GSH and is readily converted to GSH after transport. The conversion of GSH from GSH-ester does not require energy and the levels of cellular GSH achieved is not inhibited by feed back inhibition. It is also reported as a radioprotector and can elevate the level of GSH much higher than its normal status (Puri and Meister 1983; Anderson and Meister 1989).

The concentration used in in vitro studies was 20 mM. The solution was prepared fresh in sterilized distilled water prior to use.

3. Buthionine Sulfoximine (BSO; Sigma, USA): It has already been described in the Materials and Methods section of Chapter I. In this experiment, BSO was used only in in vivo study and the concentration was 4 mg kg^{-1}.

4. Radiation: Described in Materials and Methods section of Chapter II.

Experimental system

Similar as described in Materials and Methods section of Chapter I.

Methods

Experimental procedure

a. *Treatment of GSH*

It was injected/added just after γ-irradiation. In mice, it was injected intraperitoneally for in vivo studies. For in vitro studies, GSH was added in 1ml aliquot of blood samples, soon after irradiation, mixed properly and kept for 3h at 4°C before culture was set up.

b. *Treatment with GSH-ester*

This was used only in in vitro studies. 20mM of GSH-ester added into 1ml aliquot of blood sample, soon after irradiation and kept for 3h at 4°C before setting up of culture.

c. *Irradiation*

For in vivo studies, mice were irradiated in a similar manner as already described in the same section of Chapter II. Mice were irradiated only with 2Gy. For in vitro studies, 1ml

aliquot of blood was taken in a sterilized small 25 ml flat bottom glass beaker. The lymphocytes were exposed to 2 and 3 Gy of ϒ-irradiation either at room temperature or in ice. Samples which were irradiated in ice were kept in ice for 30 min before irradiation. Blood samples, which were irradiated at room temperature were kept in 37^{0}C and those irradiated on ice, were kept at 4^{0}C for 1h after irradiation.

d. *Culture procedure*

The methodology for collection of blood and setting up of culture was similar as described in Chapter II.

e. *Chromosome preparation, staining and scoring*

Similar as described in Chapter II.

f *Calculation and Statistics*

The method of calculation and formulae applied for statistics are desribed in Chapter II.

RESULTS

GSH-posttreatment after ϒ-irradiation to BSO treated and untreated mice

ϒ-rays induced CAs have been studied in mouse BMCs as positive control to GSH posttreated samples in each experiment. GSH post treatment has also been performed after ϒ-irradiation in BSO treated mice. All their results are presented both individually and in pooled form in table 3.1. Only one radiation dose was used (2Gy). At least two mice were used in each step. Individual data of each type of aberrations and aberrant metaphases did not show any appreciable difference between 2 Gy and 2Gy + GSH samples, however, the pooled data showed that there was a reduction in the fequency of Iso-chd. and Chd. bks. without any change in frequencies of aberrant metaphases.

GSH posttreatment to BSO treated mice showed that there was a significant increase in the frequency of rearrangement type of aberrations, while significant reduction in the frequency of Iso-chd. bk. and Chd. bk. was observed without any change in the frequency of aberrant metaphases (table 3.1). 2Gy irradiation to BSO treated mice showed higher sensitivity to irradiation with respect to individual aberration types except for exchanges, where frequency was reduced significantly.

GSH and GSH- ester posttreatment to γ-irradiated lymphocytes in vitro

Two different sets of experiments were performed, one with 2Gy and the other with 3Gy of irradiation Since, these experiments were performed separately, therefore two different dose rates had to use. Data from experiments of both the sets are pesented individually in table 3.2 and pooled in table 3.3

These experiments were conduted by irradiating (2 and 3Gy) HPBLs in Go stages both at room temperature and at 4^0C Then GSH and GSH-ester was added immediately after irradiation Since irradiation at 4^0C block the repair of irradiated Go cells, therefore, one would expect rise in the frequency of aberrations in sample irradiated at 4^0C. The data presented in table 3.2 and 3.3 showed that there was a significant rise in the frequency of deletion without affecting the frequency of aberrant metaphases in samples irradiated at 4^0C. However, the frequency of rearrangements was significantly reduced.

In GSH and GSH-ester treated samples, the frequency of deletion was reduced significantly and simultaneously the frequency of rearrangements was increased considerably However, There was no appreciable change in the frequency of aberrant metaphases, in GSH added samples whereas considerable reduction in the frequency of aberrant metaphases was observed when GSH-ester was used.

The effect of GSH and GSH-ester alone in HPBLs have also been studied and the data indicate that there was no induction of any aberration in the system.

Table 3.1 Effect of GSH-posttreatment on chromosome aberrations induced by Gamma irradiation in normal and BSO treated (4 mg kg^{-1}) mouse bone marrow cells.

Exptl. Cond.	TM	Abt. M %	Aberrations/cell			
			Exch.	Isochd. bk.	Chd. bk.	SCU
Radiation Dose Rate: 18.6 Gy/min						
2Gy	117	91	0.36	0.71	3.66	0.50
	088	90	0.28	0.63	3.88	0.31
Pooled	*205*	*91*	*0.32*	*0.67*	*3.77*	*0.39*
2Gy + GSH	124	84	0.31	0.65	3.30	0.05
	102	85	0.33	0.68	2.88	0.26
	093	90	0.25	0.44	3.28	0.14
	319	*86*	*0.30*	*0.59*	*3.15***	*0.15*
BSO + 2Gy	101	88	0.04	0.85	3.94	0.00
	134	80	0.10	0.83	2.34	0.06
	083	77	0.07	1.05	4.36	0.02
	318	*82*	*0.07*	*0.91*	*3.55*	*0.03*
BSO + 2Gy	043	84	0.40	0.35	2.42	0.35
+ GSH	097	87	0.35	0.55	3.46	0.25
	107	92	0.36	0.69	3.78	0.33
	101	83	0.24	0.66	3.53	0.19
	348	*87*	*0.34**	*0.56***	*3.30*	*0.28**

* $p<0.05$, ** $p<0.001$ X^2-test at df=2

Table 3.2 Effect of GSH(20 mM) and GSH ester (20 mM) posttreatment on chromosome aberrations in Gamma irradiated human peripheral blood lymphocytes in vitro.

Expt. condn	Abt M (%)	TM	Aberrations/cell			
			Dicen	Delu	chd. bk.	Ring
Radiat. Dose Rate: 12.39 Gy/min						
Control	2	101	0	0.005	0.01	0
	2	102	0	0.005	0.01	0
Control(4^0C)	3	098	0	0.01	0.02	0
GSH	3	111	0	0.01	0.02	0
	1	110	0	0.01	0.02	0
GSH-ester	3	125	0	0.01	0.01	0
	3	101	0	0.01	0.01	0
2 Gy	77	109	0.10	0.74	0.05	0.05
	69	091	0.13	0.70	0.05	0.02
	77	093	0.16	0.78	0.04	0.03
2 Gy(4^0C)	74	092	0.10	0.99	0.08	0.01
	68	101	0.09	0.91	0.07	0.01
	60	086	0.06	0.86	0.06	0.00
2 Gy(4^0C)	61	056	0.20	0.59	0.00	0.05
+ GSH	67	071	0.24	0.58	0.03	0.04
	65	129	0.29	0.52	0.06	0.07
	61	092	0.19	0.51	0.04	0.06
Radiation Dose Rate: 8.19 Gy/min						
3 Gy	53	108	0.45	0.67	0.05	0.06
	66	085	0.41	0.49	0.02	0.07
	61	103	0.32	0.46	0.07	0.07
3 Gy(4^0C)	45	116	0.22	0.46	0.01	0.03
	70	080	0.26	0.85	0.11	0.02
3 Gy(4^0C)	68	060	0.58	0.37	0.05	0.12
+ GSH	67	096	0.50	0.35	0.09	0.03
	40	143	0.29	0.12	0.01	0.04
3 Gy(4^0C)	13	068	0.39	0.16	0.04	0.01
+ GSH-ester	36	108	0.24	0.16	0.09	0.04
	57	103	0.31	0.37	0.06	0.06
	64	064	0.51	0.48	0.02	0.05

Table 3.3 Effect of GSH (20 mM) and GSH ester (20 mM) posttreatment on chromosome aberrations in Gamma irradiated human peripheral blood lymphocytes in vitro.

Rad dose(Gy)	GSH	Ester	Abt M (%) ± SEM	TM (n)	Aberrations/cell + SEM D + R	Deletion	Chd. Bk
0 (RT)	-	-	2 ± 0	203 (2)	0	0.005 + 0	0.01 ± 0
	+	-	2 ± 1	221 (2)	0	0 01 + 0	0.02 + 0
	-	+	3 ± 0	226 (2)	0	0.01 + 0	0.01 + 0
0 (4^0C)	-	-	3 ± 0	098 (1)	0	0.01 + 0	0.02 + 0
Dose Rate: 12.39Gy/min							
2 (RT)	-	-	74 ± 03.0	293 (3)	0 18 + 0.01	0.74 ± 0.02	0.05 ± 0
2 (4^0C)	-	-	67 ± 04 0	279 (3)	0 09 + 0.01*	0.92 ± 0.04**	0.07 + 0.01
2 (4^0C)	+	-	64 ± 01 0	348 (4)	0 26 + 0 01*	0.55 ± 0.02**	0.03 ± 0.01
Dose Rate: 8.19Gy/min							
3 (RT)	-		60 + 04 0	296 (3)	0 45 + 0 04	0 54 + 0.06	0.05 + 0.01
3 (4^0C)	-	-	57 ± 12 5	196 (2)	0 24 + 0 14*	0 65 +0.18*	0.06 + 0.03
3 (4^0C)	+	-	58 ± 09.0	299 (3)	0 51 + 0.04**	0.28 ± 0.08**	0.05 ± 0.02
3 (4^0C)	-	+	48 ± 07.0	343 (4)	0.40 + 0.04**	0.29 ± 0.08**	0.05 ± 0.01

RT- Room Temperature; TM- Total Metaphases; D + R- Dicentric + Ring
* p< 0 05, ** p< 0 001 X^2-test at df=2

DISCUSSION

In contrast to the limited studies on protective effect of GSH , employing parameter of CAs, data obtained on the role of GSH in the inherent cellular radiation protection mechanism are quite rich and informative. It has been demonstrated that, GSH plays a significant role in cellular detoxification processes (Revesz and Modig 1965; Revesz et al. 1984), regulates various enzymatic pathways by acting as a cofactor (see Meister and Anderson 1983) and it is involved in cell growth and replication process (Mazia 1961; Holmgren 1979) may be relevent to the participation of GSH in repair.

Many different biochemical repair reactions are likely to occur, after the initial radical reactions but little is known about their mechanisms at the molecular level. In an attempt to clarify the possible role of GSH in biochemical repair process, the extent of the rejoining of radiation induced SSbs was determined upto 1h after exposure. These observations indicate 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 (Edgren et al. 1981; Revesz and Edgren 1984). The rejoining can be error free or error prone and therefore, the parameter like CAs, whose structural alteration reflects the pattern of rejoining of radiation induced strand breaks have been considered. If the rejoining of the strand breaks are error prone type (misrepair), then the frequency of rearrangement types of aberrations is increased Whereas, error free type of rejoining reduces the frequency of Chd. and Iso-chd. bks. or deletions. The data presented in Chapter II is clearly exhibiting the increased frequency of Chd. and Iso-chd. bks. or deletions without increasing the frequency of rearrangements in GSH-depleted cells which prompted us to undertake the present objective i.e the role of GSH in cellular repair / misrepair processes.

Two approches have been taken for this study. One was GSH-posttreatment just after Υ-irradiation to BSO treated and untreated mice and secondly, GSH or GSH-ester posttreatmet to HPBLs, irradiated at 4^0C. The rationale for this approach is based on the premise that addition of exogenous GSH increase the level of endogenous GSH and thereby involve in the repair and misrepair processes of radiation induced strand breaks.

The addition of GSH after irradiation is poorly effective in reducing the frequency of Chd. and Iso-chd bks induced by 2Gy of irradiation and the frequency of exchanges is not altered As has been outlined in the section of Introduction and Literature Review of

Chapter II, that reports are available on the protective effect of GSH-pretreatment on radiation induced CAs in different systems. Generally all radioprotectors protect cells effectively, if it is treated before irradiation (Bacq 1965; Edgren 1970). However, present mild protective effect observed in GSH-posttreated samples could be due to involvement of GSH in cellular repair processes and thereby reducing the frequency of Chd. and Iso-chd. breaks. The data obtained from GSH posttreatment to BSO treated samples clearly indicate that endogenous rise of either GSH or any other thiol compounds could contribute in cellular repair and misrepair phenomenon and thus reducing the frequency of Chd. bk. and Iso-chd. bks. and increase the frequency of exchanges respectively. However, similar increase in the frequency of exchangs by GSH posttreatment to BSO untreated sample was not observed. It is important to note that GSH posttreatment to the sample irradiated at 4°C increased the frequency of exchanges. It seems that, in the present study, exogenous addition of GSH might not increase the endogenous GSH level within 1-2h and this could be the reason for the observed poor influence on repair/misrepair phenomenon. In the sample irradiated at 4°C, 1h incubation at 4°C was allowed after GSH addition and thus helping to increase the endogenous GSH level before effective cellular repair/misrepair processes start.

The present study is mainly to assess the influence of GSH, on rejoining of radiation induced DNA dsbs, since, the parameter here is CAs It has been shown that most DNA dsbs, induced by 5-10 Gy of X-ray rejoin with a half life of about 40 min (Bradley and Kohn (1979). By this approach, it is not possible to see the influence of endogenous thiol on the repair of DNA lesion, which occurs immediately after irradiation.

In order to see the role of endogenous GSH on total repair of γ-irradiated cells, the second approach has led to studies, in which, HPBLs were irradiated on Ice, to eliminate enzymatic repair processes. Both GSH or GSH ester was added soon after irradiation and cells were kept at 4°C for 3 hours to allow these chemicals to enter into the cells. It is accepted that, GSH-ester is readily transported into the cells and is hydrolyzed intracellularly This leads to greatly increased cellular levels of GSH (Wellner et al. 1984; Puri and Meister 1983) Thus, in addition to GSH, we have also used GSH-ester for 3h to increase the intracellular GSH level. Data in this study show that exposure of cells to irradiation on ice decreased the frequency of exchange types of aberrations in contrast to

cells irradiated at room temperature. This could be attributed due to less repair activity in cells at 4°C.

When GSH or GSH-ester was added soon after irradiation, there is increase in repair and misrepair phenomena and thereby the frequency of exchanges is elevated and simultaneously the frequency of deletions is reduced. GSH-ester reduced the frequency of aberrant metaphases more effectively than GSH. Present results also indicate that this repair and misrepair phenomena in irradiated cells after addition of GSH or GSH-ester may not specifically due to increased endogenous GSH level, rather it could be due to increased endogenous thiol levels including GSH. It is most likely that, the level of endogenous GSH may be less in GSH posttreated cultures than GSH-ester posttreated cultures since ester of GSH is very effectively transported into cells than GSH itself and is readily converted to GSH after transport (Wellner et al 1984). Nevertheless, both are showing same degree of increase in the frequency of exchanges and reduction in the frequency of deletions, which implies that other thiols besides GSH can also influence post irradiation biochemical repair processes. This observation is in agreement with the reported effect of various thiol treatment, when tested on the rejoining of SSbs in $GSH^{-/-}$ and $GSH^{+/+}$ cells (Edgren et al. 1981; Revesz and Edgren 1982). The defective rejoining observed with $GSH^{-/-}$ cells when irradiated aerobically was restored to normal if the cells were treated immediately after irradiation with GSH, dithiothreitol (DTT) or mercaptopropionyl glycine (MPG). Present results with CAs and available reports on SSb rejoining indicate that GSH requirement of the biochemical processes concerned with the rejoining of the strand breaks induced by irradiation in the presence of air, therefore to be nonspecific, and some other thiols may substitute for GSH.

In considering this role of GSH, the question arises to what extent, the effect of this substance is specific. GSH is generally not considered to be transported across the cell membrane (Clark 1986). It has been observed that, there was a marked increase in intracellular cysteine concentration after GSH treatment (Wardman et al. 1991). Almost 7-10% of total cysteine may be utilized for GSH biosynthesis in human lymphocytes (Reed 1983). Therefore, initially, cysteine but then couple of hours after, concentration of endogenous GSH may be increased after GSH treatment exogenously. In the present in vivo system the single injection of GSH may not be increasing endogenous GSH in BMCs, within 1-2h after injection Moreover, exposing BSO treated cells to GSH, failed to restore

intracellular GSH, however, elevation of intracellular cysteine could take place (Wardman et al. 1991).

It seems that, in the present study, exogenous addition of GSH might not increase the endogenous GSH level within 1-2h. Therefore, the present observed effect of GSH posttreatment could be due to an enhancement of endogenous cysteine level initially and later, increased endogenous GSH level may contribute to this effect.

Hittelman and Polard in 1982 demonstrated that DNA repair continues to take place upto 60 min after irradiation, albeit at a much slower rate than that observed immediately after irradiation. Therefore, it is most likely that endogenous enhancement of thiols mainly affecting the repair of long lived DNA lesions, which are slower component of DNA repair.

It is not possible to ascertain the mechanism by which GSH or other NPSH are involved in repair and misrepair event after irradiation in this study. However, they could participate in this process at various levels. For example, GSH is known to participate in nucleotide synthesis (Holmgren 1976,1979). According to them, the rate of DNA synthesis could be reduced in these deficient cells. The amplitude of the effect would depend on the size of the nucleotide and the time of exposure, perhaps affecting the rate of repair. In addition, NPSH may participate more directly as cofactors of repair enzymes. Therefore, in this study, GSH and other NPSH may participate in both ways in the biochemical repair processes after irradiation.

CHAPTER IV

CELLULAR GSH-LEVEL AND ITS EFFECT ON BLM ACTION

LITERATURE REVIEW

The role of glutathione (GSH) on cellular metabolism has been well established and therefore it is of interest to see the effects of cellular GSH on the cytotoxicity of chemicals. Tumour cells of human origin cultured in vitro, were shown to contain extremely high levels of GSH (Biaglow et al. 1983; Mitchell et al 1985). The possible relevance of GSH in cancer chemotherapy and the development of resistance during the course of treatment was emphasized by the findings, that tumour cells made resistant to some anticancer drugs, eg., melphalan, cis-platin and adriamycin, have increased cellular GSH concentration (Green et al. 1984; Hamilton et al. 1985). For these reasons, much interest has focussed on techniques of reducing cellular levels of GSH prior to treatment with cytotoxic agents. The development of BSO, a specific inhibitor of GSH-synthesis, has removed much of these uncertainities caused due to unwanted side effects, that were associated with GSH-depleting agents, having less specificity. In vitro studies, using human tumour cell lines have shown that depletion of cellular GSH by BSO can indeed increase the cytotoxicity of a variety of anticancer drugs (Green et al. 1984; Crook et al. 1986; Lee et al. 1986). GSH is observed to inhibit the production of active Mitomycin C (MMC) species in rat liver nuclei and nuclei of mouse cell line EMT6 (Kennedy et al. 1985) When BSO was used to deplete GSH in P388 mouse leukemia cells, they became more sensititive to MMC toxicity, exhibiting significant cross resistance to MMC (Xu et al. 1922 a,b). Similar observations were made by Ono and Shrieve (1986) in EMT6 cells grown as solid tumours in mice. The ability of BSO to potentiate the antitumour activity of anticancer drugs has been demonstrated in vivo for cyclophosphamide (Ono and Shrieve 1986), bleomycin (Tsutsui et al. 1986) and cis-platin (Tsutsui et al. 1986).

However, it is not clear, whether therapeutic benifit can be obtained by selectively depleting tumour GSH contents while partly or wholly sparing the critical normal tissues. Consequently additional work is needed to asses the chemotherapeutic use of BSO in combination with these anticancer drugs in vivo. It has been demonstrated that after treatment of mice with BSO, normal tissues (liver, kidney and bone marrow) regain their GSH levels faster than normal tissues (Minchinton et al. 1984). Therefore, it is possible that a tissue differential may be obtained.

Several stuies have indicated that combining BSO with cytotoxic agents did not adversely affect normal tissue toxicity For example, Russo et al. (1986) showed no effect of BSO on

CFUs survival and peripheral WBC counts following melphalan treatment. No detectable effect on lung toxicity of cyclophosphamide was found when measured by breathing rate (Lee et al 1981)

Evidence so far thus in support of the idea that the therapeutic index of some chemotherapeutic drugs may be improved by BSO. It must be emphasized, however, that much more indepth studies using normal tissue toxicity models best suited for each particular cytotoxic drug should be carried out before combination therapy.

In this investigation bleomycin (BLM) was selected as a radiomimetic agent, an antitumour antibiotic. The aim of the study is to establish a relationship between BLM-induced DNA damage and endogenous GSH status, since it is accepted that GSH plays an important role in cellular defence against cytotoxic insults. The present study mainly emphasizes the effect of BLM on normal cells, with or without BSO both in vivo and in vitro systems. BLM has shown to produce CAs comparable to those induced by X-rays, producing CAs in Go and G1 cells and chromatid type in G2 cells (Ohama and Kadotani 1970; Scott and Zampetti-Bosseler 1985; Chatterjee and Jacob-Raman 1986). As in the case of indirect action of rdiation BLM is also known to induce DNA breaks, through the production of free radicals (Sausville et al. 1984; Takeshita et al. 1978). Russo et al. (1984) reported that depletion of cellular GSH concentration enhanced the cytotoxicity of BLM-treatment and protection was observed when the GSH-level was elevated. Certain other protective chemicals such as AET, HCT have been shown to have an anticlastogenic effect against BLM-induced CAs in human lymphocytes (Gebhart 1978). By allowing simultaneous presence of GSH and BLM during the treatment Chatterjee et al.(1989) demonstrated that the presence of GSH potentiates the clastogenic action of BLM in muntjac lymphocytes in vitro. They attributed that the potentiation could be due to GSH acting as a reducing agent in reactivating oxidized BLM. However, results of some earlier biochemical studies also showed an enhanced reduction in the molecular size of DNA, when BLM treatment was coupled with mercaptoethylamine (MEA) pretreatment in the case of synthetic DNA (Nagai et al. 1969) and in DNA isolated from HeLa or *E. coli* (Suzuki et al. 1969). Sausville et al.(1978) interpreted the enhanced degradation of DNA, when treated with BLM in presence of MEA, as due to the latters role as a reducing agent in the redox cycling of the Fe(III).BLM allowing further radical production. Ekimoto et al.(1980) studied the kinetics of

the reaction of BLM-Fe(II).O_2 with DNA in the absence or presence of 2-mercaptoethanol (2ME). The total number of bases released in the presence of 2-ME increased 6.5 times. They also demonstrated that the reaction is biphasic; the first one is due to BLM.Fe(II), which is originally present and the second is due to BLM.Fe(II), produced by the reduction of BLM.Fe(III) with 2-ME. In another study (Nagai et al. 1969), involving the measurement of the melting temperature of isolated DNA, treated with BLM alone or pretreated with MEA or DTT, it was found that the presence of these reducing agents decreased the melting temperature; the author suggested an alternative hypothesis that BLM can bind better to a DNA-helix destabilized by reducing agents. Dose dependent potentiation by cationic thiol radioprotector WR-1065 in the induction of micronuclei by BLM in Go lymphocytes is also reported (Hoffman et al. 1993).

All these ressults clearly indicate that thiol-radioprotectors like GSH are not acting as a protector against BLM induced damages. Therefore, it is interesting to see the effect of BLM on the cells having low level of GSH, since depletion of cellular GSH made tumour cells more susceptible to irradiation and certain chemotherapeutic agents (Meister and Griffith 1979).

MATERIALS AND METHODS

Materials

1. Bleomycin (BLM, Nippon Kyaku Co. Ltd, Japan): This is a glycopeptide, an antitumour antibiotic. Umezawa et al. (1966) discovered it, which was isolated from the microorganism *Streptomycis verticellus*. About 200 types of BLM are known, which are made up of a peptide and disaccharide and differ in their terminal amine. The clinically used BLM is a mixture of BLM-A2, the most abundant component (60-70%). Two ampules of commercially used "Bleocin" were used in this study, cotaining 15 mg potency of BLM-hydrochloride. Stock solution (3 mg ml^{-1}) was prepared in sterilised double distilled filtered water. In case of in vivo studies, the same solution was used. In in vitro studies fresh working solution (300 μg ml^{-1}) was prepared and three concentrations (15, 45 and 60 μg ml^{-1}) were used.

2. L- Buthionine S-R Sulfoximine (BSO, Sigma USA): It has already been described in the Materials and Methods section of Chapter I.

3. Glutathione (GSH, Sigma, USA): The description is given in Materials and Methods section of Chapter III.

4. Glutathione-ethyl ester (GSH-ester, Sigma USA): This has already been described in the Materials and Methods section of Chapter III.

Experimental Systems

Both in vivo mouse bone marow cells (BMCs) and in vitro human peripheral blood lymphocytes (HPBLs) were used in this study. The detail description of the systems are given in the Materials and Methods section of Chapter II.

Methods

Experimental procedure

a) Treatment of BSO

In in vitro experiments, 1 and 5 mM of BSO was added into 1 ml aliquot of blood for 5h.

b) Treatment with BLM

BLM (15, 45 and 60 $\mu g\ ml^{-1}$) was added and incubated at 37°C for 2h in both BSO-treated and untreated blood. In BSO treated sample BLM was added 3h after BSO-treatment. Two hours after BLM-treatment, blood sample was washed off twice with culture medium. For in vivo study 10-80 mg kg^{-1} BLM was injected intraperitoneally.

c) Culture Procedure

Similar as discussed in Materials amd Methods section of Chapter II. Cultures were terminated at 48h.

d) Chromosome preparation, staining and scoring

For in vivo experiment cells were fixed 13h after BLM treatment. The detail procedure has been described in Methods section of Chapter II.

e) Calculation and Statistics

The formulae used for statistical calculations are mentioned in the same portion of the chapter II.

RESULTS

In vivo treatment of BLM (10-80 mg kg^{-1} body weight) did not show any type of CAs induction in mouse BMCs

In in vitro studies BLM-induced CAs were studied in HPBLs as positive control to BSO-treated in each experiment and their results are represented in table 4.1. The data are also plotted in Graph (Fig 4.1). The negative control value is presented from 2 cultures. A minimum of 2 samples were used per point. Similar to Υ-ray, BLM produced both chromosome and chromatid type of aberrations. Aberrations were scored in four categories:- i) Dicentrics and Rings, both with and without fragments ii) Deletions (including both terminal and interstitial) and iii) Chromatid breaks. The microphotographs of these aberrations are shown in Fig 2.8 b and c. Translocations were not scored. The frquencies of exchanges (dicentrics and rings) at 15, 45 and 60 μg ml^{-1} were 2, 10 and 9 per cent and for chromatid breaks, it was 25, 38 and 42 percent. However, the data of individual experiment showed a good degree of variation in the frequencies of all types of aberrations from sample to sample. Dose dependent increase was very prominent for deletions and chromatid breaks also.

BLM-treatment to BSO-treated cells did not sensitize the cells with respect to the fequencies of aberrant metaphases and individual types of aberrations (Table 4.1). This is also clear from individual sample data. Both BSO 1 and 5 mM showed almost same degree of reduction. The category of aberration showing maximum frequency and consistent reduction with BSO-treatment was deletions. The second major group of aberration was chromatid breaks which also showed reduction although it was not significant in all cases. From the individual data it is clear that the frequency of exchanges did not show any appreciable reduction after BSO-treatment, although there was an indication of reduction in their frequency at 45 μg ml^{-1} of BLM. However, it is worth noting that one out of four samples with 45 μg ml^{-1} BLM alone showed 0.25 exchange per cell which was very higher frequency than others and that was the reason for the higher frequency of exchanges showed i.e 0.10 per cell in Table 4.1.

Two experiments were performed with GSH (10mM) and GSH-ester (10mM) as pretreatment to BLM (60 mM) at Go stages of HPBLs. Results of individual experiments are presented in table 4.2. GSH-ester treatment for 3h prior to BLM-treatment enhanced the frequency of CAs (Fig 4.2). Similar enhancement was not there for aberrant metaphases and deletions with GSH. From the pooled data it is very clear that GSH-ester pretreatment potentiated the frequency of BLM-induced CAs much more efficiently than GSH-pretreatment.

Table 4.1 Effect of BSO-treatment on chromosome aberrations induced by Bleomycin (BLM) in human peripheral blood lymphocytes in vitro.

BLM (ug ml^{-1})	BSO (mM)	Abt M (%)	TM	Aberrations/cell ± SEM			Sample[b] No.
				Exchange[a]	Deletion	Chd.bk.	
0	0	02	101	0	0 005	0.00	A
		02	102	0	0.005	0.00	B
0	5	03	113	0	0 01	0.02	A
		02	110	0	0.01	0.02	B
15	0	13	145	0.01	0.04	0.12	A
		22	124	0.04	0.08	0.32	B
		08	072	0 01	0 15	0.32	C
		14 ± 4.09	*341*	*0.02 ± 0.01*	*0.09 ± 0.03*	*0.25 ± 0.06*	
	1	17	070	0 00	0 14	0.14	A
		14	205	0.01	0.10	0.08	B
		16 ± 4.09	*275*	*0.01 ± 0.005*	*0.12 ± 0.02*	*0.11 ± 0.01**	
	5	03	113	0.00	0.02	0.02	A
		10	227	0.01	0.04	0.09	B
		07	070	0.06	0.11	0.03	C
		07 ± 2.09†	*410*	*0.02 ± 0.01*	*0.06 ± 0.05*	*0.05 ± 0.02***	
45	0	54	084	0.25	0.39	0.50	A
		30	147	0.07	0.18	0.26	B
		37	128	0.04	0.33	0.44	C
		22	128	0 01	0 11	0.20	D
		38 ± 9.24	*487*	*0.10 ± 0.01*	*0.28 ± 0.08*	*0.38 ± 0.09*	
	1	09	066	0 00	0.06	0.26	B
		30	150	0 03	0.29	0.23	D
		20 ± 10.5††	*216*	*0.02 ± 0.02**	*0.18 ± 0.11**	*0.25 ± 0.02**	
	5	11	184	0.01	0.08	0.16	B
		24	053	0.04	0.19	0.38	C
		36	099	0.03	0.24	0.42	D
		31 ± 7.24†	*336*	*0.02 ± 0.01**	*0.14 ± 0.05***	*032 ± 0.09**	

† $p<0.01$, †† $p<0.001$ 2 x 2 contingency X^2-test ;
* $p<0.05$, ** $p<0.01$, ***$p<0.001$ X^2-test at df=2
b Sample number representing individual blood sample. a Exchanges = Dicentric + Ring
TM Total metaphases

table contd. Table 4.1

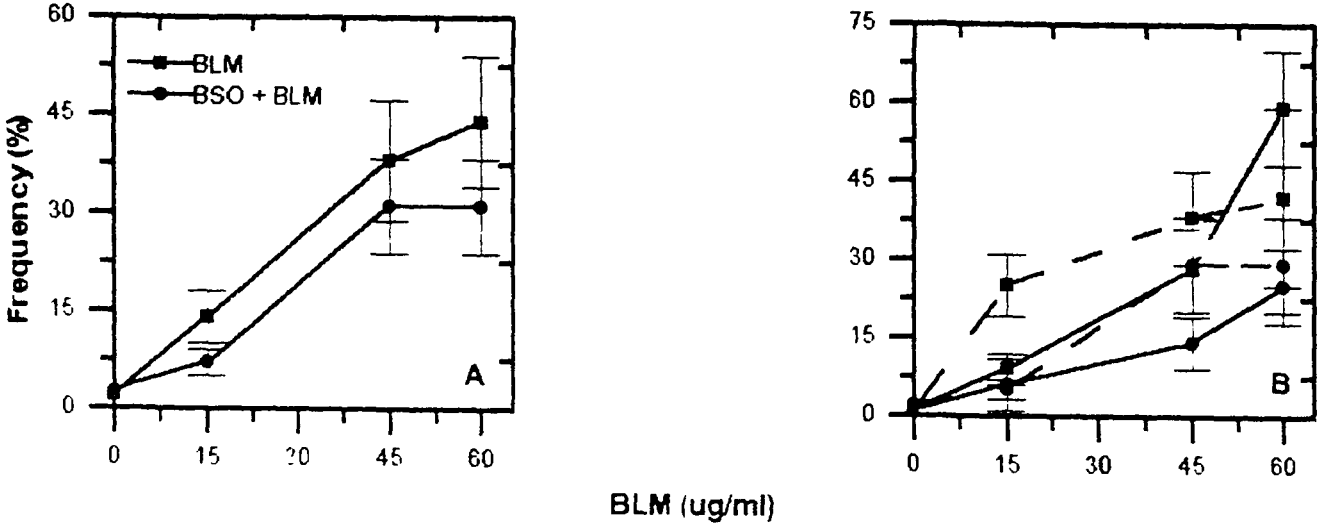
BLM (ug ml^{-1})	BSO (mM)	Abt. M. (%)	TM	Aberrations/cell + SEM			Sample[b] No
				Exchanges[a]	Deletion	Chd.bk.	
60	0	63	123	0 13	0 52	0.76	D
		39	179	0 12	0 82	0.29	E
		29	150	0 03	0 43	0.20	F
		44 ± 10.08	*452*	*0.09 ± 0.03*	*0.59 ± 0.11*	*0.42 ± 0.17*	
	1	39	142	0 05	0 46	0.41	E
		28	178	0 04	0.12	0.30	H
		34 ± 5.86[+]	*320*	*0.05 ± 0.03*	*0.29 ± 0.07*[***]	*0.36 ± 0.08*	
	5	48	118	0 15	0 38	0 44	D
		35	106	0.09	0.37	0.20	E
		14	055	0.04	0.09	0.07	F
		25	197	0.04	0.15	0.45	H
		31 ± 7.24[++]	*476*	*0.08 ± 0.03*	*0.25 ± 0.07*[***]	*0.29 ± 0.09*[**]	

+ $p<0.01$, ++$p<0.001$ 2 x 2 contingency X^2-test ;
* $p<0.05$, ** $p<0.01$, ***$p<0.001$ X^2-test at df=2
b Sample number representing individual blood sample. a Exchanges = Dicentric + Ring
TM Total metaphases

Table 4.2 Influence of GSH and GSH ester pretreatment on Bleomycin induced chromosome aberrations in human peripheral blood lymphocytes *in vitro*.

Exptl Condition	Abt M (%)	TM	Aberrations/cell Exchange[$]	Deletion	Chd.bk.
BLM 60	29	150	0.03	0.43	0.20
	21	101	0.02	0.14	0.08
	25 ± 4.0	*251*	*0.025 ± 0.005*	*0.28 ± 0.14*	*0.14 ± 0.06*
GSH 10 +	29	76	0.06	0.10	0.46
BLM 60	29	161	0.09	0.16	0.47
	29 ± 0.0	*237*	*0.08 ± 0.015*	*0.13 ± 0.03*	*0.47 ± 0.005**
GSH-ester 10	36	072	0.06	0.38	0.47
+ BLM 60	45	244	0.02	0.52	0.75
	40.5 ± 4.5^{+}	*316*	*0.04 ± 0.02*	*0.45 ± 0.07*	*0.61 ± 0.14***

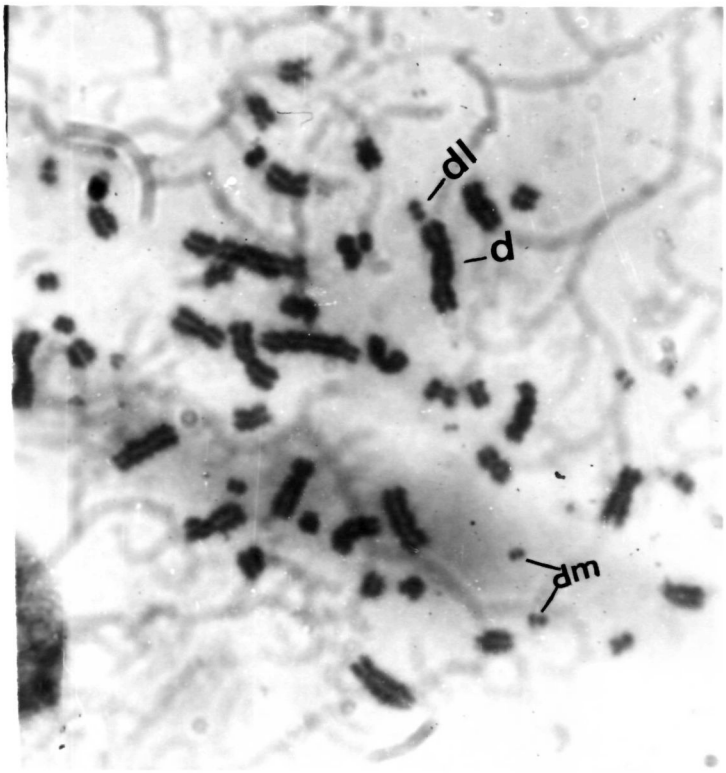
+ $p<0.001$ 2 x 2 contingency X^2-test ; * $p<0.01$, ** $p<0.001$ X^2-test at df=2



The frequency of aberrant metaphases (A) and deletion (—) and Chromatid break (- - -) (B) in BLM and BSO + BLM treated sample Mean +SEM are indicated in all cases.

Fig 4.1

Fig 4.2: Microphotograph showing Bleomycin plus Glutathione induced chromosome aberrations in first cycle metaphase of human peripheral blood lymphocytes.
d, dicentric; dl, deletion; dm, double minute.


dl
d
dm

DISCUSSION

It has previously been shown that BLM like ionizing radiation can induce CAs (Ohara and Kadotani 1970; see Vig and Lewis 1978) and the cellular target for CAs induction by BLM is DNA (Evans 1977). At equal levels of deletions and rearrangements induced by 40 μg ml^{-1} BLM and 2 Gy X-rays in muntjac lymphocytes the frequency of aberrant metaphases and the delay in cell cycle kinetics are lesser in BLM-treated cultures than in the irradiated one (Chatterjee and Jacob-Raman 1988). As outlined in the Introduction that this study was carried out to check the effect of endogenous GSH-level on the DNA-damaging ability of BLM. Studying cell survival, Russo et al (1984) reported that the depletion of cellular GSH concentration enhanced the cytotoxicity of BLM treatment and protection was observed when the level of GSH was elevated. However, there have been no studies on the effect of GSH-depletion on the clastogenic action of BLM.

In the present study, the frequency of BLM induced aberrant metaphases, deletions and chromatid breaks increased in a dose dependent manner, however, the frequencies of exchanges (dicentrics+rings) did not increase in similar manner. In fact, there are few reports regarding the absence of dicentrics, rings and triradials (Promchainnant 1975) or very few dicentrics (Bornstein et al. 1971) in human lymphocytes after treatment with BLM for 24h before harvesting. In the present study BLM was washed off after 2h. Therefore, in the present study the low frequency of exchanges might be due to low exposure time of BLM with the cells or it could be due to intrinsic feature of the test system. The nature of the primary DNA lesions induced by BLM and ϒ-rays may be different and therefore their involvement in interaction may also differ since the frequency of exchanges induced by ϒ-rays are higher than BLM.

The present results show that depletion of cellular GSH by BSO (30% or less depletion in blood GSH level) minimise the effect of BLM with respect to chromosome aberration formation. Therrefore, endogenous GSH, whose depletion could sensitize the cells against radiation induced damages (Bump et al 1982, Clark et al 1984) need not be effective in sensitizing the cells against radiomimetic drug like BLM-induced damages. This reducing effect of BLM-induced CAs with respect to GSH depletion, observed in the present study is associated with significant alteration of the frequency of aberrant metaphages, deletions and chromatid breaks. It implies that GSH depletion by BSO minimize the action of BLM at the cellular site.

Potentiation of BLM action by GSH and GSH-ester pretreatment is observed in this study. Potentiation is more pronounced in cultures with GSH-ester than GSH. GSH is generally not considered to be transported accross the cell membrane (Clark 1986). Therefore, in order to increase the intracellular level of GSH appreciably, GSH-ester was used in this study, which is readily transported into the cells and is converted to GSH, increasing its level within 3 - 4 h (Wellner et al. 1989). It is worth noting that, in this study GSH is added 3h before BLM addition for the sake of true comparison with GSH-ester treatment. GSH-ester and BLM was washed off 2h after BLM-treatment which means total period for GSH-ester treatment was 5h. It was evident that synergistic effect of BLM-action with GSH was signifiicant when GSH was added 30 min before or after BLM treatment (Chatterjee et al. 1989). It could be that very little GSH may be left in the reduced form in the cell after 3h pretreatment with GSH and thus potentiation effect is less.

In order to explain how GSH depletion is reducing BLM action, one has to consider the mechanism of BLM action itself. It is known that BLM requires a metal ion cofactor for its activity and is capable of binding with Fe(II) to yield an oxygen sensitive complex Fe(II).BLM (Sausville et al. 1976). The oxidation of this complex to Fe(III).BLM yields a radical which is responsible for DNA damage. Employing electron spin resonance technique, it has been confirmed that BLM·Fe(II)O_2 system generates superoxide and hydroxy radicals (Sugiura and Kikuchi, 1978) Generation of $O_2^{\cdot-}$ has also been confirmed by using diethyldithiocarbamate, inhibitor of Superoxide dismutase, which greatly increases the susceptibility of the cells to BLM-cytotoxicity (Lin et al. 1980). Presence of reducing agents, such as MEA, DTT enhance the breakage of DNA (Suzuki et al 1969, Umezawa et al 1973) and GSH and cysteine enhanced the CAs caused by BLM due to reducing the Fe(III).BLM to Fe(II).BLM; the mechanism allowing multiple radical production from a single iron BLM (Chatterjee et al 1989, Chatterjee and Jacob-Raman 1993). Therefore, the reducing effect of BLM on CA formation in GSH - depleted cells observed in the present study can be explained on the basis of the failure of the reactivation of the oxidized BLM by the reducing agent GSH, which is present endogenously.

In this study, the effect of BLM with respect to deletion and cromatid break formation was reduced in BSO-treated cells. Thus it appears that, GSH depletion reduces the number of strand break induction and thereby reduces the frequency of both types of aberrations. However, at higher concentration of BLM, the frequency of deletion is reduced more

efficiently than chromatid breaks This could be due to less occurrence of clustering of several single strand lesions in BSO-treated cells, which minimize the probability of formation of DNA-double strand breaks i.e. deletions.

GSH-ester mediated potentiation was more poronounced in the production of deletions and chromatid breaks and similarly BSO-mediated reduction was also effective in these two types of aberrations. It is also of interest to note that there was a significant increase in interstitial type of deletions in GSH-ester treated sample in the present study which is also in support of the earlier observation in mutjac lymphocytes treated with GSH (Chatterjee et al. 1989). If it is truely interstitial in nature, this indicates that, the BLM molecule bound to the DNA helix causes strand scissions at physically close points. This could be a reflection of the biphasic action of BLM in presence of a reducing agent, which has been discussed earlier. The frequency of exchanges, induced by BLM was not influenced by GSH-ester or by BSO treatment. This can be considered, due to the intrinsically lower production of exchanges by BLM in HPBLs. In fact, there are few reports regarding the absence of dicentrics, rings and triadials (Promchainnant 1975). However, it is interesting to note that in spite of considerable increase in BLM-induced DNA strand breaks in GSH-ester treated sample, the frequency of exchanges did not increase at all. This is not the case for muntjac lymphocytes where the frequency of exchanges was increased significantly (Chatterjee et al. 1989). Therefore, what appears apparently as a case of differential pattern of sensitization in human or in muntjac, may be only an expression of the intrinscic features of the test systems themselves.Therefore, both GSH and GSH-ester were treated for 3h before BLM addition. However it could be that very little GSH may be left in the reduced form in the cell after 3h pretreatment with GSH, therefore potentiation effect is less.

In case of in vivo studies, BLM treatment, upto 80mg/kg body weight were tested in bone marrow cells of mice. In contrast to the in vitro studies, BLM failed to induce any type of CAs in bone marrow tissue. This might be due to the tissue specificity of BLM activity, as BLM is reported to show little or no bone marrow toxicity (Kimura et al. 1972).

There is interest in using thiol compounds like WR-1065 or WR-2271 to minimize damage in nontarget tissues in cancer chemotherapy and radiotherapy (Glover et al. 1988; Yuhas et al. 1980). Though GSH and WR-1065 may protect against some cytotoxic drugs (Wolf et al. 1987; Glover et al. 1988), they enhance the clastogenicity of BLM in Go lymphocytes

(Chatterjee et al 1989; Littlefield and Hoffman 1993). Enhancement of damage in nontarget tissues may pose a risk, if thiol compounds are used in combined therapy with BLM. Therefore, the relative concentration of GSH in normal and tumour cells will be an important factor in determining therapeutic index, while using BLM as anticancer drug.

CHAPTER V

EFFECT OF GSH ON CELL CYCLE KINETICS AND SISTER CHROMATID EXCHANGES

LITERATURE REVIEW

Reduced glutathione (GSH), the major nonprotein thiol compound is present in all cells (Kosower 1976). It possess a wide range of biochemical activities and functions, largely attributable to the particular feature of the thiol group (Friedman 1973; Kosower 1976). Due to its reducing function, it reacts with free radicals and reduces the damage induced by ionizing radiation (Bacq 1965; Sasaki and Matsubara 1977). However, evidence also exists showing that thiol compounds themselves cause chromosome aberrations and polyploidy and inhibit cell growth in culture (Eker and Phil 1964; Najleti and Spencer 1969). It has been observed that 15mM of GSH reduced 2Gy of X-ray induced cell cycle delay and chromosome aberrations significantly in cultured cells, although such a treatment to unirradiated cells caused a remarkable delay in cell progression without inducing any CAs (Chatterjee and Jacob-Raman 1986). A similar observation was also made with another thiol radioprotector, L-cysteine (Chatterjee and Jacob-Raman 1993). Mutagenecity of GSH and cysteine was also determined in bacteria by the Ames test in the presence of mammalian subcellular preparations (Glatt et al. 1983). Reports were also available regarding the biphasic cytotoxicity pattern for cysteamine, WR-1065 and related SH-compounds. The SH-compounds are more toxic at lower concentrations (0.5 - 1 mM) than at higher concentrations (10mm) (Issels et al. 1984; Mori et al. 1983). The toxicity of the SH-containing radioprotective agent dithiothreitol (DTT) has also been studied using Chinese hamster V79 cells. The results suggested that toxicity results from autoxidation of DTT to produce H_2O_2, which in turn reacts via the metal catalyzed Fenton reaction to produce the ultimate toxin, $OH^{\cdot}$ radicals (Held and Melder 1987).

Sister Chromatid exchanges (SCEs) are considered to be a sensititive indictor of DNA damage. McRae and Stich (1979) Speit et al. (1980a) and Speit and Vogel (1982) demonstrated the induction of SCEs by thiol radioprotectors. However, there are contradictory findings, when reduced GSH was tested using two closely related cell lines of Chinese hamster (V79 & CHO) (McRae and Stich 1979; Speit et al. 1980a). It was proposed that, the difference in the induction of SCEs by GSH in these two cell lines was due to difference in the ability of the cells to degrade H_2O_2 , which is generated in the process of autoxidation of SH-compounds (Friedman 1973; Issels et al. 1984). In the report of the Gene-Tox pregramme Tucker et al. (1993), listed GSH in the category of chemicals which failed to induce SCEs but are inadequately tested.

Therefore, it is necessary to examine its SCE-inducing ability in mammalian cells, particularly in an in vivo system.

Aminothiol radioprotectors, in general have been reported to bind with DNA and slow down the strand separation for replication (Brown 1967). Such inhibition of DNA synthesis is known to lead to SCE-induction (Schneider 1978; Natarajan and Mullenders 1987). Therefore, in the present study, we have analyzed both cell cycle kinetics and SCE frequency.

At present much research involves adding exogenous thiols or depleting intracellular thiols in biological models Therefore, in order to deplete endogenous GSH, we have used buthionine sulfoximine (BSO), a potent inhibitor of GSH-synthesis by inhibiting the action of γ-glutamyl cysteine synthetase (Griffith et al. 1979; Griffith and Meister 1979) with the aim to see the the effect of exogenously added GSH on the induction of SCEs in normal and GSH-delpleted mice. This approach would help to understand the role of exogenously added GSH in SCE induction.

Thiols are known to produce H_2O_2 and free radicals in the presence of oxygen and metal catalysts (Hanaki and Kamide 1971) and also by its autoxidation (Friedman 1973). Therefore, an attempt was made to determine the effect of catalase, an enzyme which catalyses the decomposition of H_2O_2 , on SCE induction in mouse exposed to GSH.

MATERIALS AND METHODS

MATERIALS

1. Reduced Glutathione (GSH; Sigma; USA): As described in Materials and Methods sections of chapter III.
2. Buthionine Sulfoximine (BSO; Sigma; USA): Described in Materials and Methods section of chapter I.
3 Catalase (from Bovine liver; Sigma; USA): It is an enzyme, which catalyzes the divalent reduction of H_2O_2 to H_2O using H_2O_2 as an electron donor.

$$H_2O_2 + H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

4. 5 - bromo- 2- deoxyuridine tablet (BUdR - tablet; Boeringer Mannheim; Germany): Paraffin coated tablets, 50mg in weight was implanted subcutaneously.

Experimental systems

This study has been carried out in mouse bone marrow cells (BMCs) in vivo. Details are mentioned in chapter I

Methods

Experimental Procedure

a *Treatment of animals and isolation of bone marrow cells*

Reduced GSH of different concentrations (300, 400, 500 and 800 mg kg^{-1} body weight) was injected intraperitoneally (i p) 45 min before subcutanious implantations of a BUdR tablet BSO (4 and 200 mg kg^{-1}) was dissolved in phosphate buffer solution (pH 7 4) with the working solution of 1-10 mg ml^{-1} concentration and 10h after BSO-treatment (i p) reduced GSH (400 or 800 mg kg^{-1}) was injected. When catalase was used, it was injected (i p) 30 min after BUdR tablet implantation

Cells were fixed at 17 or 20h after GSH treatment, each preceded by 2h colchicine (15 mg kg^{-1}) treatment After the animals were killed by cervical dislocation, the femurs were dissected out and the bone marrow cells were obtained by injecting 2ml of prewarmed (37°C) 0 075M KCL (hypotonic solution) into one end with 26 gauge needle

b *Preparation of metaphases and differential staining*

Similar to that described in Materials and Methods section of chapter II.

c *Scoring*

The slides were coded and randomized 25 - 85 cells with differentially stained sister chromatids from each mouse were studied for SCEs For scoring cell cycle kinetics, metaphases were categorised as in first, second and subsequent division cycle based on their differential staining pattern

d *Calculation and Statistics*

The cell cycle data are presented as average generation time (AGT), which is a ratio of BUdR duration(h) and replication index (RI), where

$$RI = (1 \times M1 + 2 \times M2 + 3 \times M3) / \text{number of cells}$$

The BUdR durations were 16 25h and 19 25h for the cells fixed at 17h and 20h respectively To analyze the distribution of SCEs among cells, The dispersion coefficient was analyzed, based on responses in individual animals

The SCE frequencies and AGTs in different groups were compared using Student's t-test (parametric statistical analysis)

RESULTS

GSH and BSO induced SCEs and their effect on normal cell cycle kinetics have been studied in mouse bone marrow cells and their results are presented in table 5.1.

Microphotographs representing different cell cycle metaphases and sister chromatid exchanges are presented in Fig.2 1.

It was possible to distinguish unequivocally the number of divisions in the presence of BUdR, as well as the number of SCEs in second replication cycles. Table 5.1 shows that GSH, at all concentrations induced a significant level of SCEs in BMCs, harvested at either 17 or 20h. At the highest concentration of GSH (800mg kg^{-1}), not enough cells were available for SCE analysis at the 17h fixation time due to considerable delay in cell proliferation. However, cells fixed at 20h gave more second cycle cells for SCE analysis Similar levels of SCEs were observed at 400 and 800 mg kg^{-1} of GSH (Fig. 5.1). The frequencies of SCEs in the untreated controls did not differ significantly from each other at the two sample times (table 5.1).

Mice treated with BSO also showed an increased frequencies of SCEs per cell. Both 4 and 200 mg kg^{-1} of BSO induced same degree of SCEs in vivo. Treatment of GSH to BSO- treated mice enhanced the frequency of SCEs further and brought it to the same level shown by GSH treatment of normal mice (Fig 5.2).

Table 5.2 illustrates that the presence of catalase reduces the ability of GSH to induce SCEs. Catalase treatment alone also increased the frequencies of SCEs, but it was not statistically significant with respect to the control (Fig 5.1). In contrast to the GSH-treated normal mice, catalase absolutely failed to reduce the frequency of SCEs induced by GSH in BSO-treated mice. Table 5.3 represents the distribution of SCEs among cells in various treated samples.

In addition to an analysis of the frequency of SCEs, the cell cycle kinetics was also studied in GSH treated mice. The fluorescence plus Giemsa staining technique facilitated the scoring of GSH-induced cell cycle delay in terms of reduction in the frequency of second and subsequent division metaphases following GSH treatment. The percentage of first cycle metaphases (M1) was higher indicating a delay in cell progression in a dose dependent manner (Table 5.1). Although the basic cell cycle progression varied considerably among individuals in each group, the extent of delay by GSH increased in a dose dependent manner. The AGT was significantly increased in the groups treated with 400, 500 and 800 mg kg^{-1} of GSH in normal mice. BSO treated mice did not show any delay in cell proliferation after GSH treatment.

Table 5.4 represents the induction of CAs in BSO treated mice. Although both 4 and 200 mg kg^{-1} of BSO induced same degrees of SCEs/M (Table 5.1), but 200 mg kg^{-1} induced significant amount of aberrations in chromosomes than 4 mg kg^{-1} of BSO.

Table 5.1 Effects of GSH and BSO alone or in combination on cell cycle kinetics and the frequency of SCEs in mouse bone marrow cells in vivo.

Dose mg kg^{-1}	TM	MI (%)	AGT (h)	Mean AGT	Cells scored	SCE/ metaphase	SCE/metaphase/mouse $\bar{X} \pm$ SEM
Fixation at 17H							
0	302	49	10 62		44	2 52	–
	160	34	08 13	–	41	2 80	–
				9.37			2.66 ± 0.10
300	137	54	11 13	–	26	4 00	–
	178	31	09 10	–	47	3 78	–
	201	50	10 83	–	44	3 68	–
				10 35			3.82 ± 0.07*
500	267	42	10 28	–	50	6 12	–
	269	64	11 95	–	47	6 68	–
	147	83	13 89	–	16	6 30	–
	248	89	14 77	–	–	–	–
				12.72*			6.36 ± 0.13**
800	452	63	11 86	–	73	5 02	–
	292	92	14 91	–	–	–	–
	121	84	14 00	–	–	–	–
	248	89	12 50	–	11	6 90	–
				13.32*			5.96 ± 0.66**
BSO	178	58	07 88		65	3 40	--
200	200	47	07 19		72	3 84	--
				07 53			3.63 ± 0.30
Fixation at 20 H							
0	178	08	08 56	–	37	2 37	–
	080	28	09 92	–	35	2 77	–
	156	24	09 62	–	65	2 38	–
	130	29	09 62	–	41	2 90	–
				09.43			2.60 ± 0.12
400	149	23	09 82	–	42	5 14	–
	231	43	11 66	–	55	6 36	–
	218	61	13 85	–	60	6 32	–
	160	38	11 19	–	46	4 72	–
				11.63**			5.64 ± 0.36**
800	248	55	08 55	–	34	5 55	–
BSO	096	06	08 26	–	58	2 62	–
004	125	12	08 48	–	39	3 43	–
	121	22	09 30	–	46	4 48	–
	135	27	09 58	–	42	3 81	–
				08.90			3.59 ± 0.33**
BSO +	107	07	08 48	–	58	5 21	–
GSH	117	10	08 40		48	8 19	--
400	072	06	08 26		30	5 07	
	095	22	09 39		36	6 28	
				08.63			6.19 ± 0.62**
BSO +	083	06	08 59	–	34	6 58	–
GSH	107	13	08 76	–	48	6 58	–
800	115	21	09 12		47	3 13	–
				08.82			5.43 ± 0.93**

* $p < 0.01$, ** $p < 0.001$ compared to the respective control, Student's t-test

Table 5.2 Effect of Catalase on SCE-induction by GSH-treatment in normal and BSO-treated mice in vivo (20 h)

Dose mg kg^{-1}	TM	MI %	Cells scored for SCE	SCE/M	SCE/M/Mouse $\bar{X} \pm$ SEM
GSH	178	08	37	2.37	
0	080	28	35	2.77	
	156	24	65	2.38	
	130	29	41	2.90	**2.60 ± 0.12**
400	149	23	42	5.14	
	231	43	55	6.36	
	218	61	60	6.32	
	160	38	46	4.72	**5.64 ± 0.36****
Catalase					
100	253	20	68	3.39	
	127	12	55	4.65	
	096	19	47	2.10	
	057	05	40	2.43	**3.14 ± 0.38**
GSH +					
Catalase	156	55	25	2.52	
	140	26	60	4.08	
	324	20	84	4.92	
	160	09	51	4.33	
	065	20	40	2.40	
	102	16	60	2.43	**3.45 ± 0.46**** a
BSO	096	06	42	2.62	
004	125	12	39	3.43	
	121	22	46	4.48	
	135	27	42	3.81	**3.59 ± 0.33****
BSO +	108	07	58	5.21	
GSH	117	10	48	8.19	
	116	18	36	6.60	
	071	04	30	5.07	**6.19 ± 0.62***
BSO +	067	04	51	5.82	
GSH +	079	05	62	6.27	
Catalase	035	03	31	5.71	
					5.93 ± 0.17*

* $p<0.01$; ** $p<0.001$ Students t-test ; a Between GSH and GSH + Catalase.

Table 5.3 Distribution of SCEs in the cells treated with GSH alone or in combination with either catalase or BSO (20h)

Dose mg kg^{-1}	Cells Scored	SCE per M	SCE X ± SEM	SCE Range [a]	Dispersion coefficient [b]
GSH					
0	37	2.37	2.60 ± 0.12	0--7	1.21
	41	2.90		0--7	1.34
	65	2.38		0--8	1.45
	35	2.77		0--8	1.06
400	42	5.14	5.64 ± 0.36	1--13	1.58^{+}
	55	6.36		1--16	1.75^{+}
	60	6.32		2--18	1.69^{+}
	46	4.72		1--11	1.28^{+}
Catl	68	3.39	3.14 ± 0.38	0--10	
100	55	4.65		0--11	
	23	2.10		0--05	
	40	2.43		0--06	
GSH+Catl	25	2.52	3.45 ± 0.46^{**c}	0--9	1.59^{+}
400 100	40	2.40		0--6	1.37
	68	3.39		0--10	1.27
	50	4.65		0--11	1.12
	84	4.92		0--15	1.90^{+}
	53	2.75		0--6	0.35
BSO	39	3.43	3.59 ± 0.33*	0--8	1.05
004	42	2.62		0--7	1.38
	46	4.48		0--10	1.58^{+}
	42	3.81		0--11	2.18^{+}
BSO+GSH	58	5.21	6.19 ± 0.62*	0--15	1.18
	48	8.19		1--19	1.50^{+}
	30	5.07		0--12	1.48
	36	6.28		2--17	2.03^{+}
BSO+GSH	51	5.82	5.93 ± 0.17*	0--13	
+ Catl	62	6.27		0--19	
	31	5.71		0--20	

[a] Range of SCE values

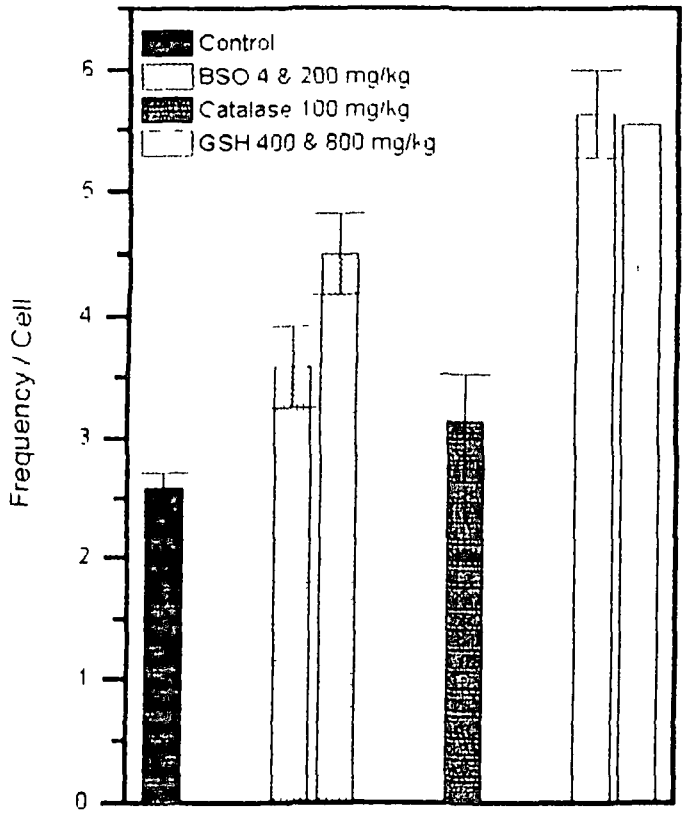
[b] Disperson coefficient = Variance/mean

$^{+}$ Significantly different at $\alpha = 0.05$ from Poison distribution

* $p<0.01$; ** $p<0.001$ Student's t-test. c Between GSH and GSH+Catalase

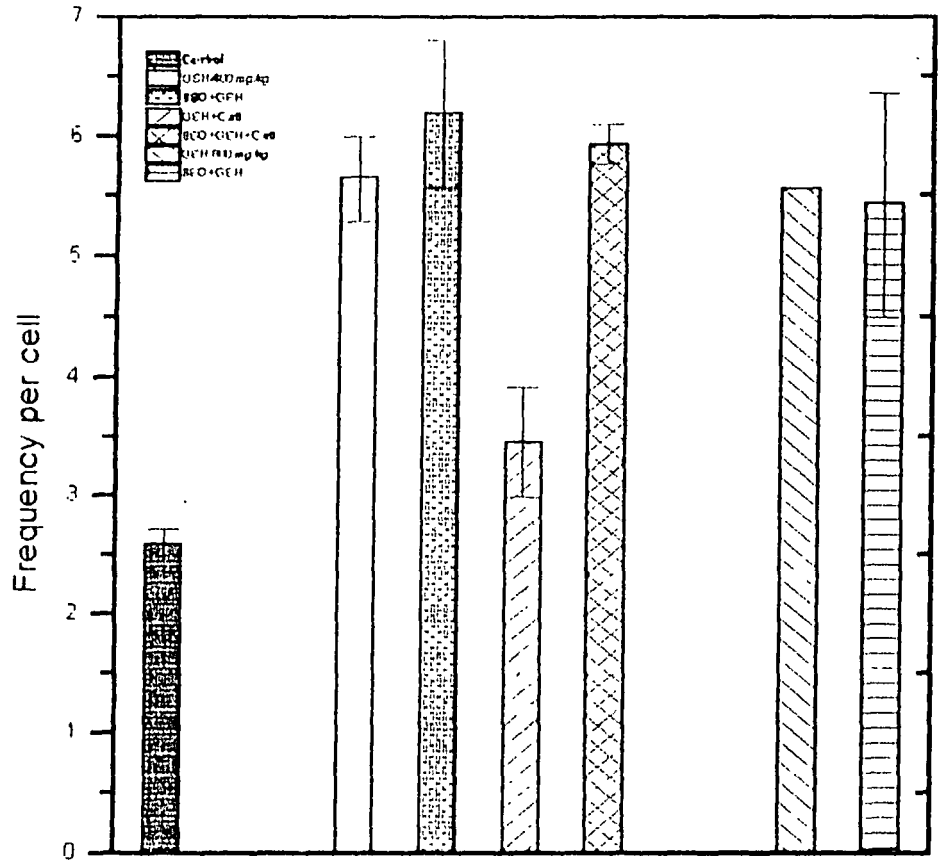
Table 5.4 Effect of BSO alone on chromosome aberration in mouse bone marrow cells (17h)

BSO ($mg\ kg^{-1}$)	Abt. M %	TM	Aberrations/Cell	
			Isochd. bk	Chd. bk
000	**02**	**462**	**0**	**0.03**
004	03	096	0.01	0.02
	02	125	0	0.02
	03	121	0.01	0.02
pooled	**03**	**342**	**0.01**	**0.02**
200	15	095	0	0.18
	06	103	0	0.08
	10	**198**	**0**	**0.12**



Effect of GSH, BSO and Catalase alone on the frequency of SCEs in mouse bone marrow cells in vivo

Fig. 5.1



Effect of GSH alone or in combination with BSO and Catalase on the frequency of SCEs in mouse bone marrow cells in vivo

Fig. 5.2

DISCUSSION

SCEs have proved to be a sensititive indicator for DNA damage in many investigations (Latt et al. 1980) Reports available on SCEs induction by GSH in in vitro systems are contradictory (McRae and Stich 1979 ; Speit et al 1980a). To the best of our knowledge, no study has been done on the effect of GSH on SCE induction in vivo. The present in vivo study shows that exogenous addition of GSH induces SCEs significantly in bone marrow cells of mice The range of SCE values per cell is increased significantly in GSH treated mice compared to untreated controls. McRae and Stich (1979) suggested that the formation of H_2O_2 is the reason for SCE induction by thiols. This is supported by our present in vivo study, since in the presence of catalase, the frequencies of SCEs induced by GSH reduced significantly. However, it is important to note that catalase brings the GSH induced SCE frequency down to the catalase alone level Therefore, the entire effect exerted by GSH on SCE induction could be due to H_2O_2 formation. A novel aspect of the present study is the analysis of the influence of BSO on the induction of SCEs Treatment with BSO produces a rapid decrease in the GSH level of the various tissues (Griffith and Meister 1979). It has been reported (Lee et al 1987) that after a dose of 2 5 mmol kg^{-1} BSO, GSH nadirs were obtained by approximately 5h for the liver and kidney, 8h for bone marrow and lungs The degree of depletion was greatest for the kidney (80%), liver (74%) and bone marrow (83%) In this investigation 4 and 200 mg kg^{-1} of BSO was injected for 10h. The concentration of BSO, we used was on the lower side but was based on our CAs study where mice treated with both 4 and 200 mg kg^{-1} showed significantly increased sensitivity to ionizing radiation in comparison to BSO untreated mice (Chapter II). It has also been demonstrated in Chapter I that 200mg kg^{-1} BSO could deplete endogenous GSH level in much higher degree than 4mg kg^{-1} BSO treatment. The higher degree of GSH depletion by 200mg kg^{-1} of BSO could be due to better depletion occurred in both cytoplasmic as well as nuclear compartment of the cell It is worth noting that 200 mg kg^{-1} also induced CAs, mostly chromatid breaks, which was not seen by 4 mg kg^{-1}. Therefore, present results suggest an important protective role of endogenous GSH in the cells against peroxides and free radicals which are formed by normal metabolic pathways (Griffith and Meister 1979) The demonstration of elevated frequency of SCEs after exposing BSO treated mice to GSH and the failure of catalase to reduce this

suggest the non involvement of H_2O_2 in SCE induction in BSO treated mice It appears that the exogenous addition of GSH in BSO treated mice could not have increased the endogenous GSH level due to impairment of GSH synthesis by the single BSO treatment This impression is further consolidated by the cell proliferation data where GSH could not induce any delay in cell cycle kinetics in BSO treated mice Wardman et al (1992) demonstrated that intracellular cysteine levels are enhanced after exposing BSO treated cells to GSH Lee et al (1987) showed that the recovery rate of GSH to pretreatment values following a single dose of BSO was 72h for bone marrow Therefore addition of GSH after 10h of BSO treatment could not negate the inhibitory effect of BSO on GSH synthesis However the increased frequencies of SCEs induced by GSH treatment in this case is difficult to interpreat It could be that GSH degraded product(s) might be involved in this induction It is clear from the results that neither the generation of H_2O_2 nor the binding ability of GSH to chromatin was responsible for such SCE induction GSH induces a delay in cell cycle kinetics in a dose-related fashion, which is in agreement with earlier in vitro findings (Chatterjee and Jacob-Raman 1986) The effect of GSH on the cell cycle has also been observed by Speit et al (1980a), while studying the effect of GSH on SCE induction They mentioned that GSH at concentrations greater than 5mM inhibits cell cycle progression in V79 Chinese Hamster cells In general amino thiol radioprotectors are thought to bind with DNA and slow down strand separation for replication (Brown 1967) Therefore, the cell cycle delay effect of GSH observed in the present in vivo study may involve the binding of the chemical to chromatin

Reduced GSH a naturally occurring cellular component, can induce genetic damage if SCEs are taken as a measure of DNA damage This induction of SCEs is largely due to generation of H_2O_2 after autoxidation of GSH, since catalase brings down the GSH - induced SCEs to catalase alone level However, the induction of SCEs by GSH in BSO treated mice indicated the involvement of an unknown route by which the degraded products of exogenously added GSH might induce SCEs Therefore, the involvement of an unknown route or direct action of GSH to chromatin can not be ruled out in a small fraction of SCEs induced by GSH in normal mice in vivo besides the major contribution of H_2O_2. The findings presented here indicate that the mechanism resulting in SCE formation by GSH may not be simple and more study is needed to develop a comprehensive hypothesis

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SUMMARY

The study is basically intended to establish a correlation between the intensity of cellular radio and chemosensitivity measured by means of quantitation of different cytogenetical end-points and the endogenous cellular level of reduced glutathione (GSH), a sulphydryl tripeptide known as radioprotector. The work has been carried out in *mouse bone marrow cells as in vivo system and human peripheral blood* lymphocytes as in vitro system

The detail account of the work is being mentioned in term of the objective and the interpretation/discussion is mentioned after brief description of the results.

Objective Measurement of endogenous glutathione (GSH) before or after BSO-treatment

Quantitative analysis of GSH in mouse bone marrow cells and human blood has been performed by UV-visible spectrophotometer The reagent dithiobis or DTNB has been used and OD of sample has been measured at 412 nm relative to a blank containing DTNB, buffer and TCA At each point we have used a minimum of 6 mice and 5 human donors for this measurement

Results

- Following a single dose of 4 and 200 mg kg $^{-1}$ BSO, the GSH content of mouse bone marrow cells were depleted significantly to a level of about 69 and 45% of the BSO-untreated mice respectively
- The blood GSH concentration showed a range between 720 and 813 μmol ml^{-1} with an average of 759 4 $\pm$ 33 93 μmol ml^{-1} in the BSO-untreated human blood. Significant depletion (70% of the control value) was achieved by 5mM BSO

Objective Evaluation of the influence of normal and modified level of endogenous GSH in the induction of chromosome aberrations (**CAs**) induced by radiation in mammalian cells both in vivo and in vitro.

Results

- Lower concentration of buthionine sulfoximine (BSO) showed protection with 1.5 and 2 Gy.
- BSO (4 and 200 mg kg^{-1}) sensitized the cells significantly against all the radiation doses.
- The increment of the frequency of CAs is particularly prominent for chromatid breaks and deletions, however, the frequency of rearrangements were either reduced or not altered.
- BSO also sensitized the cells against radiation in vitro and like in vivo the frequency of exchanges were reduced in spite of increasing the frequency of deletions and chromatid breaks.

GSH-depletion by BSO-treatment sensitized the cellular radiosensitivity both in vivo and in vitro systems and in spite of increasing in the number of strand breaks the frequency of exchanges were reduced significantly. This indicates the probable involvement of endogenous-GSH in DNA repair / misrepair phenomenon.

Objective: To explore whether cellular-GSH plays any role in repair in irradiated cells.

GSH was added soon after irradiation to normal and BSO-treated mouse. Human PBL was treated with γ-radiation at 4^0C and then GSH or GSH-ester has been added and subsequently raised the temparature to 37^0C. Cultures were set up and harvested after 48h of growth

RESULTS:

- GSH post-treatment in BSO + 2Gy samples increased the frequency of exchange aberrations.
- Reduced frequency of chromosomal exchanges and elevated frequency of deletions were observed without any significant change in the frequency of aberrant metaphases in the cells irradiated at 4^0C
- Addition of either reduced-GSH or GSH-ester at 4^0C after irradiation decreased the frequency of aberrant metaphases and deletions and increased the frequency of exchanges significantly.

The present results indicate the involvement of GSH either directly or indirectly in cellular repair/misrepair processes after damages induced by irradiation. Since the GSH increased the cellular repair ability, the frequency of misrepair has also been increased simultaneously and therefore, there is an elevated frequency of exchanges while the frequency of deletions and aberrant metaphases were dropped down.

Objective: Evaluate the influence of normal and modified level of endogenous-GSH in the induction of CAs induced by Bleomycin (BLM) in mammalian cells both in vivo and in vitro.

Results

- BLM (10 to 80 mg kg^{-1}) did not induce any significant CAs in bone marrow cells of mouse
- Cells treated with BSO (1 and 5 m M) reduce the effect of BLM with respect to all kinds of CAs in human PBLs
- The potentiating effect of BLM was distinct when cells treated with GSH-ester 3h before BLM-treatment It is not true when GSH was added in the same way.

In vivo effect of BLM supports earlier observation made by Kimura et al (Cancer, 29, 58-60, 1972) that BLM shows little or no bone marrow toxicity. In vitro data implies that endogenous GSH could act as reducing agent and might be the cause for potentiation of BLM-action.

Objective : Effect of reduced-GSH on sister chromatid exchanges (SCEs) and cell cycle kinetics in normal and BSO-treated mouse.

Results :

- GSH (400-800 mg kg^{-1}) induced delay in cell cycle kinetics and also increased the frequency of SCEs significantly in mouse bone marrow cells.
- Presence of catalase reduced the ability of GSH to induce SCEs.
- Following a single dose of GSH (400 mg kg^{-1}) to BSO-treated mouse increased the frequency of SCEs ; however, catalase failed to reduce the frequency of SCEs in this circumstances
- Presence of BSO (4 and 200 mg kg^{-1}) alone induced SCEs, however, 200 mg kg^{-1} BSO also induced chromosome aberrations mainly chromatid breaks.

Data suggest that the formation of H_2O_2 due to autoxidation of reduced-GSH could be the causative factor for SCE induction by GSH. Induction of SCEs in BSO-treated mouse indicates the important role of endogenous-GSH in the cells against peroxides and free radicals which are formed in normal metabolic pathways. Since catalase failed to reduce GSH induced SCEs in BSO-treated mouse, it indicate the involvement of an unknown route or direct action of GSH to chromatin cannot be ruled out in a small fraction of SCEs induced by GSH in normal mice in vivo besides the major contribution of H_2O_2.

The present investigation has provided certain positive informations in the field of Radiation Genetics and Mutagenesis and indicated the role of endogenous GSH in defining cellular radio and chemosensitivity. The inducing ability of reduced GSH with respect to sister chromatid exchanges but not to chromosome aberrations supporting the idea that the mechanisms of induction of SCEs and CAs are different. The probable role of cellular endogenous GSH in cellular repair/misrepair phenomenon with respect to cytogenetical parameters is an important contribution of this investigation The observation of decreasing effect of bleomycin in buthionine sulfoximine treated cells implies that endogenous GSH could act as reducing agent and might be the cause for the potentiation of BLM-action while GSH or GSH-ester is added externally

Since the manipulation of GSH-levels as a means of modifying radio and chemotherapeutic response has been given considerable attention in the field of cancer therapy therefore, this findings may have some applications and implication in the field of cancer biology

ELSEVIER

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Fundamental and Molecular Mechanisms of Mutagenesis

Effect of glutathione on sister-chromatid exchanges in normal and buthionine sulfoximine-treated mice

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Abstract

Based on their ability to induce sister-chromatid exchanges (SCEs) it is evident that thiol-containing radioprotectors can induce DNA damage. However, there were contradictory findings when reduced glutathione (GSH) was tested using two cell lines. The present study demonstrated that GSH can induce SCEs and also delay in cell proliferation in mouse bone marrow cells in vivo. The presence of catalase significantly reduced GSH-induced SCE frequency down to catalase alone levels. An attempt was made to evaluate the effect of GSH treatment in buthionine sulfoximine (BSO)-treated mice (GSH-depleted mice) and the data indicate that induction of SCEs takes place without inducing a delay in cell proliferation or the generation of hydrogen peroxide. Probably, some unknown route is involved by which GSH-degraded product(s) induce SCEs in BSO-treated mice. Therefore, the induction of SCEs by GSH in normal mice may be largely due to hydrogen peroxide generation; however, the involvement of the binding ability of GSH to chromatin and the probable (unknown) route by which GSH-degraded product(s) may cause smaller fraction of SCEs cannot be ruled out.

Keywords: Sister-chromatid exchange; Glutathione; Cell cycle delay; Buthionine sulfoximine

1. Introduction

Reduced glutathione (GSH), the major non-protein thiol compound present in all cells (Kosower, 1976). It possesses a wide range of biochemical activities and functions largely attributable to the particular feature of the thiol group (Friedman, 1973; Kosower, 1976). Due to its reducing function, it reacts with free radicals and reduces the damage induced by ionizing radiation (Bacq, 1965; Sasaki and Matsubara, 1977). However, evidence also exists showing that thiol compounds themselves cause chromosome aberrations and polyploidy and inhibit cell growth in cultures (Eker and Phil, 1964; Najleti and Spencer, 1969). We have observed in our earlier study (Chatterjee and Jacob-Raman, 1986) that 15 mM of GSH reduced 2 Gy of X-ray-induced cell cycle delay and chromosome aberrations significantly in cultured cells, although such a treatment to unirradiated cells caused a remarkable delay in cell progression without inducing any chromosome aberrations. A similar observation was also made with another thiol radioprotector, L-cysteine (Chatterjee and Jacob-Raman, 1993).

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Mutagenicity of GSH and cysteine was also determined in bacteria by the Ames test in the presence of mammalian subcellular preparations (Glatt et al., 1983).

Sister-chromatid exchanges (SCEs) are considered to be a sensitive indicator of DNA damage. McRae and Stich (1979), Speit et al. (1980a) and Speit and Vogel (1982) demonstrated the induction of SCEs by thiol radioprotectors. However, there were contradictory findings when reduced GSH was tested using two closely related cell lines of Chinese hamster (V79 and CHO) (McRae and Stich, 1979; Speit et al., 1980a). It was proposed that the difference in the induction of SCEs by GSH in these two cell lines was due to differences in the ability of the cells to degrade hydrogen peroxide, which is generated in the process of autoxidation of SH compounds (Friedman, 1973; Issels et al., 1984). In the recent report of the Gene-Tox program Tucker et al. (1993) listed GSH in the category of chemicals which failed to induce SCEs but were inadequately tested. Therefore, it is necessary to examine its SCE-inducing ability in mammalian cells, particularly in an in vivo system.

Aminothiol radioprotectors, in general, have been reported to bind with DNA and slow down strand separation for replication (Brown, 1967). Such inhibition of DNA synthesis is known to lead to SCE induction (Schneider et al., 1978; Natarajan and Mullenders, 1987). Therefore, in the present investigation, we have analyzed both cell cycle kinetics and SCE frequency.

At present, much research involves adding exogenous thiols or depleting intracellular thiols in biological models. Therefore, to deplete endogenous GSH, we used buthionine sulfoximine (BSO), which is a potent and selective inhibitor of GSH synthesis by inhibiting the action of γ-glutamyl cysteine synthetase (Griffith et al., 1979; Griffith and Meister, 1979), with the aim of seeing the effect of exogenously added GSH on the induction of SCEs in normal and GSH-depleted mice. We hoped that this approach would help to understand the role of exogenously added GSH in SCE induction.

Thiols are known to produce hydrogen peroxide and free radicals in the presence of oxygen and metal catalysts (Hanaki and Kamids, 1971) and also by its autoxidation (Friedman, 1973). Therefore, an attempt was made to determine the effect of catalase, an enzyme which catalyses the decomposition of hydrogen peroxide, on SCE induction in mouse exposed to GSH.

2. Materials and methods

Male BALB/c mice, aged 2–3 months and weighing about 25–30 g (maintained in the laboratory in community cages in a room under controlled temperature (20°C ± 2°C) and lighting (12 h light and 12 h dark) conditions) were used in all experiments. Reduced glutathione (CAS No. 70-18-8), buthionine sulfoximine and catalase (from bovine liver) were obtained from Sigma Chemical Company (USA). 5-Bromodeoxyuridine tablets (50 mg) were obtained from Boehringer-Mannheim, Germany. The aqueous drug solutions were freshly prepared and used.

Treatment of animals and isolation of bone marrow cells

Reduced GSH of different concentrations (300, 400, 500 and 800 mg/kg body weight) was injected intraperitoneally (i.p.) 45 min before subcutaneous implantation of a BrdU tablet. BSO (18 mM/kg) was dissolved in phosphate buffer solution (pH 7.4) and 10 h after BSO treatment (i.p.) reduced GSH (400 or 800 mg/kg) was injected. When catalase (100 mg/kg) was used, it was injected (i.p.) 30 min after BrdU tablet implantation.

Cells were fixed at 17 or 20 h after GSH treatment, each preceded by 2 h colchicine (15 mg/kg body weight) treatment. After the animals were killed by cervical dislocation, the femurs were dissected and the bone marrow cells were obtained by injecting 2 ml of 0.075 M KCl (hypotonic solution) into one end with a 26-gauge needle.

Preparation of metaphases

A single-cell suspension was made in hypotonic solution and incubated at 37°C for 15 min, and centrifuged at 1200 rpm for 5 min. The cells

Table 1
Effects of GSH and BSO alone or in combination on cell cycle kinetics and the frequency of SCEs in mouse bone marrow cells in vivo

Dose (mg/kg)	TM	MI (%)	AGT (h)	Mean AGT	Cells scored	SCE/metaphase	SCE/metaphase/mouse (X ± SEM)
Fixation at 17 h							
GSH							
0	302	49	10.62	–	44	2.52	–
	160	34	08.13		41	2.80	–
				9.37			2.66 ± 0.10
300	137	54	11.13	–	26	4.00	–
	178	31	09.10	–	47	3.78	–
	201	50	10.83	–	44	3.68	–
				10.35			3.82 ± 0.07 *
500	267	42	10.28	–	50	6.12	–
	269	64	11.95	–	47	6.68	–
	147	83	13.88	–	16	6.30	–
	248	89	14.77	–	–	–	–
				12.72			6.36 ± 0.13 **
800	452	63	11.86	–	73	5.02	–
	292	92	14.91	–	–	–	–
	121	84	14.00	–	–	–	–
	057	70	12.50	–	11	6.90	–
				13.32			5.96 ± 0.66 **
Fixation at 20 h							
0	178	08	08.56		37	2.37	–
	080	28	09.92	–	35	2.77	–
	156	24	09.62	–	65	2.38	–
	130	29	09.62	–	41	2.90	–
				09.43			2.60 ± 0.12
400	119	23	09.82		12	5.11	
	231	13	11.66		55	6.36	–
	218	61	13.85	–	60	6.32	–
	160	38	11.19		46	4.72	–
				11.63 **			5.64 ± 0.36 **
800	248	55	08.55	–	34	5.55	–
BSO							
004	096	06	08.26	–	42	2.62	–
	125	12	08.48	–	39	3.43	–
	121	22	09.30	–	46	4.48	–
	135	27	09.58	–	42	3.81	–
				08.90			3.59 ± 0.33 *
BSO + GSH							
004 + 400	107	07	08.48	–	58	5.21	–
	117	10	08.40	–	48	8.19	–
	072	06	08.26	–	30	5.07	–
	095	22	09.39	–	36	6.28	
				08.63			6.19 ± 0.62 **
004 + 800	083	06	08.59	–	34	6.58	–
	107	13	08.76	–	48	6.58	–
	115	21	09.12	–	47	3.13	–
				08.82			5.43 ± 0.93 **

TM: total metaphases
* $p < 0.01$ ** $p < 0.001$ compared to the respective control. Student's t test

were fixed in two changes of methanol acetic acid (3:1) for 30 min and 10 min respectively, resuspended in 0.5 ml of fixative, and dropped onto chilled slides and flame dried

Differential staining for sister chromatids

The method of Goto et al (1975) was followed. The slides were treated for 10 min with Hoechst 33258 (50 μg/ml) at room temperature, rinsed in distilled water and air dried. These slides were then mounted in 2 × SSC (pH 6.8) and exposed to sunlight in moist condition for 30–40 min depending on the intensity of the sunlight. Slides were rinsed twice in distilled water and stained in 2% Giemsa (BDH Chemicals Ltd. UK) for 3–4 min and mounted in DPX.

Scoring

The slides were coded and randomized. 25–85 metaphase cells with differentially stained sister chromatids from each mouse were studied for evidence of SCEs. For scoring cell cycle kinetics, metaphases were categorized as in first, second or subsequent division cycle based on their differential staining pattern. The cell cycle data were presented as average generation time (AGT) which is a ratio of BrdU duration (h) and replicative index (RI) where RI = (1 × M1 + 2 × M2 + 3 × M3)/number of cells. The BrdU durations were 16.25 h and 19.25 h for the cells fixed at 17 and 20 h respectively. To analyze the distribution of SCE among cells the dispersion coefficient was analyzed based on responses in individual animals. Data were subjected to parametric statistical analysis.

3. Results

It was possible to distinguish unequivocally the number of divisions in the presence of BrdU, as well as the number of SCEs in second replication cycle cells. Table 1 shows that GSH at all concentrations induced a significant level of SCEs in bone marrow cells harvested at either 17 or 20 h. At the highest concentration of GSH (800 mg/kg) not enough cells were available for SCE analysis at the 17 h fixation time due to considerable delay in cell proliferation. However, cells fixed at 20 h gave more second cycle cells for SCE analysis. Similar levels of SCEs were observed at 400 and 800 mg/kg of GSH. The frequencies of SCEs in the untreated controls did not differ significantly from each other at the two sample times. Mice treated with BSO also showed an increased frequency of SCEs per cell. Treatment with GSH

Table 2
Effect of catalase on SCE induction by GSH treatment in normal and BSO treated mice in vivo (20 h)

Dose (mg/kg)	Number of mice	Cells scored	SCE/metaphase/mouse (X ± SEM)	SCE range
GSH				
0	4	178	2.60 ± 0.12	2.37–2.90
400	4	203	5.64 ± 0.36	4.72–6.36
Catalase				
100	4	186	3.14 ± 0.38	2.10–4.65
GSH + Catalase				
400 + 100	6	320	3.15 ± 0.46 **	2.40–4.92
BSO				
001	4	169	3.59 ± 0.33 *	2.62–4.48
BSO + GSH				
001 + 100	1	172	6.19 ± 0.62 *	5.07–8.19
BSO + GSH + Catalase				
004 + 400 + 100	3	114	5.93 ± 0.17 *	5.71–6.27

* $p < 0.01$ ** $p < 0.001$ Student's t test † Compared to GSH

Table 3
Distribution of SCEs in cells treated with GSH alone or in combination with either catalase or BSO (20 h)

Dose (mg/kg)	SCE mean ± SEM [a]	SCE range [b]	Cells scored	Dispersion coefficient [c]
GSH				
0	2.37 ± 0.26	0–7	37	1.21
	2.90 ± 0.31	0–7	41	1.34
	2.38 ± 0.23	0–8	65	1.45
	2.77 ± 0.29	0–8	35	1.06
400	5.14 ± 0.44	1–13	42	1.58 *
	6.36 ± 0.45	1–16	55	1.75 *
	6.32 ± 0.42	2–18	60	1.69 *
	4.72 ± 0.36	1–11	46	1.28
GSH + Cat	2.52 ± 0.28	0–9	25	1.56 *
400 + 400	2.40 ± 0.21	0–6	40	1.37
	3.39 ± 0.17	0–10	68	1.27
	1.65 ± 0.20	0–11	50	1.12
	4.92 ± 0.19	0–15	84	1.90 *
	2.75 ± 0.13	0–6	53	0.35
BSO				
001	3.43 ± 0.30	0–8	39	1.05
	2.62 ± 0.29	0–7	42	1.38
	4.48 ± 0.39	0–10	46	1.58 *
	3.81 ± 0.41	0–11	42	2.18 *
BSO + GSH				
004 + 400	5.21 ± 0.33	0–15	58	1.18
	8.19 ± 0.51	1–19	48	1.50 *
	5.07 ± 0.50	0–12	30	1.48
	6.28 ± 0.59	2–17	36	2.03 *

[a] Animal mean SCE/cell frequency ± standard error of the mean among observed cells
[b] Range of SCE values
[c] Dispersion coefficient = variance/mean
* Significantly different at $\alpha = 0.05$ from Poisson distribution

of BSO-treated mice enhanced the frequency of SCEs further and brought it to the same level shown by GSH treatment of normal mice

Table 2 illustrates that the presence of catalase reduces the ability of GSH to induce SCEs. Catalase treatment alone also increased the frequency of SCEs but the increase was statistically non-significant with respect to control In contrast to GSH-treated normal mice, catalase absolutely failed to reduce the frequency of SCEs induced by GSH in BSO-treated mice

Table 3 represents the distribution of SCEs among cells in various treated samples In the GSH-treated sample the dispersion coefficient values are significantly higher indicating the SCE values are spread out considerably compared to control When catalase was added, the range of SCE values came down towards the control level The higher values of the dispersion coefficient in both BSO- and BSO + GSH-treated samples indicated the greatest spread in number of SCEs per cell

In addition to an analysis of SCE frequencies, cell cycle kinetics was also studied in GSH-treated mice. The fluorescence plus Giemsa staining technique facilitated the scoring of GSH-induced cell cycle delay in terms of reduction in the frequency of second and subsequent division metaphases following GSH treatment The percentage of first cycle metaphases (M1) was higher indicating a delay in cell progression in a dose-related fashion (Table 1) Although the basic cell cycle progression varied considerably among individuals in each group, the extent of delay by GSH increased in a dose-dependent manner. The AGT was significantly increased in the groups treated with 400, 500 and 800 mg/kg of GSH compared to control In contrast to the delay induction by GSH in normal mice, BSO-treated mice did not show any delay in cell proliferation after GSH treatment.

4. Discussion

SCEs have proved to be a sensitive indicator for DNA damage in many investigations (Latt et al., 1980). Reports available on SCE induction by GSH in in vitro systems are contradictory (McRae and Stich, 1979; Speit et al., 1980a). To the best of our knowledge, no study has been done on the effect of GSH on SCE induction in vivo. The present in vivo study shows that exogenous addition of GSH induces SCEs significantly in bone marrow cells of mice. The range of SCE values per cell is increased significantly in GSH-treated mice compared to untreated controls. McRae and Stich (1979) suggested that the formation of hydrogen peroxide is the reason for SCE induction by thiols This is supported by our present in vivo study since in the presence of catalase, the frequency of SCEs induced by GSH is reduced significantly However, it is important to note that catalase brings the GSH-induced

SCE frequency down to the catalase alone level. Therefore, the entire effect exerted by GSH on SCE induction could be due to hydrogen peroxide formation.

A novel aspect of the present study is the analysis of the influence of BSO on the induction of SCEs. Treatment with BSO produces a rapid decrease in the GSH levels of the various tissues (Griffith and Meister, 1979). It has been reported (Lee et al., 1987) that after a dose of 2.5 mM/kg BSO, GSH nadirs were reached by approximately 5 h for the liver and kidney, 8 h for lung and bone marrow. The degree of depletion was greatest for the kidney (80%), liver (74%) and bone marrow (83%). In this investigation we injected 18 mM/kg BSO (4 mg/kg) for 10 h. The concentration of BSO we used here was on the high side but was based on our chromosome aberration study where mice treated with 18 mM BSO/kg showed significantly increased sensitivity to ionizing radiation in comparison to normal mice (unpublished results). Therefore, in the present study BSO treatment for 10 h should be able to reduce endogenous GSH levels adequately and the induction of SCE by GSH depletion suggests the important protective role of endogenous GSH in the cell against peroxides and free radicals which are formed by normal metabolic pathways (Meister, 1979).

The demonstration of elevated frequency of SCEs after exposing BSO-treated mice to GSH and the failure of catalase to reduce this suggest the non-involvement of hydrogen peroxide in SCE induction in BSO-treated mice. It appears that the exogenous addition of GSH after BSO treatment could not have increased the endogenous GSH level due to impairment of GSH synthesis by the single BSO treatment. This impression is further consolidated by the cell proliferation data where GSH could not induce any delay in cell kinetics in BSO-treated mice. Wardman et al. (1992) demonstrated that intracellular cysteine levels are enhanced after exposing BSO-treated cells to GSH. Lee et al. (1987) showed that the recovery rate of GSH to pretreatment values following a single dose of BSO was 72 h for bone marrow. Therefore, addition of GSH after 10 h of BSO treatment could not negate the inhibitory effect of BSO on GSH synthesis. However, the increased frequency of SCEs induced by GSH in this case is difficult to interpret. It could be that GSH-degraded product(s) might be involved in this induction. It is clear from the results that neither the generation of hydrogen peroxide and nor the binding ability of GSH to chromatin was the factor responsible for such SCE induction.

GSH induces a delay in cell cycle kinetics in a dose-related fashion, which is in agreement with our earlier in vitro findings (Chatterjee and Jacob-Raman, 1986). The effect of GSH on the cell cycle has also been observed by Speit et al. (1980a) while studying the effect of GSH on SCE formation. They mentioned that GSH at concentrations greater than 5 mM inhibits cell cycle progression in V79 Chinese hamster cells. In general, aminothiol radioprotectors are thought to bind with DNA and slow down strand separation for replication (Brown, 1967). Therefore, the cell cycle delay effect of GSH observed in the present in vivo study may involve the binding of the chemical to chromatin.

Reduced GSH, a naturally occurring cellular component, can induce genetic damage if SCEs are taken as a measure of DNA damage. This induction of SCEs is largely due to generation of hydrogen peroxide after autoxidation of GSH since catalase brings down the GSH-induced SCEs to the catalase alone level. However, the induction of SCEs by GSH in BSO-treated mice indicated the involvement of an unknown route by which the degraded products of exogenously added GSH might induce SCEs. Therefore, the involvement of an unknown route or direct action of GSH to chromatin cannot be ruled out in a small fraction of SCEs induced by GSH in normal mice in vivo besides the major contribution of hydrogen peroxide.

The findings presented here indicate that the mechanism resulting in SCE formation by GSH may not be simple and more study is needed to develop a comprehensive hypothesis.

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