EFFECT OF CYCLOPHOSPHAMIDE-INDUCED
GENERATION REACTIVE OXYGEN FORMS
ON ULTRASTRUCTURE OF THE LIVER AND LUNG

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The aim of the present study was to investigate the correlation between ultrastructural
changes of the liver and lung, intensity of lipid peroxidation (measured by the level of
malondialdehyde (MDA)) in liver and lung tissue homogenates and the alterations in the
activities of superoxide dismutase (Cu,Zn-SOD) and glutathione reductase (GSSG-R) following
cyclophosphamide (CP) administration. Treatment with CP caused an increase in MDA level in
liver and lung tissue homogenates. No statistical differences, however, were noted in GSSG-R
and SOD activities in liver and lung homogenates. The paper discusses a potential link between
the findings of biochemical analysis and the morphological changes found within the liver and
lung, especially the apoptosis-like changes.

Key words: rats, liver, lung, cyclophosphamide, reactive oxygen forms,
ultrastructure.

Cyclophosphamide (CP), an alkylating agent is one of the widely used
cytostatic drugs. The mechanism of action of alkylating agents consists in the
conversion of an active hydrogen atom from the biologically active molecules (DNA,
RNA, enzymes, mucopolysaccharides). The alklation concerns carboxyl groups,
amino-terminals, phosphate groups and others. The alklation of the biologically active
molecules causes an impairment of their functions. Cyclophosphamide requires
metabolic activation by the cytochrome P-450 mixed function oxygenase (MFO)
system to exert its therapeutic and toxic effects. This metabolism produces
hydroxylated primary active metabolites: acrolein, phosphoramid mustard and
nitrogen mustard. Alternately, CP can be metabolized to the non-toxic metabolites.
Tissue differences in detoxifying enzyme activity have been postulated to explain the
organ-specific toxicity of CP. Since aldehyde oxidase and aldehyde dehydrogenase -
the detoxifying enzymes - are absent in the lung, CP causes selective pulmonary injury.
MFO-mediated metabolism of CP is an important, but not exclusive pathway to bioactivate various xenobiotics. The involvement of other metabolic pathways, such as cooxidation via prostaglandin H synthase (PHS) in the toxicity of CP has been postulated (2, 9). In contrast to MFO-s, found in the highest concentrations in the liver, PHS and lipoxygenase activities are relatively high in the lung and bladder, sites of major CP-induced toxicity (2). The effects of inhibitors of MFO or PHS activity substantiate the conclusion that activation of CP occurs, at least partially, by different enzyme systems in the lung and liver (9). Therefore, MFO inhibitors decrease alkylating activity in the liver, but not in the lung, while inhibitors of PHS are effective in the lung, but not in the liver.

The aim of the present study was to evaluate the effect of CP on the intensity of lipid peroxidation in the liver and lung, the changes in the activities of superoxide dismutase (SOD), and glutathione reductase (GSSG-R) as well as correlation between biochemical changes and ultrastructural changes after CP-induced damage.

Material and Methods

Wistar rats, divided into two experimental groups (10 animals in each) were used. Group I was given cyclophosphamide in a single intraperitoneal dose of 150 mg/kg b.w. Group II (control) received PBS instead of cyclophosphamide. The rats were sacrificed 24 h (subgroups I-1, II-1) and 7 days (subgroups I-7, II-7) after CP (or PBS) administration.

For ultrastructural examinations, 1 mm³ samples were collected from the central part of the right liver lobe, and left upper lobe of the lung. They were fixed in 3.6% solution of glutaraldehyde in 1M cacodylic buffer, pH 7.4, at 4°C for 2 h, refixed in 2% osmium tetroxide in Millonig’s buffer, pH 7.4, and after dehydration embedded in Epon 812. Ultrathin sections were contrasted with uranyl acetate and lead citrate and evaluated using a transmission electron microscope (TEM) Opton 900PC.

For biochemical analysis the liver and lung were weighed and homogenized in 9 ml of ice-cold 0.25 M sucrose and 0.15 M NaCl with addition of 6 µl of 250 mM BTH in ethanol (to prevent formation of new peroxides during the assay). Homogenization procedure was performed under completely standardized conditions. Twenty per cent homogenates were centrifuged at 10 000 x g for 15 min at 4°C and the supernatant was kept on ice until it was assayed. This procedure was used in 7 rats of each experimental group. The supernatant was used to analyse superoxide dismutase (Cu,Zn-SOD), glutathione reductase (GSSG-R), and malondialdehyde (MDA).

Cu,Zn-SOD (EC.1.15.1.1) activity in lung homogenates was determined by the method of Misra and Fridovich (6) as modified by Sykes et al. (16), which provides the measurement of the activity of cytosolic SOD. A standard curve for SOD activity was made using SOD from bovine erythrocytes (Sigma, St Louis, MO). One unit of SOD was defined as the amount of the enzyme which inhibited the oxidation of epinephrine to adrenochrome by 50%. Enzyme activity was expressed as units/mg of protein (U/mg.prot.) for lungs and serum.

Glutathione reductase (GSSG-R; EC.1.6.4.2) activity was measured by monitoring the oxidation of NADPH at 340 nm. Enzyme activity was expressed as units/mg of protein (U/mg.prot.) for lungs and serum.

The extent of lipid peroxidation in the lungs was assayed with thiobarbituric acid (TBA). Chromogenous condensation product of TBA with malondialdehyde
(MDA) (thiobarbituric acid-reactive substances - TBA-rs) was extracted from the aqueous phase into butanol and then an absorption at 532 nm was monitored. The concentration of MDA in lung homogenates was expressed in nmoles TBA-rs/g of pulmonary tissue (nmol/g.p.t.). The concentration of MDA in blood serum was expressed in nmoles TBA-rs/ml of serum (nmol/ml.b.s.).

Data are presented as means ± standard deviation (SD) of seven observations. Comparisons between the means of the control and treated subgroups were made using Mann-Whitney U-test for non-parametric data. In all cases, a probability level of P<0.05 was considered to indicate a significant difference. Statistical calculations were performed with Statistica 5.0 programme.

Results

Ultrastructural analysis of hepatocytes from animals of group I revealed no significant destructive changes. The most pronounced alterations were found 24 h after CP administration. The examination revealed marked polymorphism of the mitochondria and condensation of their matrix, segmentary blurring of the structure of the surrounding membranes, the presence of osmophilic intramitochondrial bodies and paracrystalline structures usually arranged along the organelles. Golgi complexes were stimulated. The rough endoplasmic reticulum was focally degranulated, while the smooth endoplasmic reticulum appeared considerably proliferated. Seven days after CP administration the ultrastructure of hepatocytes showed a regression of changes and gradual normalization. A detailed description of morphological and ultrastructural changes in hepatocytes was presented in our earlier paper (18).

The structure of the lungs in control group revealed no pathological changes 24 h following CP administration, the capillary endothelium of interalveolar septa showed features of focal damage or/and oedema of varied degree. Sporadically apoptosis-like changes were seen (Fig.1). The vascular lumen displayed numerous monocytes, and more rarely blood platelets, neutrophilic granulocytes and phagocytes with apoptotic bodies (Fig. 2). Type II of alveolar epithelial cells (EP II) had features of considerable damage, particularly within their intracellular structures. Damage to mitochondria prevailed, including discontinuation of mitochondrial membranes. Emptiness or/and changes in the structures of lamellar bodies of EP II as well as fusion of emptied bodies and pseudocyst formation within the cytoplasm of EP II were observed. Necrosis of EP II and their disintegration were rare.

Seven days after CP administration the capillary lumen of interalveolar septa displayed, in addition to inflammatory cells, numerous blood platelets. The platelets frequently combined with one another or/and with vascular endothelium. The areas of lung parenchyma with platelet accumulation frequently showed megakaryocytes or their fragments. The highest differentiation of morphological changes was observed within EP II. The cells had empty or misshapen lamellar bodies, or contained very large dense and giant lamellar bodies which occasionally combined with one another. In places, proliferation of EP II was observed in the alveolar lumen. These cells contained a varied number of usually typical lamellar bodies. No features of damage to mitochondria were noticed in the majority of cells. Severe damage to EP II, including their disintegration or apoptosis-like changes, was focally seen. Intensified fibroplasia processes were found to occur in the interstitium of interalveolar septa.
Fig. 1. The nucleus (N) of an interstitial cell with chromatin condensed under the nuclear membranes. Subgroup I-1, TEM, 7000x.

Fig. 2. Apoptotic bodies are seen within phagocyte in the capillary lumen. Subgroup I-1, TEM, 3000x.
Results of the analysis of antioxidant enzymes and changes in the levels of MDA are presented in Tables 1 and 2.

Table 1
The mean levels ± standard deviation (SD) of MDA, activities of Cu,ZnSOD and GSSG-R in liver tissue homogenates
Statistical differences - x: P<0.002 (comparison between subgroups I-1 and II-1);
y: P<0.002 (comparison between subgroups I-7 and II-7)

<table>
<thead>
<tr>
<th></th>
<th>Group I-1</th>
<th>Group I-7</th>
<th>Group II-1</th>
<th>Group II-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>Mean</td>
<td>1.42^x</td>
<td>1.33</td>
<td>0.69^y</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>± 0.43</td>
<td>± 0.38</td>
<td>± 0.21</td>
</tr>
<tr>
<td>SOD</td>
<td>Mean</td>
<td>13.40</td>
<td>13.14</td>
<td>12.55</td>
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<td></td>
<td>SD</td>
<td>± 2.04</td>
<td>± 2.08</td>
<td>± 1.87</td>
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<tr>
<td>GSSG-R</td>
<td>Mean</td>
<td>13.69</td>
<td>14.28</td>
<td>13.80</td>
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<tr>
<td></td>
<td>SD</td>
<td>± 2.82</td>
<td>± 1.07</td>
<td>± 1.42</td>
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Table 2
The mean ± standard deviation (SD) levels of MDA, activities of Cu,Zn-SOD and GSSG-R in lung tissue homogenates
Statistical differences: between subgroups I-7 and II-7 y – P=0.035;
between subgroups I-1 and I-7 z – P=0.00174

<table>
<thead>
<tr>
<th></th>
<th>Group I-1</th>
<th>Group I-7</th>
<th>Group II-1</th>
<th>Group II-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>Mean</td>
<td>1.13</td>
<td>0.84</td>
<td>0.84^z</td>
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<td></td>
<td>SD</td>
<td>± 0.10</td>
<td>± 0.10</td>
<td>± 0.07</td>
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<tr>
<td>SOD</td>
<td>Mean</td>
<td>8.60</td>
<td>8.24</td>
<td>9.00</td>
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<tr>
<td></td>
<td>SD</td>
<td>± 1.08</td>
<td>± 0.70</td>
<td>± 1.06</td>
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<tr>
<td>GSSG-R</td>
<td>Mean</td>
<td>13.12</td>
<td>11.60</td>
<td>14.17</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>± 0.67</td>
<td>± 0.43</td>
<td>± 2.29</td>
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</table>

Intraperitoneal CP administration in a single dose of 150 mg/kg b.w., resulted in an increase in lipid peroxidation products (MDA) in liver and lung homogenates. In subgroup I-1 (24 h following CP administration) as well as in subgroup I-7 (7 d), the increase in MDA level was statistically significant. No statistically significant differences were found in the activity of Cu,Zn-SOD and GSSG-R in liver tissue homogenates 1 and 7 d after CP administration (Table 1).
Discussion

Most of the changes observed in ultrastructural study in hepatocytes 24 h after CP administration can be classified as adaptive or compensatory alterations resulting from the enhanced cell metabolism expressed by, among other things, condensation of the mitochondrial membrane. The ATP-synthetizing mitochondria assume a condensed form, reducing the space occupied by the mitochondrial matrix. Changes of that type within mitochondria have been reported after administration of chloramphenicol, halothane and in carbon tetrachloride intoxication (1). In the present study, most of these changes were observed in subgroup I-1. The changes in group I-7 were sporadic. Like mitochondrial damage, the changes were reversible and subsided 7 d after cyclophosphamide administration. The mechanism of cyclophosphamide action causing lesions in the rat liver is a complex problem. Metabolism of cyclophosphamide occurs in the hepatocytes in the presence of NADPH and oxygen, and the proliferation of the smooth endoplasmic reticulum elements is regarded as the ultrastructural exponent of its biotransformation (4). CP has been proved to induce significant alterations in the activity of NADH and NADPH₂, likely to be responsible for the ultrastructural changes within mitochondria. However, we observed features of mitochondrial damage with disruption of mitochondrial membranes. We assume that an increase in MDA level in liver homogenates may be a biochemical evidence of these changes. Further studies are required to confirm this assumption.

Pulmonary injury induced by cyclophosphamide leads to changes in epithelial cell structure, as well as alveolocapillary permeability, and surfactant recovery as revealed in the present study. The little attention has been directed towards participation of the type II alveolar epithelial cells (EP II) in the pathogenesis of oxidant-induced pulmonary injury (11, 12). In biochemical studies focusing on the antioxidant characteristics of EP II, either freshly isolated or cultured EP II have been used. Our evaluation of Cu,Zn-SOD and GSSG-R in lung tissue homogenates revealed no major changes in the activity of these enzymes. Only in subgroup I-1 a slight increase in the activity of GSSG-R was noted. In other studies (7, 17), a decrease in the activity of Cu,Zn-SOD and GSSG-R was found. Our results appear to be rather consistent with those obtained by Patel and Block (7) following the administration of acrolein - a CP metabolite.

It seems that the results of the study have been also influenced by constriction of the bronchial lumen observed already 24 h after CP administration. Constriction of the bronchial lumen, especially when considerable, leads to alveolar hypoxia. Halliwell and Gutteride (3) demonstrated that hypoxia could induce the synthesis of SOD in the rat lungs. On the other hand, Russel et al. (8) revealed a decrease in Mn-SOD expression in type II cells isolated from the nonventilated and hypoperfused lungs. Alveolar hypoxia and fibrosis observed within interalveolar septa may lead to an increase in the number of EP II 7 d following CP administration. Intensification of fibroplasia processes in the interstitium of interalveolar septa is a likely manifestation of repair or adaptive processes (10). It may be also associated with an inflow and accumulation of monocytes, blood platelets and alveolar macrophages in the lungs with the subsequent production of factors stimulating the production of connective tissue fibres, particularly FGFs (fibroblast growth factor), PDGF (platelet-derived growth factor), TNF-α and Interleukin-1 by these cells (5, 15). The role of EP II in the development of the changes is not fully understood. The earlier studies (13, 14)
reported the possibility of active participation of EP II in the processes of fibrosis due to acute lung tissue damage and in details discussed the role of apoptosis in lung tissue rebuilding during its damage. We think that further studies should be undertaken to elucidate this problem, however, according to the current study results, the role of reactive oxygen forms in that process seems to be most important.

References