Modulation of the tissue defense system by squalene in cyclophosphamide induced toxicity in rats

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Abstract

Introduction: Antineoplastic drug, Cyclophosphamide (CP), is a widely used drug that causes toxicity through its metabolites, phosphoramide mustard and acrolein. Squalene (SQ), an intermediate in the cholesterol metabolism has antioxidant and membrane stabilizing property. In the present study, the protective role of SQ towards the tissue defense system of the liver and kidney in the toxicity induced by CP was assessed.

Material and methods: Normal Wistar albino rats were administered CP in a dose of 150 mg/kg b.wt., i.p., twice, for 2 consecutive days to induce toxicity. SQ, in a dose of 0.4 ml/day/rat p.o. was used to treat the toxicity induced by CP.

Results: Significantly decreased activities of enzymic antioxidants [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-s-transferase (GST) and glutathione reductase (GR)], decreased levels of reduced glutathione and increased levels of thiobarbutric acid reactive substance (TBARS) were observed. These pathological alterations were significantly normalized during the treatment of SQ.

Conclusions: CP toxicity increased the free radical levels in the tissues and affected the activities of the enzymic antioxidants. Increased levels of TBARS [a measure of lipid peroxidation (LPO)] and decreased levels of GSH (due to utilization for detoxification process) evidenced the damage to these tissues. Protection exerted by SQ could be due to free radical quenching, providing additional alkylation site to CP metabolites and by inducing enzymic antioxidant production in these tissues. In conclusions improved antioxidant defense system in the liver and kidney of the experimental rats confirms the protective role of SQ against CP induced toxicities.

Key words: enzymic antioxidants, TBARS, reactive oxygen species, free radicals, liver, kidney.

Introduction

A free radical is any atom or molecule, capable of independent existence that has one or more unpaired electrons. If uncontrolled by the protective mechanisms, it leads to a pathological effect which causes damage to cellular membranes, proteins and nucleic acids [1]. The major limitation of cancer chemotherapy is the injury of the normal tissue, leading to multiple organ toxicity [2, 3]. CP is an alkylating agent, the most commonly used anticancer and immunosuppressant drug. It is used for the treatment of chronic and
acute leukemias, multiple myeloma, lymphomas, and rheumatic arthritis and also in the preparation for bone marrow transplantation [4, 5]. High-dose chemotherapy administered in bone marrow transplantation regimens commonly induces nausea, emesis, oropharyngeal inflammation, abdominal pain and diarrhea [6]. Phosphoramide mustard and acrolein are the two active metabolites of CP formed by hepatic microsomal cytochrome P450 mixed functional oxidase system [7]. CP's antineoplastic effects are associated with the phosphoramide mustard, while the acrolein is linked with its toxic side effects [8]. Chemotherapy and radiation therapy are associated with increased formation of reactive oxygen species (ROS) and depletion of critical plasma and tissue antioxidants [9]. Two reactive moieties of acrolein, an aldehyde group and an unsaturated bond, have the potential to cross-link to nucleophilic groups on two different proteins [10]. Acrolein is a relatively long-lived molecule that might diffuse some distance before reacting with proteins [11]. Acrolein interferes with the tissue antioxidant defense system [12], produces highly reactive oxygen free radicals [13] and is mutagenic to mammalian cells [14]. Due to the highly reactive nature, free radicals can readily combine with other molecules, such as enzymes, receptors, and ion pumps, causing oxidation directly, and inactivating or inhibiting their normal functions [15].

Liver disorders were observed in the elevated therapeutic dose of CP [16-19]. The nephrotoxicity of CP was evidenced by the proximal tubular damage, a significant reversible depolarization and a decrease in conductance [20, 21]. CP induced urinary bladder inflammation has demonstrated alterations in neurochemical [22] electrophysiological properties [23] of the bladder.

Effects due to CP toxic metabolites could be avoided by detoxifying with agents which are able to conjugate or quench these toxic metabolites. An antioxidant agent like amifostine has a cytoprotective action against platinum-induced renal toxicity, was avoided due to its toxicities like hypocalcaemia, anxiety and hypotension [24, 25]. Other cytoprotective agents such as sodium thiosulphate, mesna and procainamide are not approved for wide clinical use due to lack of efficacy and non-selective cytoprotection against toxicity induced by platinum and alkylating agents in tumour tissues [26]. In a recent work, it has been concluded that hydralazine prevents the protein cross-linking against acrolein mediated toxicity [27]. Therefore, there is a need for a novel agent, which would protect the normal tissue from chemotherapy-induced toxicity without tumour protection and tumour growth stimulation properties.

SQ, the intermediate of the cholesterol metabolism, is an isoprenoid compound having six isoprene units. SQ has been reported to possess antioxidant and membrane stabilizing properties [28-30]. In vitro experimental evidence indicates that the SQ is a highly effective singlet oxygen scavenging agent [31]. The protective activity of SQ against radiation-induced injury was demonstrated in a mouse model [32]. Several experimental models demonstrated the detoxifying activities of SQ against a wide range of chemicals and a sink for highly lipophilic xenobiotics, assisting in their elimination from the body [33-35]. In our previous studies, it has been proved that the toxicity induced within 10 days by the administration of CP was attenuated by the treatment of SQ [36, 37]. The present study was designed to evaluate the protective efficacy of SQ towards the antioxidant defensive mechanism in CP induced toxicity in the liver and kidney of the experimental rats.

Materials and methods

Chemicals and drugs

Cyclophosphamide (Ledoxan®) was purchased from Dabur Pharma Limited, New Delhi, India. Squalene (≥97% by GC) was procured from Sigma Chemicals Company, St. Louis, MO, USA. All other chemicals and solvents used were of the highest purity and analytical grade.

Experimental design

Male albino Wistar rats (150±10 g) procured from Tamilnadu University for Veterinary and Animal Sciences, Madhavaram, Chennai, India were used for the study. Animals were fed with commercially available standard rat pellets and water. Male albino Wistar rats (25±2°C) and were acclimatized to 12:12 h light:dark cycles. Animal experiments were conducted according to the guidelines of the Institutional Animal Ethics Committee (Approval No. 01/006/06).

The rats were divided into four groups of six animals each, Group I served as the vehicle (normal saline) treated controls. Group II animals received CP intraperitoneally dissolved in saline, in a dose of 150 mg/kg b.wt., twice, for two consecutive days. Group III animals received SQ orally in a dose of 0.4 ml/day/rat on all the days of the experimental period (12 days) {Various doses of SQ (0.2, 0.4, 0.6, 0.8 and 1.0 ml/day/rat) were administered orally to the CP intoxicated animals to optimize the SQ dose for a maximum efficacy in the minimum dose, determined by the levels of serum marker enzymes for tissue damage (data not shown). It was found that 0.4 ml/day/rat of SQ have the maximum protective efficacy in the minimum dose. This dose of SQ has the protective efficacy against CP induced toxicities in the rats [36, 37]. Group IV animals were co-treated with CP (as in Group II) and SQ (as in Group III) for the first two days and SQ treatment was followed continuously daily for ten days up to the end of the experimental period.
At the end of the experimental period, all the animals were sacrificed by cervical decapitation. The liver and kidney were immediately excised and rinsed with ice-cold physiological saline. These were homogenized in 0.1 M Tris-HCl buffer (pH 7.4) and aliquots of this homogenate were used for the assays. SOD was assayed by the method of Misra and Fridovich [38]. The degree of inhibition of auto oxidation of epinephrine at an alkaline pH by SOD was used as a measure of enzyme activity. CAT was assayed by the method of Takahara et al. [39]. The amount of hydrogen peroxide consumed by the enzyme was used as a measure of the enzyme activity. GPx was assayed by the method of Rotruck et al. [40]. The enzyme activity was assessed in terms of utilization of the glutathione and is based on the remaining glutathione after the reaction, which forms a complex with 5,5-dithio-bis {2-nitrobenzoic acid} (DTNB). GST was assayed by the method of Habig et al. [41]. GR that converts oxidized glutathione (GSSG) to the reduced form (GSH) was assayed by the method of Staal et al. [42]. GSH was determined by the method of Moron et al. [43]. The level of lipid peroxidation was assayed by the method of Ohkawa et al. [44] and was expressed as nmoles of TBARS g⁻¹ of tissue.

**Statistical analysis**

The results were expressed as the mean ± standard deviation for six animals in each group. Differences between the groups were assessed by the analysis of variance (ANOVA) using the SPSS 10.0 software for Windows. Post-hoc testing was performed using the least significance difference (LSD) test.

**Results**

Severe biochemical changes in the liver and kidney of the experimental animals were observed due to oxidative damage during the intraperitoneal administration of CP. Table I shows the abnormally decreased activities of enzymic antioxidants in the liver of the experimental animals that indicate cellular damage caused by CP. The activities of SOD, CAT, GPx, GST and GR were decreased by 25.8, 31.7, 32.2, 41.5 and 47.3 %, respectively, in Group II animals when compared with the control (Group I). Activities of these antioxidant enzymes were restored to near normalcy after SQ administration p.o. to the CP intoxicated rats. No significant alterations were observed in any of these parameters in the SQ alone (Group III) administered rats except the level of GR, which was significantly elevated than the control. Toxicity of CP in the liver was also confirmed by the 1.26 fold increase in the level of TBARS (Figure 1) and 45% decrease in the level of GSH (Figure 2) in the group II animals when compared with the control. These abnormal alterations were reverted to significantly normal during the treatment with SQ. No significant changes were observed in the SQ alone administered group, which showed the non-toxic nature of the SQ.

Table I shows the abnormally decreased activities of enzymic antioxidants in the kidney of the experimental animals. Results are expressed as mean ± S.D. for six rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (Control)</th>
<th>Group II (CP)</th>
<th>Group III (SQ)</th>
<th>Group IV (CP + SQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>9.3±0.57</td>
<td>6.9±0.78***</td>
<td>9.6±1.03**</td>
<td>8.4±0.79**</td>
</tr>
<tr>
<td>CAT</td>
<td>65.12±4.64</td>
<td>44.49±5.78***</td>
<td>70.66±8.41**</td>
<td>63.41±8.12***</td>
</tr>
<tr>
<td>GPx</td>
<td>103.87±9.44</td>
<td>70.43±8.91***</td>
<td>101.34±7.24**</td>
<td>92.72±8.31**</td>
</tr>
<tr>
<td>GST</td>
<td>11.34±0.97</td>
<td>6.63±0.69***</td>
<td>11.47±1.27**</td>
<td>8.35±0.74**</td>
</tr>
<tr>
<td>GR</td>
<td>1.65±0.08</td>
<td>0.87±0.07**</td>
<td>1.75±0.07*</td>
<td>1.33±0.12***</td>
</tr>
</tbody>
</table>

Units – SOD: 50% inhibition of epinephrine auto oxidation min⁻¹; CAT: nmoles of H₂O₂ decomposed min⁻¹ mg⁻¹ protein; GPx: nmoles of GSH oxidized min⁻¹ mg⁻¹ protein; GST: Units min⁻¹ mg⁻¹ protein; GR: nmoles of NADPH oxidized min⁻¹ mg⁻¹ protein. Comparisons are made between Control (group I) with CP (group II), CP with CP+SQ (group IV) and Control with SQ (group III). Statistical significance: *P≤0.05, **P≤0.01 and ***P≤0.001

NS – not significant
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### Table II. Activities of enzymic antioxidants in the kidney of the experimental animals. Results are expressed as mean ± S.D. for six rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (control)</th>
<th>Group II (CP)</th>
<th>Group III (SQ)</th>
<th>Group IV (CP+SQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>4.35±0.38</td>
<td>3.21±0.42***</td>
<td>4.29±0.35**</td>
<td>3.94±0.4*</td>
</tr>
<tr>
<td>CAT</td>
<td>10.33±1.12</td>
<td>6.98±0.83***</td>
<td>10.38±0.96**</td>
<td>8.94±0.71**</td>
</tr>
<tr>
<td>GPx</td>
<td>57.49±4.91</td>
<td>42.75±5.32***</td>
<td>55.94±6.31**</td>
<td>55.79±4.4***</td>
</tr>
<tr>
<td>GST</td>
<td>9.48±1.04</td>
<td>6.47±0.75***</td>
<td>9.12±0.83**</td>
<td>8.38±0.94**</td>
</tr>
<tr>
<td>GR</td>
<td>0.78±0.09</td>
<td>0.59±0.05**</td>
<td>0.8±0.07**</td>
<td>0.72±0.08**</td>
</tr>
</tbody>
</table>

Units – SOD: 50% inhibition of epinephrine auto oxidation min⁻¹; CAT: nmoles of H₂O₂ decomposed min⁻¹ mg⁻¹ protein; GPx: nmoles of GSH oxidized min⁻¹ mg⁻¹ protein; GST: Units min⁻¹ mg⁻¹ protein; GR: nmoles of NADPH oxidized min⁻¹ mg⁻¹ protein. Comparisons are made between Control (group I) with CP (group II), CP with CP+SQ (group IV) and Control with SQ (group III). Statistical significance: *P≤0.05, **P≤0.01 and ***P≤0.001 NS – not significant.

Discussion

High doses of CP can cause an acute form of lethality within 10 days of its administration [45]. In experimental animals, the activities of SOD, CAT, GPx, GST and GR were decreased by 26.2, 32.4, 25.6, 31.8 and 24.4%, respectively, in Group II animals when compared with the control (Group I). Activities of these antioxidant enzymes were restored to near normalcy after SQ administration p.o. to the CP intoxicated rats. No significant alterations were observed in any of these parameters in the SQ alone (Group III) administered rats. 157 fold increase in the level of TBARS (Figure 3) and 43.78% decrease in the level of GSH (Figure 4) in the group II animals when compared with the control also confirm the CP nephrotoxicity. These abnormal alterations were reverted to significantly normal during the treatment with SQ. No significant changes were observed in the SQ alone administered group.

![Figure 2](image2) Levels of GSH in the liver of the experimental animals. Results are given as mean ± S.D. for six rats. Comparisons are made between Control (group I) with CP (group II), CP with CP+SQ (group IV) and Control with SQ (group III). Statistical significance: *P≤0.05, **P≤0.01 and ***P≤0.001

![Figure 3](image3) Levels of TBARS in the kidney of the experimental animals. Results are given as mean ± S.D. for six rats. Comparisons are made between Control (group I) with CP (group II), CP with CP+SQ (group IV) and Control with SQ (group III). Statistical significance: *P≤0.05, **P≤0.01 and ***P≤0.001

![Figure 4](image4) Levels of GSH in the kidney of the experimental animals. Results are given as mean ± S.D. for six rats. Comparisons are made between Control (group I) with CP (group II), CP with CP+SQ (group IV) and Control with SQ (group III). Statistical significance: *P≤0.05, **P≤0.01 and ***P≤0.001
our previous studies, it has been proved that the toxicity induced by CP within 10 days of the CP administration was attenuated by the treatment of SQ [36], evidenced by the significant normalization of clinical chemistry parameters and histopathological studies of the liver and kidney. Administration of intermittent massive doses of CP has been found to be advantageous in the chemotherapy [46]. The cellular mechanism of toxicity is mediated by an increase in the free radicals through intracellular phosphoramide mustard and acrolein, the principle alkylating metabolites of CP [47]. Increased free radical production stimulates lipid peroxidation and is the sources for the degradation of DNA, lipids and carbohydrates [48]. ROS can affect many kinds of proteins, interfering with the enzyme activity and the functions of the structural proteins [49]. The antioxidant enzymes SOD, CAT and GPx act in coordination to combat the formed ROS. Cellular defense against the intermediates of dioxygen reduction (superoxide radical, hydroxyl radical and hydrogen peroxide) were done by these enzymic antioxidants. A decrease in the activities of the antioxidant enzymes of CP administered rats was due to the inactivation of these enzymes by ROS [13]. This causes further elevation in the levels of ROS which severely decrease the activities of SOD, CAT and GPx. This is consistent with the previous report [50]. Yoshiyuki Kohno et al. stated that the rate constant of quenching of singlet oxygen by SQ was similar to that of α-tocopherol [51]. The SQ treated group (Group IV) showed improved activities of SOD, CAT and GPx than the CP group. These evidenced the low ROS level and ROS mediated inactivation of enzymes were prevented by SQ protecting the hepatocytes and nephrocytes from damage.

GST isozymes catalyse the conjugation of glutathione to several electrophilic compounds, including polyaromatic hydrocarbon [52]. CP administration decreased the activities of GSH metabolizing enzymes, GR and GST. GR contains one or more sulphhydryl group residues, which are essential for the catalytic activity and are vulnerable to free radicals [53]. Another possible mechanism for the decreased activity of GR could be due to the selective reaction of acrolein with the active site sulphhydryl cysteine [54]. These might be the reason for the decreased activity of GR in CP administration. This, in turn reduces the regeneration of GSH from GSSG by GR. The decreased availability of GSH partly might be responsible for the decreased activity of GR and also because of its oxidative modification in its protein structure. SQ treatment enhanced the activities of GR and GST. This reveals the attenuation of CP toxicity by SQ. It can also be hypothesized that SQ might have provided an additional alkylation site for CP toxic metabolites and exert its protection. The activity of GR was significantly increased in the SQ alone administered group than the control, which could be due to the antioxidant and the membrane stabilizing potential of SQ.

Increased lipid hydroperoxides and thiobarbituric acid-reactive substances also suggest oxidative stress in patients receiving chemotherapy [55]. Pathological changes associated with the significant increase in LPO, depletion in non-protein sulfhydryl groups and decrease in CAT activity reflects many functional alterations such as drop in the glomerular filtration rate, glomerular capillary damage and tubulotoxicity [56]. Due to the lipid peroxidation induced by CP, the levels of TBARS increased in the liver and kidney. This might be due to the increased production of free radicals or decreased enzymic antioxidant defense system. Following the administration of SQ, the levels of TBARS were maintained to the near normal status which indicates the reduced level of LPO. From this, it could be concluded that the oxidative stress induced by CP was attenuated by SQ due to its antioxidant property [29] and membrane stabilizing property [30]. This is consistent with the previous reports of our laboratory [37].

Glutathione, the non-enzymic antioxidant is an important scavenger of electrophiles such as acrolein [40, 57] and has been shown to be a critical factor in the toxicity of CP to hepatocytes [58]. GSH is known to protect renal and neuronal cells from cisplatin induced toxicity [59]. Depletion of plasma and tissue GSH appears to contribute to chemotherapy induced organ toxicity [60, 61]. In the present study, oxidative stress due to CP decreased the GSH levels in the liver and kidney. SQ treatment increased the levels of GSH (might be indirectly by increasing the GR activity), which is essential for detoxification processes and exerts protection for these tissues.

Conclusions

In the present study, the toxicity produced by CP, evidenced by decreased activities of enzymic antioxidants, elevated levels of TBARS and decreased GSH levels in the liver and kidney was attenuated by the treatment of SQ to the experimental rats. From these observations, it is possible to conclude that SQ was found to be effective in normalizing these pathological changes by modulating the antioxidant defense system through enzymic antioxidants.

Reference


