Modulation of rat erythrocyte antioxidant defense system by buthionine sulfoximine and its reversal by glutathione monoester therapy

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Received 9 September 2003; received in revised form 7 November 2003; accepted 11 November 2003

Abstract

The protective effects of glutathione monoester (GME) on buthionine sulfoximine (BSO)-induced glutathione (GSH) depletion and its sequel were evaluated in rat erythrocyte/erythrocyte membrane. Animals were divided into three groups ($n=6$ in each): control, BSO and BSO + GME group. Administration of BSO, at a concentration of 4 mmol/kg bw, to the albino rats resulted in depletion of blood GSH level to about 59%. GSH was elevated several folds in the GME group as compared to the control ($P<0.05$) and BSO ($P<0.001$) groups. Decreased concentration of vitamin E was found in the erythrocyte membrane isolated from BSO-administered animals. Antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) were also found to be altered due to BSO-induced GSH depletion in blood erythrocytes. The SOD and CAT activities in BSO group were significantly lower ($P<0.001$) than the other groups. Lipid peroxidation index and malondialdehyde (MDA) levels in erythrocytes and their membranes were increased to about 45% and 40%, respectively. The activities of Ca\textsuperscript{2+} ATPase, Mg\textsuperscript{2+} ATPase and Na\textsuperscript{+}K\textsuperscript{+}ATPase were lower than those of control group ($P<0.05$), whereas the activities of these enzymes were found to be restored to normal followed by GME therapy ($P<0.05$). Cholesterol, phospholipid and C/P ratio and some of the phospholipid classes like phosphatidylcholine (PC), lysocephatidylcholine (LPC) and sphingomyelin were significantly ($P<0.05$) altered in the erythrocyte membranes of BSO-administered rats compared with those of control group. These parameters were restored to control group levels in GME-treated group. Oxidative stress may play a major role in the BSO-mediated gamma glutamyl cysteine synthetase ($\gamma$-GCS) inhibition and hence the depletion of GSH. In conclusion, our findings have shown that antioxidant status decreased and lipid peroxidation increased in BSO-treated rats. GME potentiates the RBC and blood antioxidant defense mechanisms and decreases lipid peroxidation.

Keywords: Buthionine sulfoximine; Antioxidant; Glutathione monoester

1. Introduction

The clinical use of buthionine sulfoximine (BSO) during chemo- and radiotherapy against drug and radiation resistant tumors has been practiced in the recent years [1,2]. For instance, the exposure of cells and tissues to BSO elicits a number of effects including hepatic dysfunction, renal calcification, LDL oxidation, etc. Studies by Meister’s group have demonstrated the toxic effects of BSO on liver [4] and LDL oxidation (unpublished data).

The vital role of structural modification in sulfhydryl groups in protein has demonstrated in abnormal cellular functions, including transport of organic cations [5,6]. Glutathione (GSH), a tripeptide, the major non-protein thiol in mammalian cells, performs various functions ranging from cellular metabolism to transport and also protection against oxygen free radicals [7,8]. It has been reported that alteration of endogenous GSH is associated with mitogenic stimulation and GSH may regulate DNA synthesis. GSH depletion decreases the rate of cell proliferation and inhibits cancer growth [9]. It has also been reported that chemical depletion of intracellular GSH resulted in a decline of cellular pH in Ehrlich tumor cells, possibly due to changes in the Na\textsuperscript{+}/H\textsuperscript{+} antiporter activity [10]. Another study indi-
cates that the decrease in GSH content resulted in inhibition of neutrophil function as measured by decrease in superoxide generation, Na⁺ and Ca⁺ influx and lysosomal enzyme release [11].

Lipids are involved in a wide spectrum of cellular functions. Studies have shown changes in cholesterol and phospholipid pattern associated with malignancy and cell differentiation [12–14]. Our recent studies with BSO have also demonstrated the increased levels of lysophosphatidylcholine (LPC) with corresponding decrease in phosphatidylcholine (PC) of oxidatively modified LDL (unpublished data). We have also observed a decrease in the cellular GSH associated with oxidation of lipids and proteins (apo-B) of rat LDL in vivo. In view of this, we investigated the effect of BSO on the erythrocytes and their membranes that are always exposed to high partial pressure of oxygen.

In light of the above, the optimal use of BSO to sensitize the resistant tumor cells requires a detailed knowledge on its effects to the normal cells and it is also important to design the protocols to protect the normal cells and macromolecules from oxidative damages. Erythrocyte membranes are very rich in polyunsaturated fatty acids (PUFA) and are more vulnerable to lipid peroxidation due to constant exposure to oxygen. In the present study we have depleted GSH content using BSO and its effects were investigated. Therapeutic trials were also made with glutathione monoester (GME) supplementation to the BSO-treated animals.

2. Materials and methods

2.1. Chemicals

All the fine chemicals including BSO, bovine serum albumin (BSA), reduced glutathione (GSH), oxidised glutathione (GSSG), dithionitrobenzoic acid (DTNB), bathophenanthroline, etc., were purchased from Sigma Chemical Co. USA. All other chemicals used were of good quality and analytical grade.

2.2. Animals

Male albino Wistar rats (100 ± 10 g body weight) purchased from Fredrick Institute of Plant Protection and Environmental Toxicology (FIPPET), Padappai, Chennai, were fed ad libitum chow containing 5% fat, 21% protein, 55% nitrogen free extract, 4% fiber (w/w) and adequate mineral and vitamin contents.

2.3. Experimental design

The rats were divided into three groups; each group consisted of six animals.

Group I: (Control) Rats were given physiological saline (0.5 ml) intraperitoneally (i.p.) twice a day.

Group II: (BSO) Rats were intraperitoneally given BSO (0.5 ml, 4 mmol/kg body weight) twice a day. The dosage was fixed on the basis of Meister’s report [3].

Group III: (BSO + GME) Intraperitoneal injection of BSO (0.5 ml, 4 mmol/kg body weight) twice a day. GSH-monoester (5 mmol/kg body weight) injection (0.5 ml) was given 30–45 min after BSO administration based on Meister’s report [3].

2.4. Isolation of erythrocytes and erythrocyte membranes

Erythrocytes and their membranes were isolated from control and experimental groups according to the method of Dodge et al. [15] with slight modifications. Packed cells were washed with isotonic saline to remove the buffy coat. Different aliquots of packed cells were thoroughly washed with tris-buffer, 0.31 M, pH 7.4. These were used for the determination of several biochemical parameters. Then the another aliquot of packed cells was subjected to hemolysis by adding hypotonic tris-buffer, 0.015 M, pH 7.2. After 4–6 h, the erythrocyte ghosts were sedimented by high speed centrifugation at 12000 rpm, for 40–45 min, at 4–6 °C. The supernatant, hemolysate, was used for the analysis of antioxidants. The erythrocyte membrane pellets were suspended in 0.02 M tris-buffer, pH 7.2, and subjected to various biochemical investigations.

2.5. Estimation of protein

The protein content of the erythrocyte membranes was estimated according to the method of Lowry et al. [16].

2.6. Determination of antioxidants

2.6.1. Glutathione estimation

GSH was estimated from the hemolysate after sedimenting the erythrocyte membrane (EM) fraction. An aliquot of lysate was subjected to assay with DTNB and the color produced was read immediately at 412 nm [17].

2.6.2. α-Tocopherol estimation

Erythrocyte membrane content of α-tocopherol was determined after extracting with hexane. The hexane extracts were processed according to the method of Desai [18] using bathophenanthroline, which forms complex with ferrous ions.

2.7. Determination of antioxidant enzymes

2.7.1. Catalase (CAT)

CAT activity of erythrocytes was assayed following the method of Beers and Seizer [19]. The breakdown of hydrogen peroxide on addition of enzyme source was
followed by observing the decrease in OD of peroxide solution in ultraviolet (240 nm) region.

2.7.2. Superoxide dismutase (SOD)

The activity of SOD in the hemolysate was determined according to the method of Misra and Fridovich [20]. It is based on the inhibition of epinephrine adrenochrome transition by the enzyme.

2.7.3. Glutathione peroxidase (GPX)

Activity of GPX from hemolysate was assayed according to the procedure of Rotruck et al. [21] with slight modifications. This procedure is based on the reaction between the glutathione remaining in the following reaction with DTNB to form a compound which absorb light maximally at 412 nm.

\[
2\text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{GPx}} \text{GSSG} + 2\text{H}_2\text{O}
\]

2.8. Determination of lipid peroxides by high performance liquid chromatography (HPLC)

Lipid peroxides were measured as malondialdehyde (MDA) products, using the HPLC acetonitrile precipitation assay [22]. Briefly, aliquots of erythrocytes and membranes suspended in buffer were mixed with equal volume of acetonitrile, homogenized and centrifuged to precipitate the proteins. The supernatants were then filtered through millipore membrane (0.2 μm) and 20 μl of samples was injected by Hamilton syringe in the HPLC for detection of MDA. The MDA was identified by comparing the retention time (Rt) with the standards, tetra methoxy propane run under identical conditions. Diene conjugates, indices of LPO initiation, were measured in the lipid extracts of erythrocyte membranes by following the method of Klein [23].

2.9. Determination of membrane-bound ATPases

Activities of Na⁺K⁺, Ca²⁺ and Mg²⁺ ATPases from erythrocyte membranes were determined by following the method of Bonting [24], Hjerten and Pan [25] and Ohinishi et al. [26], respectively. The activities were indirectly measured by estimating the phosphorous liberated after the incubation of EM in a reaction mixture containing the substrate ATP with the co-substrate elements at 37 °C for 15 min. The reactions were arrested by adding 1.0 ml of 10% TCA. The phosphorus content from the TCA supernatants was then determined by the method of Fiske and Subbarow [27].

2.10. Lipid extraction and analysis

The erythrocyte membrane pellets obtained from 1.0 ml of packed cells were subjected to lipid extraction with chloroform–methanol (1:2, v/v) and purified by the method of Folch et al. [28]. Lipid extract from 1.0-ml packed cells was applied to a silicic acid (Sigma) column and sequential elution of neutral, glyco- and phospholipids was done by adding the respective solvent chloroform (10 column volumes), acetone + 0.03% water (2 column volumes) and methanol (10 column volumes). All the fractions were collected individually and dried under nitrogen atmosphere. Thin layers of silica gel-H with 0.5-mm thickness (used for the purpose of quantitative analysis of phospholipids than is generally used for qualitative analysis) were made using TLC appicator; the plates were allowed to dry at room temperature for 1–2 h and then activated at 110 °C for 1 h, just before use. Samples (40 μl, from 2.0 ml of stock extract containing phospholipids of 1.0-ml packed cells) were loaded on to the gel using capillary tubes. Chromatograms were developed with chloroform – methanol – con.ammonia– water (70:30:3:2, by volume). After the run, air dried plates were exposed to iodine vapor and the spots were detected, marked and then the iodine was evaporated. Various fractions with silica gel were scrapped off and taken in test tubes. The silica gel was then removed by centrifugation after adding 2.0 ml solvent to each tube. The supernatant solvent with individual phospholipid class was subjected to perchloric acid digestion; phosphorous content was determined by following the method of Fiske and Subbarow [27]. Erythrocyte membrane total cholesterol [29] and total phospholipids [30] were estimated from the lipid extract as described.

2.11. In vitro studies with H₂O₂

Percentage maximal release of MDA, a product of PUFA, was measured following incubation of erythrocytes or membranes with two different concentrations of hydro-

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>BSO</th>
<th>BSO + GME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysate total GSH (μmol/g Hb)</td>
<td>9.87 ± 0.68 a</td>
<td>4.29 ± 0.62 b</td>
<td>9.41 ± 1.46 a</td>
</tr>
<tr>
<td>α-Tocopherol (EM) (μg/mg Protein)</td>
<td>2.56 ± 0.23 a</td>
<td>1.42 ± 0.12 b</td>
<td>2.48 ± 0.11 a</td>
</tr>
<tr>
<td>Catalase (EM) μmol H₂O₂ cons/min/mg protein</td>
<td>3.21 ± 0.44 a</td>
<td>1.74 ± 0.15 b</td>
<td>2.51 ± 0.22 a</td>
</tr>
<tr>
<td>Superoxide dismutase (hemolysate, units/mg Hb)</td>
<td>3.31 ± 0.29 a</td>
<td>1.21 ± 0.34 b</td>
<td>3.03 ± 0.27 a</td>
</tr>
<tr>
<td>Glutathione peroxidase (hemolysate, μmol GSH cons./min/mg protein)</td>
<td>39.87 ± 3.19 a</td>
<td>28.92 ± 1.50 b</td>
<td>37.53 ± 2.64 a</td>
</tr>
</tbody>
</table>

Values represent the group means ± S.D. for six animals. Different superscripts within a row show significant variations between groups, \( P < 0.05 \).
In vitro studies on H\textsubscript{2}O\textsubscript{2}-induced LPO (maximal release of MDA) in erythrocytes and erythrocyte membranes isolated from control and experimental groups

<table>
<thead>
<tr>
<th>Source</th>
<th>Groups</th>
<th>Untreated cells</th>
<th>Treated with 3% H\textsubscript{2}O\textsubscript{2}</th>
<th>Treated with 0.75% H\textsubscript{2}O\textsubscript{2} + Na-azide</th>
<th>%Maximal release of MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocyte (pmol/mg Hb)</td>
<td>Control</td>
<td>6.21 ± 0.09\textsuperscript{b}</td>
<td>6.48 ± 0.23\textsuperscript{b}</td>
<td>96.28 ± 3.49\textsuperscript{b}</td>
<td>6.66 ± 0.43\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td>BSO</td>
<td>11.16 ± 1.23\textsuperscript{a}</td>
<td>15.29 ± 1.02\textsuperscript{a}</td>
<td>112.28 ± 4.9\textsuperscript{a}</td>
<td>13.6 ± 1.08\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>BSO + GME</td>
<td>7.48 ± 0.21\textsuperscript{b}</td>
<td>6.98 ± 0.86\textsuperscript{b}</td>
<td>101.81 ± 4.3\textsuperscript{b}</td>
<td>6.86 ± 0.72\textsuperscript{b}</td>
</tr>
<tr>
<td>Erythrocyte membrane (nmol/mg protein)</td>
<td>Control</td>
<td>0.92 ± 10.06\textsuperscript{b}</td>
<td>1.08 ± 0.06\textsuperscript{b}</td>
<td>3.69 ± 0.43\textsuperscript{b}</td>
<td>29.27 ± 2.43\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td>BSO</td>
<td>2.11 ± 0.12\textsuperscript{a}</td>
<td>3.23 ± 0.16\textsuperscript{a}</td>
<td>5.62 ± 0.48\textsuperscript{a}</td>
<td>57.47 ± 4.12\textsuperscript{a}</td>
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<tr>
<td></td>
<td>BSO + GME</td>
<td>1.06 ± 0.08\textsuperscript{b}</td>
<td>1.16 ± 0.12\textsuperscript{b}</td>
<td>3.83 ± 0.61\textsuperscript{b}</td>
<td>30.29 ± 2.38\textsuperscript{b}</td>
</tr>
</tbody>
</table>

Values represent the group means ± S.D. for six animals. Different superscripts within a column show significant variations between groups, \( P<0.05 \).

### 3. Results

#### 3.1. Body weights

There were no statistically significant changes in the body weights of animals noted during the study (data not given).

#### 3.2. Antioxidants/enzymes status

Table 1 indicates the antioxidant status of blood and erythrocyte membranes. The level of blood glutathione (\( P<0.001 \)) and the activities of SOD (\( P<0.001 \)) and GPX (\( P<0.01 \)) were diminished significantly. Decreased levels of erythrocyte membrane \( \alpha \)-tocopherol and CAT activity (\( P<0.01 \)) were observed in the BSO-induced GSH-depleted rats. Administration of GME is found to have a protective effect on the above parameters as shown in Table 1.

#### 3.3. Levels of MDA determined by HPLC

Elevated concentrations of MDA (\( P<0.001 \)) were recorded in erythrocytes and erythrocyte membranes isolated from BSO-treated rats when compared to the control and GME-supplemented groups (Table 2). BSO treatment to the rats resulted in 82% and 63% increase in the concentration of MDA of erythrocytes and erythrocyte membranes, respectively, when compared to controls. Increased absor-
bance ($A_{233}/A_{215}$) ratio by the diene conjugates was observed from erythrocyte membrane ($P<0.05$) in the BSO-administered animals. The levels of TBARS and diene conjugates were normalised in the GME-treated rats (Table 2). GME therapy to the BSO-treated animals reduced the MDA concentration to 23% and 13% in the erythrocytes and erythrocyte membranes, respectively, when compared to controls (Table 2).

3.4. In vitro lipid peroxidation

Table 3 depicts the basal and $H_2O_2$-induced LPO in erythrocytes and erythrocyte membranes obtained from control and experimental animals, in vitro. The level of LPO significantly increased ($P<0.05$) in both the erythrocytes and membranes of BSO group when compared to control. On incubation with $H_2O_2$ and Na-azide, a maximum release of MDA was recorded in thiol-depleted rats. GME-supplemented rats showed the reverted levels of membrane lipid peroxides.

3.5. Erythrocyte membrane (EM) lipid profile

The levels of cholesterol and phospholipids extracted from BSO-administered rat erythrocyte membrane were altered (Table 4) significantly ($P<0.01$). The levels of phospholipid had decreased in BSO-treated animals, whereas the cholesterol and cholesterol/phospholipid ratio had increased ($P<0.05$). GME therapy to the BSO-treated animals resulted in restoration of GSH levels and prevented the above changes in the erythrocyte membrane.

3.6. Membrane-bound ATPases

The activities of the membrane-bound enzymes were also found to diminish on BSO-induced GSH depletion (Fig. 1).
Erythrocyte membrane-bound enzymes, Ca\(^{2+}\)ATPase (\(P < 0.001\)), Mg\(^{2+}\)ATPase (\(P < 0.01\)) and Na\(^+\)K\(^+\)ATPase (\(P < 0.05\)) activities were decreased in BSO injected rats, but they were normalized upon GME-supplementation along with BSO (Fig. 1).

3.7. EM-phospholipid profile

Fig. 2 indicates the concentrations of phospholipid classes in erythrocyte membrane of rats treated with BSO and BSO + GME. Statistically significant decrease in the levels of PC (\(P < 0.01\)) and sphingomyelin (\(P < 0.01\)) and increase in the levels of LPC (\(P < 0.01\)) and phosphatidyl ethanolamine (\(P < 0.05\)) were noted in BSO-treated animals, whereas no significant changes were seen in the levels of amino phospholipids. GME-supplementation resulted in bringing back all these parameters to near control values (Fig. 2).

3.8. Correlation analysis

Fig. 3 shows correlations in all the groups between (a) GSH and TBARS, (b) GSH and vit. E, (c) vit. E and total ATPases, and (d) C/P ratio and total ATPases. There were negative correlations between (a) \(r = -0.908, P < 0.01\) and (d) \(r = -0.868, P < 0.05\). Conversely, there were positive correlations between (b) \(r = 0.987, P < 0.01\) and (c) \(r = 0.994, P < 0.01\).

4. Discussion

In general, the erythrocytes in normal physiological conditions are resistant to oxidative damage because of their efficient protective mechanisms. The interior of the cell is rich in antioxidant enzymes, such as CAT, SOD, GPX and glutathione reductase [34,35]. Erythrocyte membrane is also rich in \(\alpha\)-tocopherol concentration [36]. However, under oxidative stress condition the erythrocytes may be susceptible to oxidative damage due to the presence of haeme-iron, PUFA and oxygen, which may initiate the reactions that induce oxidative changes in red blood cells. Ney et al. [37] had reported that erythrocytes and their membranes are very sensitive to oxidative damage due to their content of unsaturated fatty acids, which are continuously exposed to high concentration of oxygen.

Our recent findings and others indicate that abnormalities induced by BSO administration to the normal cells may be associated with the enhanced production of reactive oxygen species [4,38,39]. The results from the current study showed that BSO caused a significant increase in erythrocyte and its membrane TBARS formation, and this clearly demonstrates that the erythrocytes are exposed to the oxidative stress environment. It has been reported that ROS, superoxide anions, hydroxyl radicals and hydrogen peroxide enhance the oxidative process and induce lipid peroxidative (LPO) damage in cell membranes and disturb the biochemical and
physiological functions within the erythrocytes, thereby affecting membrane integrity [40–42].

In the current study, decreased levels of total GSH in the blood of BSO-treated animals were recorded and these can be correlated with the decreased biosynthesis of GSH by hepatic tissues [4]. Due to the lesser availability of GSH, an oxidative stress condition might have resulted in the circulatory system. Probably, this could be the reason for the decreased MDA levels recorded in the erythrocytes and their membranes isolated from the BSO-treated rats. In general, glutathione (GSH) is synthesized in liver and transported to other tissues using gamma-glutamyl transpeptidase. Plasma GSH levels arise largely from the liver; cells of the kidney and other cells that are well equipped with transpeptidase utilize plasma glutathione [43]. In our previous study [4], it has been demonstrated that intraperitoneal administration of GME to the BSO-treated rats resulted in remarkable restoration of hepatic glutathione level. In accordance with this, in the current study also GME supplementation to the GSH-depleted rats was found to increase the blood GSH levels tremendously, which indicates an effective uptake of GME and transport of GSH by the liver tissue.

Another interesting observation in this study was the decreased levels of erythrocyte membrane α-tocopherol in the BSO group. This is well associated with the declined GSH content and increased LPO. The decline of GSH could be ascribed to this effect [44] and should be considered seriously since the membranes are always rich in α-tocopherol that protects them from toxic damages due to oxidative stress.

In erythrocyte membranes, the concentrations of α-tocopherol in diabetic rats were significantly lower than those in control rats [45]. Vitamin E is the major lipid antioxidant in mammalian erythrocyte membranes, where it plays an important role in the suppression of free radical-induced LPO [46]. The present study is well consistent with the above and it may be proposed that the regulation of α-tocopherol concentrations in membranes is critically important to maintain erythrocyte membrane structure and function. Further, the decreased erythrocyte vitamin E levels can be attributed either to increased consumption through the destruction of oxygen-free radicals and/or to a reduction in the regeneration of α-tocopherol from tocopheroxy radicals, which requires GSH for the conversion [47].

Chronic administration of BSO to the rats depleted GSH drastically and in turn resulted in the altered activities of the antioxidant enzymes. In the present investigation, BSO treatment decreased the activities of CAT, SOD and GPX. It has been also seen that CAT activity was inhibited by free radicals such as singlet oxygen and superoxide and peroxyl radicals [48,49]. In vitro studies by Gultekin et al. [50] observed significant reduction in the activity of SOD in erythrocytes exposed to chlorpyrifos-ethyl mediated toxicity. In this study, the in vivo increase of TBARS mediated by BSO indicates overproduction of ROS and was presumably associated with enhanced LPO, confirming the fact that the singlet oxygen and peroxyl radicals inhibited the activities of CAT and SOD.

Sodium potassium ATPase (Na⁺K⁺ATPase), calcium ATPase (Ca²⁺ATPase) and magnesium ATPase (Mg²⁺ATPase) are responsible for the transport of Na⁺, K⁺, Ca²⁺, Mg²⁺ ions, respectively, across the membrane at the expense of ATP hydrolysis [51]. The activities of these enzymes markedly decreased (Fig. 1) in the erythrocyte membranes isolated from BSO-treated rats. The activities of these enzymes were found to be decreased in ethanol-intoxicated rat erythrocyte membranes [52]. Our results are consistent with the above findings and the decreased erythrocyte membrane-bound enzyme activities can be attributed to altered membrane fluidity, enhanced LPO and declined antioxidant defense status during the course of BSO administration. Lipid peroxidation of biological membrane results in changes in its fluidity [53] and in the activities of membrane-bound enzymes [54,55].

Membrane stability is vital for the normal functioning of membrane lipids and proteins, which include receptors and enzymes. Alteration in the lipid levels disturbs the equilibrium of erythrocyte membrane and this affects the maintenance of asymmetric ionic concentration between intra- and extracellular matrix [56]. In the present study, the observed increase in membrane cholesterol, decreased phospholipids and increased C/P ratio in BSO-treated animals may be due to impaired exchange of cholesterol and phospholipids from the plasma and lipoproteins. It can also be stated that the decreased levels of phospholipids might have affected the exchange of cholesterol. An increased C/P ratio resulted due to the alteration in the levels of cholesterol and phospholipids. Changes in C/P ratio could affect the membrane viscosity and other physical properties.

The main function of the phospholipids, particularly, PC, is to maintain the structure of cellular membrane. In the current investigation, interestingly, elevated LPC was noted in the erythrocyte membrane of rats exposed to BSO treatment with the corresponding decline in the levels of PC, which suggests an impairment in esterification in thiolethanolamine residue in PE using methionine adenosyl transferase (MAT) as the methyl donor. MAT is a thiol-dependent enzyme and it has been shown that intraperitoneal injection of BSO in normal rats led to GSH depletion, inhibiting MAT activity [57]. The decrease in PC and increase in PE in BSO-treated rats may be due to decreased availability of the methyl donor, S-adenosyl methionine. All these lipid changes in BSO-treated rats were reversed by the simultaneous treatment with GSH ester. This demonstrates that the above alterations are due to thiol depletion. Moreover, previous studies by Jain et al. [58] had reported a decrease in the levels of PE in the lung and alveolar fluid of glutathione-deficient mice. In vitro study also demonstrates that glutathione modulation in HT-29 cells resulted in altered concentrations of PC and LPC [59].
In vitro studies reveal that the erythrocytes and erythrocyte membranes of BSO-treated rats are more susceptible to hydrogen peroxide attack and consequently release the maximum amount of MDA when compared to the other groups. In this context, it can be suggested that the red blood cells incubated with sodium azide, an inhibitor of CAT, resulted in higher release of TBARS in vitro, reflecting impaired CAT activity in erythrocytes, and this also confirms that the erythrocytes and erythrocyte membranes isolated from BSO-treated rats were already exposed to an oxidative environment caused by BSO-induced GSH depletion.

4.1. Antioxidant therapy

Administration of GME at a dose of 5 mmol/kg bw, i.p., twice a day, for 30 days, to the BSO-treated rats resulted in remarkable restoration of blood GSH levels. It has been reported that GSH monoethylester may be used to supplement cellular pools of GSH, in vitro and in vivo [60,61]. GSH monoesters are those in which the glycine carboxyl group is esterified and they can be readily deesterified intracellularly by esterase, into GSH and the corresponding alcohol. Restoration of GSH has normalized the level of vitamin E and also the antioxidant enzyme activities. The observed increased activity of GPX in GME-supplemented rats is due to the excess availability of GSH, which is the substrate for GPX [62]. Although BSO administration causes GSH depletion in both the normal and tumor cells, therapeutic efficacy can be achieved due to their differential requirements of GSH [3]. It is clear from the studies by Teicher et al. [63] that external supply of GME shows high specificity in protecting the normal tissues. In the current study, the excess availability of GSH in circulation, upon GME supplementation, facilitates RBCs to enrich the GSH content, which might have prevented the LPO, accumulation of H₂O₂, thereby protecting the antioxidant enzymes from toxic effects due to severe oxidative stress during BSO treatment. Since GSH efficiently reacts reversibly with thiol groups of proteins, peptides and cysteine molecules, it can protect the structural elements of the protein and regulate several enzyme activities [64]. The reason for the enhanced activities of CAT and SOD upon GME treatment may be due to the decreased oxidative stress and LPO, which is evident from the diminished production of MDA.

In the present study, GME supplementation resulted in normal values in all the parameters that were altered during the course of BSO administration. Thus, the therapeutic advantage of GME along with BSO would be effective in protecting the erythrocytes and their membranes from oxidative stress-mediated damages. In conclusion, our results indicate that the altered profiles of membrane cholesterol, C/P ratio, and membrane-bound enzyme activities are well associated with decreased vitamin E and enhanced lipid peroxides. This study also suggests some interesting possibilities, such as the increase in the levels of vitamin E by regenerating from tocopheroxy radical upon GSH supplementation as one possible mechanism that might protect the erythrocyte membranes from oxidative stress by interrupting the LPO process.

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