Protective Effects of Montelukast and L-Carnitine on Cyclophosphamide-Induced Lung Injury

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Abstract

The major aim of this work was to study the protective effects of the cysteinyl-leukotriene antagonist montelukast (ML) and L-Carnitine (LC) against cyclophosphamide (CP)-induced pulmonary injury in experimental rats. Thirty adult male albino rats were categorized at random into five groups of 6 rats each. The first group served as normal control group. All other groups were injected once with CP (150 mg/kg, i.p.). The second group was kept as CP toxicity control. The third group was injected with ML (10 mg/kg/day, i.p.) once daily for 10 consecutive days before CP injection. The fourth group received LC (250 mg/kg/day, i.p.) once daily for 10 consecutive days before CP injection. The fifth group received ML plus LC once daily for 10 consecutive days before CP injection. The protective effects of these drugs were evaluated by assessment of lung glutathione reduced (GSH), thiobarbituric acid reactive substances (TBARS), catalase (CAT), glutathione-S-transferase (GST), superoxide dismutase (SOD), and nitrite (NO2-) levels as oxidative and nitrosative stress biomarkers. This is in addition to assessment of serum levels of cyclooxygenase-II (COX-II) and lipoxygenase (LOX) as inflammatory parameters. Histopathological study was performed to confirm results of biochemical estimations. Pretreatment with ML, LC and their combinations effectively increased the levels of GSH and antioxidant enzyme GST and SOD. Pretreatment with ML, LC and their combinations decreased the serum activities of inflammatory enzymes COX-2 and LOX. Pretreatment with ML, LC and their combinations alleviated histopathological changes in lung tissue induced by CP. The results of this investigation show that ML, LC and their combination are true protective agents against CP-induced pulmonary injury in rats and may be promising for further clinical trials.

1 Introduction

The alkylating agent cyclophosphamide (CP) is a very popular cytotoxic drug used in most cancer chemotherapy and immunosuppressive protocols. Its neoplastic activity is evident against different types of human malignancies. However, CP is potentially cytotoxic against normal cells as well. It is bioactivated in the liver to active metabolites, like acrolein, that interact with cellular macromolecules, leading to DNA damage, apoptosis and oxidative stress. Most susceptible organs include lung, liver and kidney.

Oxidative stress and reactive oxygen species (ROS) generation caused by CP exposure can negatively modify the components of both healthy and neoplastic cells. Lack of detoxifying enzymes in the lungs is a cause of selective CP toxicity to lung tissue. CP is well known to cause various histopathological patterns of lung injury, including diffuse interstitial and alveolar edema as well as inflammatory changes that progress to lung fibrosis in both animals and humans. Lung toxicity by CP is probably induced directly on pulmonary tissue or indirectly via pulmonary inflammatory cell activation. Both cyclooxygenase II (COX-II) and 5-lipoxygenase (LOX) are involved in oxidative and inflammatory acute lung injury.
Properly, protective agents should be considered to alleviate severe tissue injury caused by antineoplastic agents like CP, but considering not to interfere with their beneficial anticancer effect. Montelukast (ML) is bronchodilator anti-inflammatory drug used in management of bronchial asthma. It is a selective antagonist on cysteiny-leukotriene receptor thus reducing eosinophilic inflammation of the airways. The non-protein amino acid L-carnitine (LC; β-hydroxy-γ-trimethyl-amino-butyric acid) is synthesized from the essential amino acids lysine and methionine. It participates in the metabolism of branched chain amino acids, beta oxidation of fatty acids, and cell membrane stabilization. LC was reported by many in vitro and animal studies to have free radical scavenging potential scavenger, thus protecting antioxidant enzymes from oxidative damage. It also has proved to possess anti-inflammatory activity.

This study was designed to examine the possible protective effect of ML, LC and their combinations against CP-induced lung injury in experimental rats. The protective effects of these drugs were evaluated by assessment of lung tissue glutathione reduced (GSH), thiobarbituric acid reactive substances (TBARS), catalase (CAT), glutathione-S-transferase (GST), superoxide dismutase (SOD), and nitrite (NO²⁻) contents as oxidative and nitrosative stress biomarkers, in addition to serum levels of cyclooxygenase-II (COX-II) and lipoxygenase (LOX) as inflammatory parameters.

2 Materials and method

2.1 Animals

Adult male albino rats weighing 180-200 g were procured from central animal house, Faculty of Medicine, Assiut University (Assiut, Egypt). Animals were kept in the animal room at 25±2°C with 12-hour light/12-hour dark cycles and fed with standard diet and water ad libitum. All animal handling and treatments were conducted according to the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23).

2.2 Experimental design

Thirty adult male albino rats were categorized at random into five groups of 6 rats each. The first group received only vehicles and served as normal control group. All other groups were injected once with CP (150 mg/kg, i.p.), where the second group was kept as toxicity control group and received vehicles of drugs only. The third group was injected with ML (10 mg/kg/day, i.p.) once daily for 10 consecutive days before CP injection. The fourth group received LC (250 mg/kg/day, i.p.) once daily for 10 consecutive days before CP injection. The fifth group received ML plus LC once daily for 10 consecutive days before CP injection.

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Twenty-four hours after the last dose of the specific treatment, rats were anesthetized with light ether and blood samples withdrawn directly from heart for preparation of serum. Serum was separated following centrifugation (Beckman GS-6 centrifuge, USA) at 4000 rpm for 10 minutes at 4°C and stored at -20°C prior to analysis. Soon, rats were opened then lungs were removed, washed three times in ice-cooled saline, blotted individually on ash-free filter paper and used for preparation of tissue homogenates.

2.3 Chemicals

Cyclophosphamide was obtained as pharmaceutical drug (Endoxan vial 200 mg) and dissolved in saline. Montelukast was obtained from SEDICO, (Cairo, Egypt). L-carnitine was purchased from Sigma-Aldrich (St. Louis, MO, USA). GSH [5,5’-dithio-bis-(2-nitrobenzoic acid) (DTNB)], thiobarbituric acid (TBA), N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) and pyrogallol were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.4 Preparation of lung tissue homogenate

Tissue homogenates (10% w/v) were prepared in ice-cooled phosphate buffered saline (PBS) by the aid of a homogenizer (Cole-Parmer instrument Company, USA) using glass tube and Teflon pestle. The obtained homogenate was centrifuged at 4°C for 10 minutes in a speed of 4000 rpm, where the obtained supernatant was used for estimation of different biochemical parameters.

2.5 Biochemical estimations

2.5.1 Estimation of oxidative stress parameters

Glutathione reduced (GSH) content was measured using the method of Beuter. Lipid peroxidation was estimated as TBARS according to the method described by Mihara and Uchiyama. Antioxidant enzymes CAT, SOD and GST were estimated by the methods of Abie, Marklund and Habig, respectively. NO²⁻ was estimated by the method of Montgomery and Dymock.

2.5.2 Estimation of inflammatory mediators

Inflammatory enzymes including COX-II and LOX were assayed using ELISA kit, obtained from Glory Science Company (St. Del Rio, USA) according to the method of Van Weemen and Schuurs.

2.6 Histopathological examination

Autopsy samples were taken from lung of rats in different groups and fixed in 10% formalin solution in saline for twenty four hours. Samples were washed with distilled water followed by dehydration using serial dilutions of alcohol. Xylene was used to dehydrate specimens, which were then embedded in paraffin for 24 hours in a
hot air oven at 56 °C. Tissue blocks 4 μ thick were prepared by slidge microtome using paraffin bees wax, then slides were deparaffinized and stained by hematoxylin and eosin as described by Bancroft and Steven16.

2.7 Statistical analysis

Results are presented as mean ± SEM. All the statistical analyses were performed using one way analysis of variance (ANOVA) test with turkey’s post hoc comparison test applied across all groups. Significance was based on p value < 0.05. Data analysis was accomplished using the computer software program statistical package for the social sciences (SPSS, version 20).

3 Results

Mean lung GSH level showed significant decrease in rats treated with CP as compared with normal control rats. Pretreatment with ML, LC or ML plus LC showed significant increases in lung GSH levels in comparison to CP control group. Alternatively, lung TBARS and NO2 significantly increased in rats treated with CP as compared with normal control group. Protection with ML, LC or ML plus LC significantly corrected the three parameters as compared to CP-treated rats. Combined effect of ML and LC was significantly better than either drug effect alone regarding both GSH, TBARS and NO2.

In addition, the activities of lung GST and SOD were decreased in CP control group as compared to normal control group. Administration of ML, LC or ML plus LC resulted in marked increases in tissue activities of GST and SOD as compared to CP-treated group. Alternatively, activity of lung CAT in CP rats showed no significant alteration as compared to normal control group (Table 1).

Serum levels of LOX and COX-II were significantly increased in rats treated with CP as compared with normal control rats. Pretreatment with ML, LC or ML plus LC showed significant decreases in serum levels of COX-II and LOX as compared to CP-treated group. Combination of ML and LC resulted in significantly lower levels of serum COX-II and LOX as compared to either drug alone (Table 2).

Histopathological examination of CP-treated rats showed hyperplasia of bronchioles with prebronchiolar leukocytic inflammatory cell infiltration. Alternatively, pretreatment with ML, LC or ML plus LC showed notable protection regarding histopathological lesions, returning to more or less normal architecture except for mild emphysema (Fig. 1-5).

4 Discussion

Most currently used anticancer chemotherapeutic agents like CP adversely affect normal cells, which may seriously affect patients’ quality of life, regardless of their great curative effects17. Therefore, there is a necessity to develop adjuvant therapy which may be used in conjunction with cancer chemotherapy to improve the efficacy of the treatment or reduce the associated undesirable side effects.

In the present study, CP is concerned due to its wide popularity as well as serious hazardous effects on different body tissue. CP is metabolized by hepatic cytochrome P-450 enzyme system to produce two metabolites, namely phosphoramide mustard and acrolein18. Phosphoramide mustard has apoptotic cytotoxic potential on tumor cells, while acrolein is toxic to normal cells, inducing necrotic and apoptotic cytotoxicity19. These deleterious effects are mostly due to oxidative and inflammatory pathways.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) productions mediate such injury through peroxidation of membrane lipids, proteins and DNA20. CP conjugates with GSH, thus causes oxidative stress through GSH depletion. GSH depletion leads to lowered cellular defense against free radical induced cellular injury resulting in necrotic cell death21. The detrimental effects of CP may also be mediated by stimulating nitric oxide (NO) production. RNS, particular peroxynitrite (ONOO-) produced through reacting NO and superoxide (O2-), participate massively in oxidative and inflammatory tissue injury22. In agreement with the aforementioned explanations, results of the present investigation showed oxidative and nitrosative derangements in CP control group, represented as suppressed GSH levels and antioxidant enzyme activities (GST and SOD) coupled with elevated TBARS and NO2 levels when compared with normal control animals (Table 1).

Inflammatory pathway is also involved in the pathogenesis of experimental alkylating agent-induced toxicity23. Regarding inflammatory progression, several mediators may arise from the COX-II cascade involving release of biologically active prostaglandins (PGs), or may be synthesized through the action of LOX, involving leukotrienes (LT’s) which are the second main family of arachidonic acid derivatives, which also have a major role in the inflammatory process. Previous investigators reported the key roles of COX and LOX pathways in the pathogenesis as well as in the protective strategies of lung injury23,24. In harmony with these reports, CP-treated animals in the present investigation showed significantly elevated levels of serum COX-II and LOX levels in comparison with normal control animals (Table 2).

Results of the present investigation clearly show antioxidant potentials for ML and LC, alone or in combination, regarding their effect on GSH, TBARS, GST, SOD and NO2 levels. It seems that LC is a more potent antioxidant agent compared to ML, which is apparent from the significant difference between LC and ML regarding all measured oxidative parameters in the direction of LC being better (Table 1). In agreement, it was reported that LC might be a good antioxidant through different mechanisms. First, LC can

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act as a direct free radical scavenger. Second, it is an essential factor for synthesis of adenosine triphosphate (ATP) through mitochondrial fatty acid mobilization and oxidation. At this step of ATP synthesis, a large amount of oxygen is consumed, and the oxygen is reduced to water at the end of the TCA cycle. Then, oxygen concentration decreases and ROS formation is also reduced. Third, LC also might interfere with the ROS formation through chelation of ferrous ion which is a catalyst in free radical generation reactions like Fenton and Haber-Weiss reactions. Finally, LC antioxidant power may be due to its ability to increase activities of antioxidant enzymes like SOD and others\(^7\)\(^8\)\(^25\). Regarding ML, it was reported to correct lipopolysaccharide (LPS)-induced elevations in TBARS and depletion in GSH in a lung model of injury\(^26\).

Table 1: Effect of pretreatment with ML, LC and their combinations on lung GSH, TBARS, CAT, GST, SOD and NO\(_2\) levels in rats subjected to CP-induced lung injury

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (^1)</th>
<th>CP(^2)</th>
<th>ML + CP(^3)</th>
<th>LC + CP(^4)</th>
<th>(ML+ LC) + CP(^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (µmol/g)</td>
<td>2.870±0.0657</td>
<td>0.725±0.0281(^a)</td>
<td>1.440±0.0553(^b)</td>
<td>1.803±0.0571(^b)</td>
<td>2.140±0.0657(^b)</td>
</tr>
<tr>
<td>TBRAS (nmol/g)</td>
<td>20.35±0.088</td>
<td>71.68±0.529(^a)</td>
<td>42.54±0.259(^b)</td>
<td>30.45±0.356(^b)</td>
<td>26.22±0.286(^b)</td>
</tr>
<tr>
<td>CAT (U/g)</td>
<td>0.0200±0.00037</td>
<td>0.0187±0.00174</td>
<td>0.0195±0.00022</td>
<td>0.0197±0.00062</td>
<td>0.0200±0.00037</td>
</tr>
<tr>
<td>GST (U/g)</td>
<td>0.960±0.0153</td>
<td>0.427±0.0133</td>
<td>0.592±0.0154</td>
<td>0.752±0.0120</td>
<td>0.853±0.0189(^b)</td>
</tr>
<tr>
<td>SOD (U/g)</td>
<td>0.198±0.0040</td>
<td>0.045±0.0013</td>
<td>0.108±0.0040</td>
<td>0.148±0.0026</td>
<td>0.168±0.0035</td>
</tr>
<tr>
<td>NO(_2) (µmol/g)</td>
<td>15.36±0.292</td>
<td>44.90±0.414(^a)</td>
<td>38.11±0.370(^b)</td>
<td>36.01±0.461(^b)</td>
<td>33.48±0.251(^b)</td>
</tr>
</tbody>
</table>

Data are presented as mean of 6 rats ± SEM. \(^1\)Normal control group, received only vehicles, \(^2\)CP control group, administered only CP (150 mg/kg/day, i.p., single dose) and drug vehicle, \(^3\)ML group, administered ML (10 mg/kg/day, i.p., 10 days) before CP treatment, \(^4\)LC group, administered LC (250 mg/kg/day, i.p., 10 days) before CP treatment, \(^5\)ML plus LC group, administered ML and LC in the same dose regimen stated above before CP treatment. Statistical analysis was performed by using one-way ANOVA followed by Tukey-Kramer as post ANOVA test, \(^a\)Significantly different from normal control group at p < 0.05, \(^b\)Significantly different from CP group at p < 0.05, \(^c\)Significantly different from ML group at p < 0.05, \(^d\)Significantly different from LC group at p < 0.05.

Table 2: Effect of pretreatment with ML, LC and their combinations on serum COX-II and LOX levels in rats subjected to CP-induced lung injury

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control(^1)</th>
<th>CP(^2)</th>
<th>ML + CP(^3)</th>
<th>LC + CP(^4)</th>
<th>(ML+ LC) + CP(^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-II (pg/ml)</td>
<td>47.38±0.562</td>
<td>65.24±0.482</td>
<td>52.01±0.481</td>
<td>54.29±0.552</td>
<td>49.72±0.344</td>
</tr>
<tr>
<td>LOX (ng/l)</td>
<td>20.58±1.808</td>
<td>327.56±3.370</td>
<td>229.42±0.288</td>
<td>251.22±1.807</td>
<td>213.24±1.808</td>
</tr>
</tbody>
</table>

Data are presented as mean of 6 rats ± SEM. \(^1\)Normal control group, received only vehicles, \(^2\)CP control group, administered only CP (150 mg/kg/day, i.p., single dose) and drug vehicle, \(^3\)ML group, administered ML (10 mg/kg/day, i.p., 10 days) before CP treatment, \(^4\)LC group, administered LC (250 mg/kg/day, i.p., 10 days) before CP treatment, \(^5\)ML plus LC group, administered ML and LC in the same dose regimen stated above before CP treatment. Statistical analysis was performed by using one-way ANOVA followed by Tukey-Kramer as post ANOVA test, \(^a\)Significantly different from normal control group at p < 0.05, \(^b\)Significantly different from CP group at p < 0.05, \(^c\)Significantly different from ML group at p < 0.05, \(^d\)Significantly different from LC group at p < 0.05.

On the other side, investigation of anti-inflammatory potential of ML and LC in the current investigation revealed true anti-inflammatory ability of both agents represented by significant reductions of COX-II and LOX levels in sera of ML or LC-pretreated rats compared with control CP rats (Table 2). Anti-inflammatory effects of the two test agents may be attributed partly to their anti-oxidant effects as oxidative stress triggers inflammatory cascade. Aldehydes which are generated as a result of lipid peroxidation are known to mediate tissue oxidative injury. Through their role in signal transduction, lipid peroxides were reported to be responsible for inflammatory changes in the lung tissue\(^27\).

Opposite to results of oxidative parameters, ML showed more significant anti-inflammatory potential compared with LC. This is in harmony with recent investigations declaring potent anti-inflammatory potential for ML\(^28\)\(^,\)\(^29\). Besides the well-known inhibitory
action of ML on LOX, it was recently reported to directly bind and inactivate PGE₂ in vitro.²⁵

It should be mentioned that results of biochemical estimations in this search were strongly supported by histopathological examination of lung sections obtained from study groups, where inflammatory and necrotic evidences seen in CP group were almost abolished in all treatment groups (Photos 1-5).

Fortunately, the present study results showed that combination of ML with LC showed more significant protective effect than either drug alone regarding levels of tissue GSH, TBARS, GST, SOD and NO₂⁻, and levels of serum COX-II and LOX. It seems that combination has the benefits of potent anti-oxidant effect of LC and potent anti-inflammatory effect of ML.

Fig 1: A photomicrograph of lung section obtained from normal adult male albino rats, showing normal histological structure of the bronchioles (b) and air alveoli (a)

Fig 2: A photomicrograph of lung section obtained from adult male albino rats, treated with a single dose of cyclophosphamide (150 mg/kg, i.p.); showing bronchioles hyperplasia (b) with prebronchiolar leucocytic inflammatory cell aggregation (m)

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Fig 3: A photomicrograph of lung section obtained from adult male albino rats, subjected to a single dose of cyclophosphamide (150 mg/kg, i.p.) and pretreated with montelukast (10 mg/kg/day, i.p., 10 days), showing mild emphysema (p) in air alveoli

Fig 4: A photomicrograph of lung section obtained from adult male albino rats, subjected to a single dose of cyclophosphamide (150 mg/kg, i.p.) and pretreated with L-carnitine (250 mg/kg/day, i.p., 10 days), showing mild emphysema (p) in air alveoli

Fig 5: A photomicrograph of lung section obtained from adult male albino rats, subjected to a single dose of cyclophosphamide (150 mg/kg, i.p.) and pretreated with montelukast (10 mg/kg/day, i.p., 10 days) plus L-carnitine (250 m/kg/day, i.p.), showing nearly normal architecture except for mild emphysema (p) in air alveoli

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5 Conclusion

Results of the present study strongly suggest that ML and LC are good agents to protect against CP-induced lung damage, particularly through their anti-oxidant (LC is stronger) and anti-inflammatory (ML is stronger) potentials. This is promising for further clinical trials involving application of ML and LC pretreatments before CP chemotherapeutic intervention to decrease its hazardous effect on lung and probably other tissues.

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7 Competing interests

No conflicts of interest are evident.

8 Author’s contributions

Emad H.M. Hassanein: Practical work

Amira M. Abo-Youssef: Data collection and resources

Basim A.S. Messiha: Manuscript writing and submission

Ramadan A.M. Hemeida: Idea of the work and general supervision.

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