CHAPTER-3

Role of Glutathione in DNA Strand Break Joining

Aspects covered:
1. Role of GSH in induction of chromosomal aberrations
2. GSH and its role in DNA strand break joining
3.1 Review of Literature

3.1.1 Involvement of glutathione in restoration of DNA dsbs

It has been shown that an exogenous addition of GSH could effectively reduce radiation-induced micronuclei (Mazur, 2000) and chromosome aberrations in different systems (Chaudhury and Langendorff, 1968), including the muntjac lymphocyte cultures (Chatterjee and Raman, 1986). L-Buthionine sulfoximine (BSO) specifically depletes the endogenous GSH level by inhibiting the enzyme γ-glutamyl cysteine-synthetase and increases the cellular radiosensitivity (Meister, 1983). It has been demonstrated that BSO effectively depletes GSH and sensitizes normal and cancer cells to radiation (Louie et al., 1985; Chattopadhyay et al., 1999) and other chemotherapeutic agents (Fijo and Bates, 2003). In contrast to limited studies on the protective effect of GSH employing parameters of chromosome aberrations, 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 the cellular detoxification process (Revesz et al., 1984), regulates various enzymatic pathways by acting as a cofactor (Meister and Anderson, 1983), and is involved in cell growth and replication processes (Holmgren, 1979). The level of cellular radiosensitivity has been shown to be inversely correlated with the endogenous level of non-protein-bound sulfhydryls (NPSH), GSH being the major component of NPSH (Revesz et al., 1963). However, it has been proposed that GSH is critical for determining the cellular radiosensitivity when it is present within the cell nucleus, particularly close to DNA (Prise et al., 1992).
It was suggested that ionizing radiation-induced DNA dsbs are critical lesions, which, if unrepaired or misrepaired, can cause chromosome aberrations, cell death, as well as mutations and cell transformation (Iliakis, 1991). In an attempt to clarify the possible role of GSH in biochemical repair processes, the extent of rejoining of radiation-induced ssbs was determined up to 1 h after the exposure (Revesz and Edgren, 1984), and it was observed that the repair systems involved in the rejoining of oxically and hypoxically induced single strand breaks (ssbs) differed from each other and that the former was clearly dependent upon GSH. Radiation-induced chromosome exchange aberrations are thought to arise as a consequence of illegitimate reunion (misrejoining) of free ends from different DNA dsbs (Cornfroth et al., 1993). Such misrejoining may be expected to depend on the number and proximity of the breaks. It is indicated in an earlier report that reduction in GSH is associated with a decrease in DNA repair as measured by unscheduled DNA synthesis after cisplatin exposure, and is similar to the inhibition of DNA polymerase by NEM (Berger et al., 1979). This is consistent with the observation from Meister's laboratory that potentially lethal damage from irradiation in the GSH-depleted state could be reversed by treatment with GSH-ester (Wellner et al., 1984). Indeed, human fibroblasts deficient in enzymes of GSH synthesis show delayed recovery from ionizing or UV irradiation damage due to slowed repair of DNA strand breaks (Edgren et al., 1981; Deschavanne et al., 1984; Midander et al., 1986). In an attempt to clarify the probable role of GSH in refurbishing DNA damage, the extent of rejoining of radiation-induced ssbs was determined up to 1 h after the exposure (Révész et al., 1984), and it was observed that the repair systems involved in the
rejoining of oxically and hypoxically induced ssbs differed from each other and that the former was clearly dependent upon GSH.

Two major approaches were exploited for delineating the role of GSH in DNA dsbs joining:

**Approach-1: Pre or post treatment with GSH/GSH-ester:**

GSH pretreatment lessen the frequency of all types of CA and aberrant metaphases induced by 1 and 2Gy of X-rays and also decreased the tail moment, advocating radio-protection by GSH. Such uniform protection by GSH pretreatment was not seen when cells were exposed to 3 Gy or above even at higher level of endogenous GSH. As reported earlier (Chattopadhyay et al., 1999), it was observed that the frequency of radiation (3 Gy and above)-induced exchange aberrations increased and the frequency of deletions abridged in the presence of GSH. Exchange aberrations are thought to occur as a consequence of illicit reunion (mis-rejoining) of free ends involving different DNA DSBs (Cornforth et al., 1993). The idea of involvement of GSH in formation of exchange type aberration is strengthened by the observation of an increased frequency of exchange aberrations and decreased frequency of deletion in GSH/GSH-ester post-treated HPBL irradiated at 4°C (Chattopadhyay et al., 1999). The rationale for such post-treatment to irradiated cells at 4°C is based on the premise that an increased endogenous GSH level could act on radiation-induced unrepaired DNA lesions (due to 4°C incubation) after changing the temperature from 4 to 37°C. Therefore, GSH/GSH-ester was added soon after irradiation and kept for 3 h at 4°C.
**Approach-2: Interaction of bleomycin (BLM) and radiation induced DNA lesions:**

Evidences have also been gathered from combined treatment of bleomycin and radiation where induction of CA frequencies, particularly exchange aberrations and interstitial deletions, was increased significantly. Such increase in the frequencies in exchange aberrations was not seen if the cells were pretreated with BSO (Dutta et al., 2005). Therefore, it seems that endogenous GSH could modulate the efficiency of DSB rejoining.

**Combination of Bleomycin (BLM) and radiation**

The bleomycins are a group of glycopeptides showing antimicrobial properties extracted from Streptomyces verticillus. Beolmycin, at low doses, has been known to cause DNA breaks and inhibition of cell growth (Vig and Lewis, 1978). The mechanism of action of bleomycin is governed by formation of free radicals (Sausville et al., 1976) and requires an Fe$^{2+}$ ion as a co-factor upon binding to DNA through its bithiazole and terminal amine moieties for it to produce its effects.

\[
\text{BLM} + \text{Fe}^{2+} \rightarrow \text{BLM-Fe}^{2+}
\]

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

\[
\text{BLM-Fe}^{2+} + \text{O}_2 \rightarrow \text{BLM-Fe}^{2+} \cdot \text{O}_2
\]

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

\[
\text{BLM-Fe}_2 \cdot \text{O}_2 \rightarrow \text{BLM-Fe}^{3+}
\]

We have made use of the fact that if the DNA damage produced by two agents is repaired at very different rates then the probability of producing a synergistic effect...
on aberration frequency is low. On the other hand, if the damage from both agents is repaired rapidly, then there is a high probability of producing a synergistic cumulative effect (Preston, 1982). Keeping this in mind radiation and bleomycin were used since DNA lesions induced by these two agents have similar and rapid DNA repair frequency. Since, the probability of interaction between the DNA strand breaks induced by these two agents are high and therefore, it was considered appropriate to delineate the role of GSH in such interaction in HPBL system. BLM was chosen for its ability to induce CAs in an S-independent manner just like radiation does. It is known to produce double strand breaks possessing 5’-P and 3’-PG termini which are essentially blunt (Povirk and Austin, 1991).

Both the approaches were used to see whether GSH can play any role in DNA dsbs joining. Now an attempt has been made to confirm the earlier observations through a very different strategy that have not been utilized earlier. In brief, the efficiency of DNA dsbs joining was observed biochemically in cell free extract of normal tissues and cancer cell lines having different levels of endogenous GSH.

### 3.1.2 Enzymes and mechanism of DNA end joining

DSBs are considered as the most venomous type of DNA damages. It can manifest into chromosomal rearrangements like translocations and can cause cancer or cell death. DSBs can be generated by pathological or physiological agents. Pathological agents can be exogenous such as ionizing radiation, or chemotherapeutic agents like bleomycin. They can also be endogenous like replication across a nick, oxidative free radicals, mechanical shearing at anaphase bridges, inadvertent enzyme action at fragile sites, metabolic bi-products, and so forth physiological processes such as
V(D)J recombination, class switch recombination (CSR); and meiosis also introduce DSBs in our genome. DSB repair pathways in mammals can be broadly classified into two categories, namely, homologous recombination (HR) and nonhomologous DNA end joining. NHEJ needs little or no homology and is usually imprecise, while HR requires a region of extensive homology (Sharma and Raghavan, 2010).

The key players in end joining identify the broken DNA ends and advance the processing and ligation. To begin with, the DNA with DSBs is recognized by the Ku70/Ku80 hetero-dimeric complex, which then recruits DNA-PKcs in association with Artemis. DNA-PKcs and Ku complex play an important role in forming a synaptic complex that fetch the two DNA ends together. DNA-PKcs further autophosphorylates itself and phosphorylates Artemis too. Artemis-DNA-PKcs complex can now cleave 5’-overhangs and 3’-overhangs while Artemis unaccompanied can function as an exonuclease. After processing, the gaps are filled using Pol μ and Pol λ. XLF:XRCC4:DNA Ligase IV complex finally does the tailoring (Sharma and Raghavan, 2010).
Fig 28: Mechanism of DNA strand break joining reproduced with modifications from (Sharma and Raghavan, 2010)
3.1.3 End Joining (EJ) Assays

Being one of the major pathways for DSB rejoining, extensive studies have been done to understand its mechanism. Various in vitro and in vivo assays are accessible to study EJ in different cell types. The intracellular assays, although widely used, have several inadequacies e.g. the quantity of end joined products obtained are very low and thus, various end-joined products such as dimer, trimer, and other forms of multimers cannot be visualized on a gel even after Southern hybridization. PCR amplification could be used for detecting the end joined products; however, it may not be able to distinguish between different types of joined products. Besides, while performing DNA extraction, many linear products could not be recovered and therefore one could not dig up the actual efficiency of joining. Nonetheless, it is an admirable system because the role of different proteins can be studied as one could generate knockout for different genes expressing EJ enzymes in cell lines. In vitro assays, on the other hand, use a different strategy. They use a cell-free system containing active cellular proteins or selected purified proteins. Such assays have been instrumental in studying end joining both at the biochemical and molecular level. Cell-free system includes either nuclear extracts, total protein or cytoplasmic proteins prepared from cell lines or from tissues. Cell-free system is better in terms of better flexibility in choice of DSB end configurations besides, the sequences at or adjacent to DSBs can easily be maneuvered to study different junctional features of EJ. Generally, plasmids and synthetic oligomers are used as substrates in in vitro assays. In a recent study using this assay, appropriate oligomers were designed, synthesized, and annealed to generate substrates containing dissimilar DSBs. These substrates can be end-labelled with \(^{\gamma-32P}\) ATP, incubated with the extracts or
purified proteins, and then resolved on a polyacrylamide gel. All the joining products can be visualized and the efficiency of the joining between the substrates can be easily compared as it is proportional to the intensity of bands. The standard EJ products observed are dimers, trimers, multimers, and circular products (Sharma and Raghavan, 2010).

3.2 Materials and methods

3.2.1 Cellular models

For assessing the role of GSH in frequency of chromosomal exchanges HPBL from two different individuals were drawn. For DNA strand break joining experiments tissues from different organs of mice, keeping in view their GSH status, were chosen as primary models and cancer cell lines: MCF-7, HeLa & Nalm-6 were compared to the primary cells. Nalm 6 (leukemic cell line) was a kind gift from Dr. Sathees. C Raghavan, IISc Bangalore and was used strictly in collaborative terms.

3.2.2 Induction of Chromosomal aberrations

HPBL (5 ml) is distributed in 4 culture petri-dishes. The experimental conditions were Untreated, Bleomycin (BLM; Biochem Pharmaceutical Industries, Mumbai, India), 2 Gy and BLM + 2 Gy. In another set of experiment the condition was: Untreated, BLM, 4 Gy, BLM + 4 Gy, BSO + 4 Gy, BSO + BLM and BSO + BLM + 4 Gy. Irradiation is done 3 h after the administration of (20 µg/ml) BLM or 5 h after (0.2 mM) BSO treatment. Whenever (15 mM) GSH-ester was used, it was added to the cultures 30 min before the administration of BLM in all cases.
3.2.3 GSH estimation

Determination of total GSH was done according to the protocol of Akerboom & Sies 1981 (refer section 1.2 for details). Total GSH was estimated in brain, testis, thymus, spleen, lungs, heart, liver and kidney of 4 mice and in MCF-7, HeLa and Nalm-6 cancer cell lines.

3.2.4 GSH modulation in cell line

MCF-7 cell line was treated with 0.2 mM BSO for 5 h, GSH-ester at a concentration of 15 mM was added for 3 h and GSH (15 mM) was given to cell for 3 h. The experimental condition was MCF control (or untreated), BSO treated, GSH-ester treated and GSH treated. Two different DNA end joining assays were performed.

3.2.5 DNA strand break joining

We are thankful to Dr. Sathees. C Raghavan for providing us the facilities and help for execution of this Assay. For DNA strand break joining experiments the procedure of (Kumar et al, 2010) was followed. The scheme of the procedure is as follows:

Procedure:

Preparation of cell free extract-

Cell-free extracts were prepared as described earlier (Kumar et al, 2010). Briefly, cells of interest were cultured in bulk and washed in PBS. Approximately, \(8\times10^6\)cells were resuspended in 4 ml of hypotonic buffer (Buffer A: 10 mM Tris–HCl [pH 8.0], 1 mM EDTA, 5 mM DTT and 0.5 mM PMSF) and incubated for 20 min. Cells were homogenized in presence of protease inhibitors (1 μg/ml each of aprotinin, leupeptin, pepstatin) and an equal volume of ice-cold buffer B (50 mM Tris–HCl [pH 8.0], 10 mM MgCl\(_2\), 2 mM DTT, 0.5 mM PMSF, 25% sucrose and 50% glycerol) were also
added, followed by 1 ml of neutralized, saturated ammonium sulfate solution. The resulting lysate was stirred gently and centrifuged for 3 h at 32,000 rpm in ultracentrifuge at 2°C. Proteins were precipitated using ammonium sulfate (0.33 g/ml) from the supernatant, pelleted, dissolved and dialyzed in buffer C (25 mM HEPES-KOH [pH 7.9], 0.1 M KCl, 12 mM MgCl₂, 1 mM EDTA, 2 mM DTT and 17% glycerol). The clarified extract was distributed in aliquot, quick-frozen in liquid nitrogen and stored at -80°C until used. Protein concentration was determined by Bradford’s colorimetric assay.

Protein equalization & standardization of the amount of protein for end joining assay-

Densitometry was done for amount of total protein and was found that even when equal amounts of protein is added, there is difference in the actual amount of protein loaded (This may be attributed to the inaccuracy of the Bradford). Relative calculation was done from the Graph for equal loading in EJ reaction. Then to standardize the amount of protein, DNA end joining assay was done with different concentrations of proteins i.e. 0.5, 1.0, 2.0, 3 µg and we found 2 µg protein to be optimum that shows maximum joining along with minimal nuclease activity.

Oligomers- The 75 bp oligomers with compatible and non-compatible ends were gel purified as described by (Kumar et al, 2010). The 5’ end labeling of the oligomeric DNA was done using T4 polynucleotide kinase and [γ-32P] ATP, purified and stored at −20°C until use. The following oligomers were used (Kumar et al, 2010)
Preparation of DNA substrates- The oligomeric DNA substrates (75 bp) containing 5’ compatible overhangs were prepared by slow annealing of [γ- 32P] ATP labeled TSK1 with cold complementary oligomer TSK2 (1:3 ratio) in presence of 100 mM NaCl and 1 mM EDTA (Kumar et al, 2010). Similarly, double stranded DNA containing 5’-5’ and 5’-3’ non-compatible overhangs were also prepared by mixing [γ-32P] ATP labeled TSK1 with cold complementary oligomers VK11 and VK13, respectively. Then the end joining assay was carried out according to the following scheme:

| TSK1 | 5’(GATCCCTCTAGATATCGGCGCTCGATCTACTACTCGGAGCCGGCTAGCTTGATGCTCGGAGTCCTCGCAGTCTAGACCTGAG)3’ |
| TSK2 | 5’(GATCCTCAGGCTAGACTGCAGCATCGAAGCTAGCCGGCTCGAGTAGTAAGGATCGAGGGCCCGATATCTAGG)3’ |
| VK11 | 5’(AATTCTCAGGCTAGACTGCAGCATCGAAGCTAGCCGGCTCGAGTAGTAAGGATCGAGGGCCCGATATCTAGG)3’ |
| VK13 | 5’(GGCTAGACTGCAGCATCGAAGCTAGCCGGCTCGAGTAGTACCGGATCGAGGGCCCGATATCTAGG)3’ |
3.2.6 Statistics

In case of chromosome studies, significance was tested using Fischer’s exact test and simple $\chi^2$ test. The unpaired and two tailed t-test was performed for test of significance in GSH estimations.
3.3 Results

**Effect of GSH on induction of CAs by BLM or/and radiation in HPBL**

Cells were treated with BLM, 2 Gy, BLM + 2 Gy and GSH-E + BLM + 2 Gy. Chromosomal aberrations viz; exchanges, chromatid breaks, deletions were scored. The total aberration, exchanges and deletions caused by BLM alone and 2 Gy alone were showing an additive effect when these two treatments were combined (Table 4). In both the experiments, an interesting change occurred when GSH-ester was given prior to the combination of BLM + 2 Gy. The exchange was elevated from 20 to 60% in BLM + 2 Gy whereas the frequency of deletion was reduced from 146 to 60% upon GSH-ester pretreatment. A similar kind of trend was observed in the repeat experiment.

**Effect of BSO on induction of CAs by BLM or/and radiation in HPBL**

GSH was depleted by BSO-treatment and then induction of CAs were observed in various combination of treatments viz; BLM alone, 4 Gy alone, BLM + 4 Gy, BSO + 4 Gy, BSO + BLM and BSO + BLM + 4 Gy (Table 5). In the first experiment, it was found that the frequency of exchanges in BLM + 4 Gy is 134% which was reduced significantly to 29% when GSH is depleted prior to BLM + 4 Gy exposure. On the other hand, the frequency of deletions was increased from 106 to 220%. A similar observation was noted in the repeat experiment where the frequency of exchange aberrations was dropped from 131 to 32% with a concomitant rise in the frequency of deletions i.e. from 85 to 203%.
Total GSH estimation in mice organs and cell lines

Total GSH was estimated in different organs of mice and mean results were represented in “n mol GSH per mg of organ” (Fig 30-A). In brain we found 10.9 n mol GSH, testis showed 17.14 n mol, in thymus GSH was found to be 12.65 n mol, 11.3 n mol GSH was found in spleen, in lungs we noticed the GSH level to 8.5 n mol and heart, liver, kidney showed the GSH levels to be 9.2, 20.2 and 10.7 n mol respectively. Total GSH in cell lines were measured in n mol per million cells. In MCF-7 the total GSH was found to be 8.42 n mol, followed by 5.69 n mol and 4.85 n mol HeLa and Nalm 6, respectively (Fig 30-B). For preparation of cell free extracts in mice organs, testis and lungs have been chosen on the basis of their endogenous GSH. For cell lines all the three i.e. MCF-7, HeLa and Nalm 6 were chosen for cell free extract preparation.

Comparison of DNA end joining efficiencies using 5’-5’ compatible ends

Two batches of experiments were done to compare the DNA end joining efficiencies in the testis, lungs, MCF-7, HeLa and Nalm 6 (Fig 31). We observed maximum DNA end joining with visible multimers by the testis-extract than the lung cell free extract (CFE), approximately 4 times less DNA end joining than in testis. MCF-7 and HeLa showed nearly two fold less joining than CFE of lung. Joining in Nalm 6 was minimum and difficult to detect on the auto-radiogram. From here, we concluded that more the endogenous GSH (e.g. in testis) more is the DNA end joining; and end joining efficiencies in cancer cells was found to be less than primary cells.
Effect of exogenous GSH on the DNA end joining efficiency of 5’-5’ compatible ends

Two batch of experiments were done to evaluate the influence of exogenously added GSH in the CFEs of testis and lungs on DNA end joining of 5’-5’ compatible ends (Fig 32, 33). In both the experiments we observed a dose dependent decrease in the joining efficiency when the GSH concentration was increased from 0.5 mM to 20 mM and at 20 mM the joining was totally inhibited along with some of the nuclease activity.

Effect of exogenous GSH on the DNA end joining efficiency of 5’-5’ non-compatible ends

Two batch of experiments were done to see the effect of exogenously added GSH in the CFEs of testis and lungs on DNA end joining of 5’-5’ non-compatible ends (Fig 34, 35). In both the experiments, we noted a dose dependent reduction in the joining efficiency when the GSH concentration was increased from 0.5 mM to 10 mM and at 5 and 10 mM the joining could not be detected. CFE of lung did not show any joining of 5’-5’ non-compatible ends.

Effect of exogenous GSH on the DNA end joining efficiency of 5’-3’ non-compatible ends

Two separate experiments were done to determine the joining efficiency in the CFEs of testis and lungs upon addition of exogenous GSH in different concentrations. The 5’-3’ non compatible ends seem to be more difficult to join evident from no visible joining in both testis and lungs CFEs in Fig 36 and extremely low joining in testis
and no joining in lungs CFEs as shown in Fig 37. But, again there is decrease in joining as the concentration of GSH is increased (Fig 37).

**Effect of glutathione modulation on the DNA end joining efficiency**

MCF-7 was chosen for this study and the experiment was done in duplicate using 5’- 5’ compatible ends. First of all we observed that the overall joining efficiency was less in MCF-7 as evident by presence of joined circular products below 150 bp and absence of dimers at 150 bp and multimers. When GSH was depleted using 0.2 mM BSO for 5 h, we noted a ~1.5 times increase in joining. but importantly when 2 mM GSH-Ester was added for 4 h, the DNA end joining increased by more than 2 fold (Fig 38). But addition of 2 mM GSH reduced the joining efficiency as we consistently saw from Fig 32-38.
### Table 4: Effect of GSH-ester on induction of CAs by BLM or/\& radiation in HPBL

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<th>Expt condition</th>
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<th>Exchr (%)</th>
<th>Chtd brk (%)</th>
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* Significant at p<0.01  2x2 χ² -contingency test,  $ Borderline of p<0.05 2x2 χ² -contingency test,  # significant at p<0.01 simple χ² test
Table 5: Effect of BSO on induction of CAs by BLM or/and radiation in HPBL

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<th>Chtd brk (%)</th>
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* Significant at p<0.01  2x2 $\chi^2$ -contingency test, $\$ Borderline of p<0.05 2x2 $\chi^2$ -contingency test, # significant at p<0.01 simple $\chi^2$ test
Fig 30: Total GSH estimated in mice tissues (A) and cell lines (B).
Fig 31: Comparison of 5’-5’ CEJ efficiencies in primary models and cell lines
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Fig 32: Effect of exogenous GSH on joining of 5'–5' compatible ends (batch-1)
Role of Glutathione in DNA Strand Break Joining

Fig 33: Effect of exogenous GSH on joining of 5'-5' compatible ends (batch-2)
Fig 34: Effect of exogenous GSH on joining of 5'-5' Non-compatible ends (batch-1)
Fig 35: Effect of exogenous GSH on joining of 5'-5' Non-compatible ends (batch-2)
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Fig 36: Effect of exogenous GSH on joining of 5'-3' Non-compatible ends (batch-1)
Role of Glutathione in DNA Strand Break Joining

Fig 37: Effect of exogenous GSH on joining of 5'-3' Non-compatible ends (batch-2)
Fig 38: Effect of GSH modulation on CEJ efficiency of MCF-7
3.4 Discussion

The assumption that the presence of GSH has a role to play in the interaction with DNA dsbs to form exchanges was strengthened by the observation of an increased frequency of exchange aberrations and decreased frequency of deletions in GSH/GSH-ester post treated HPBL irradiated at 4°C (Chattopadhyay et al., 1999). This is in agreement with another study, where the frequencies of total aberrations and exchanges induced by BLM and radiation alone were enhanced significantly when these two treatments were combined. Interestingly, when GSH-ester was given prior to the combination of BLM + 2 Gy, the frequency of exchanges was elevated significantly and consequently the frequency of deletions was reduced. The notion about the involvement of GSH in formation of exchange type aberrations further fortified when it was found that the frequency of exchanges amplified and the frequency of deletion went down in GSH/GSH-ester pre-treated HPBL irradiated at 4°C (Chattopadhyay et al., 1999) and also supported by an earlier report (Hoffmann et al., 1993).

The idea about the involvement of GSH in exchange aberration formation is strengthened by the observation of the drastic reduction in the frequency of exchange aberrations and huge increase in the frequency of deletions after exposing the BSO-pretreated cells to the combined treatment of BLM and radiation. It is worth mentioning that BLM treatment to BSO treated cells reduced the frequency of aberrant metaphases and chromatid breaks as shown earlier (Chattopadhyay et al., 1997). This reduction in the effect of BLM in GSH-depleted cells could be explained on the basis of failure of reactivation of the oxidized BLM by the reducing agent.
GSH that is usually present endogenously. Nevertheless, BLM + 4 Gy combined treatment to BSO-treated cells showed an increased frequency of aberrations perhaps because the frequency of DNA lesions induced by BLM in BSO-treated cells was at normal level initially and failed to increase further owing to failure of reactivation of the oxidized BLM. It has been demonstrated previously that the presence of GSH or thiol radioprotector potentiates the clastogenic action of BLM (Chatterjee et al, 1989; Hoffman et al, 1993). Therefore, in this study we used GSH ester in two samples. The frequency of exchanges was increased and the frequency of deletions was reduced significantly after the cells were treated with BLM + 2 Gy. GSH-ester is readily transported into cells and converted to GSH increasing the level of endogenous GSH within 2–3 h (Wellner et al, 1974). It seems that DNA lesions induced by BLM are subjected to interact or illegitimately unite with radiation-induced DNA-dsbs and such interaction depends on the level of endogenous GSH.

Therefore, the data at hand signify that BSO-mediated GSH depletion did not permit the interaction of DNA lesions induced by BLM and radiation and thus, witnessed the increment in the frequency of terminal deletions and chromatid breaks. Thus, GSH promoting DNA dsb rejoining and exchanges has been revealed in this study and also is in agreement with earlier studies (Chattopadhyay et al, 1999; Dutta et al, 2005; Edgren et al, 1981; Révész et al, 1984). When two double-strand breaks occur on different chromosomes, then the rejoining is mostly done by end joining (EJ) (Lieber et al, 2010) which likely to appear as exchange type aberration (dicentrics and rings) at chromosomal level. In order to ascertain the role of endogenous GSH on joining of DNA dsbs, we allowed joining of the synthetically designed oligos
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(both with compatible and non-compatible ends), mimicking broken DNA strands, in cell free extracts (CEF) of normal tissues and cancer cell lines with respect to their endogenous GSH status.

In the present study, the CFE of testis showed approximately four times more DNA end joining than in lungs. This could be attributed to the higher endogenous level of GSH in testis and supports the idea on the involvement of GSH in chromosome exchange aberration formation. A comparative EJ assay was performed using testis, lungs, MCF-7, HeLa and Nalm-6 and the efficiency of joining of cohesive ends differed among all the cell types, where highest is shown in CFE of testis and cell lines showed the least joining. This is in line with the previously established fact that efficiency of mammalian testicular germ cell extract in DSB joining of complementary ends is extraordinarily high (Raghavan and Raman, 2004). It also has the ability to join non-complementary ends that require essential pre-ligation modifications; though joining of complementary ends is preferred over non-complementary ends. Even in the present study, we found that the joining was much better and higher when cohesive ends were used and such joining was decreased when 5’-5’ non-complementary ends were used and going down further when non-complementary ends with 5’-3’ ends were used. Testicular germ cell extracts encourage for the most part truthful EJ for cohesive ends possibly to preserve the genomic sequence with least possible variation (Raghavan and Raman, 2004).

Most vital information was revealed when we used GSH (directly in the CFE) and GSH-ester (in growing cells) separately in the EJ assay. Exogenously added GSH in CEF was found to decrease the EJ efficiency in a dose dependent manner while on
the other hand treatment of GSH-ester to the growing cells increased the EJ efficiency by many folds which followed our line of findings in the present study. The probable reason behind the gradual decrease in the efficiency of EJ after GSH addition directly to CEF could be due to protein glutathionylation. Besides acting as a reactive oxygen scavenger, GSH can also form mixed disulfides with proteins, a process known as S-glutathionylation or reversible protein glutathionylation. The reversible formation of mixed disulfides between the oxidized glutathione and low-pKa cysteinyl residues is not only a cellular response to a mild oxidative/nitrosative stress, but also occurs under normal physiological conditions (Fratelli et al., 2004). Cysteine is present in the active site of many proteins and in protein motifs that function in protein regulation, cellular signaling, and control of gene expression (Jacob et al., 2006). Oxidative modifications of the cysteine sulfhydryl group have received increased attention for the last decade. Such glutathionylation has been implicated as a mechanism in protection against the irreversible protein oxidation during oxidative stress (Gallogely and Mieyal, 2007). A specific posttranslational modification of the protein cysteine residues occurs not only during the oxidative stress, but also in unstressed cells by the addition of GS-. Such oxidative thiol modifications lead to a change in the structure of proteins and cause inactivation (e.g., in phospho-fructokinase (Mieyal et al., 1991) and nuclear factor kappa B (NF B) (Qanungo et al., 2007). Therefore, at this juncture it can be speculated that addition of GSH exogenously may alter some protein structure and inactivate their ability to join 5’-5’ compatible DNA strands.
Interestingly such effect was not seen due to elevation of endogenous GSH in growing cells by GSH-ester treatment, rather it favours 5’-5’ compatible DNA strands joining. It is most likely that initial enhancement of cellular GSH will take place in the cytosol of the cell and increase the ratio between GSH/GSSG. In this more reducing cellular environment, de glutathionylation is the process by which GSH moiety can be removed from protein–mixed disulfides. Enzymes capable of reducing S-glutathionylated proteins include glutaredoxins (GRX; also known as thioltransferases) and thioredoxins (TRX) (Holmgren, 2000). Glutaredoxins (GRXs) are small redox enzymes of ~100 amino acid residues, which use GSH as a cofactor. Structurally, GRXs are very similar to thioredoxins, retaining the same fold and active sites. Both the TRX and GRX families contain a conserved -Cys-X-X-Cys- active site, which is important for their redox regulatory functions (Holmgren, 2000). They catalyze the reduction of disulfide bonds and become concomitantly oxidized by forming an intramolecular disulfide in the -Cys-X-X-Cys- active site. The oxidized enzyme is then reduced by the respective TRX reductase/GSH (Giles et al, 2003). Since glutathionylation affects the function of many proteins, the reverse process—deglutathionylation of specific proteins has been implicated in regulation of cellular homeostasis in health and diseases (Dalle-Donne et al, 2008). Therefore, in the present circumstances, the increased endogenous GSH may deglutathionylate more proteins and thereby increased the efficiency of joining of 5’-5’ compatible DNA strands.
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The present data certainly conveys that GSH plays a role in DNA strand break joining thus affecting cellular radiosensitivity; besides, raised several other issues and therefore further studies are essential to address these issues.