EXPERIMENTAL STUDY

Melatonin treatment prevents carbon tetrachloride-induced acute lung injury in rats by mitigating tissue antioxidant capacity and inflammatory response

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ABSTRACT
AIM: Carbon tetrachloride (CCl4) is an organic chemical that produces different tissue-damaging effects when ingested or inhaled. Present study aims to determine whether the application of exogenous melatonin, a neurohormone with numerous biological properties, can prevent disturbances in lung tissue antioxidative capacities and arginine metabolism, tissue inflammation and oxidative damage induced by exposure to CCl4 in rats.

METHODS: The effects of melatonin on the changes occurring in rat lung tissue after an acute exposure to CCl4 were studied by monitoring alterations in antioxidant capacities, inflammatory parameters, parameters of arginine metabolism, and lipid and protein oxidative damage.

RESULTS: The results indicated that melatonin prevents CCl4-induced lung damage by mitigating tissue antioxidant capacity and preventing nitric oxide production through a shift from nitric oxide synthase to arginase. Also, melatonin partially prevented tissue inflammation and molecules' oxidative modification seen after exposure to CCl4.

CONCLUSIONS: The protective activity of melatonin can be attributed to its ability to scavenge both free radicals, as well as to its potential to increase tissue antioxidant capacity. The modulation of inflammatory response through both decrease in tissue inflammatory parameters and influence on arginine-nitric oxide metabolism might be an additional mechanism of action (Tab. 1, Fig. 2, Ref. 33).

KEY WORDS: lung injury, carbon tetrachloride, melatonin, tissue antioxidant capacity, arginine metabolism.

Introduction
Carbon tetrachloride (CCl4) is an organic industrial chemical most frequently generated in chemical production facilities that produce chlorine, hydrogen, and alkali, and while its toxic potential is well known, its production and consumption for emissive uses is believed to be fully controlled (1). In humans (e.g. workers exposed to CCl4) and in experimental animals administered with CCl4, it causes massive damage to the liver, kidneys, respiratory system, skin, etc. (2). Since around 4 % of CCl4 is eliminated by exhalation, one can expect that lung tissue might be a potential target organ for the damage caused by the exposure to this highly toxic chemical (3).

Shortly after its absorption, CCl4 is metabolized by cytochrome P-450 which is predominantly localized in liver tissue. Nevertheless, its expression is not negligible in other tissues such as lungs (4), where the products of this metabolism, highly reactive trichloromethyl (CCl3•) and trichloromethyl peroxyl radical (CCl3O2•) induce intra-alveolar septal ruptures and interstitial cell degenerations. This sequence of events takes place due to the activity of inflammatory cells (neutrophils and macrophages) attracted to the damaged tissue (6). One could also argue that the lung tissue damage after CCl4 application could be incurred by kidney damage, since a decrease in water excretion leads to lung oedema (7). Besides the increase in reactive oxygen species (ROS) production, CCl4 causes a release of inflammatory cytokines (8) and suppression in antioxidant capacities (decrease in reduced glutathione (GSH), vitamin C and E, and antioxidant enzymes), as well as brings about an increase in lipid peroxidation, protein carbonylation, and DNA damage, which lead to cellular caspase-mediated apoptosis or necrosis (9).

Melatonin (MLT, N-acetyl-5-methoxytryptamine) is a neurohormone produced mainly by pineal gland cells, but also by other tissues/cells (10). Studies revealed that MLT is able to scavenge ox-
ygen- and nitrogen-based reactants that are known to damage cell macromolecules, as well as to increase tissue antioxidant capacity (6, 10, 11). It was found that MLT acts by increasing the expression of glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) mRNAs, and mRNA of enzymes related to GSH synthesis (11). Melatonin was previously proven to ameliorate lung tissue injury induced by bleomycin (11), acute pancreatitis (12), aortic occlusion (13), lipopolysaccharide (14), phosgene (15), etc.

Having in mind that CCl₄ is a dangerous industrial pollutant still present in the working environments of many undeveloped countries, and that its inhalation might lead to lung tissue injury, we aimed to evaluate whether the application of MLT would prevent lung tissue damage induced by this hazardous chemical. The effects of MLT would be estimated based on its potential in preventing the disturbances in pulmonary non-enzymatic tissue antioxidant capacity (TAC; vitamin C and GSH) and enzymatic antioxidant capacities (GPx and CAT) produced by CCl₄. The effect of MLT on inflammatory cell infiltration would be estimated indirectly through the myeloperoxidase (MPO) activity, which represents an enzyme located within macrophages/neutrophils. The changes in arginine metabolism that follow CCl₄ application and the impact of MLT treatment are going to be assessed based on nitrate/nitrite and citrulline concentrations, as well as based on changes in arginase and iNOS activities. In the end, the potential of MLT in the prevention of lipid peroxidation (malondialdehyde (MDA) levels) and carbonyl protein modification induced by CCl₄ would be determined as well.

**Materials and methods**

**Drugs and chemicals**

All reagents, solvents and drugs used in this investigation were of analytical grade or better and were obtained from Sigma-Aldrich (St. Louis, MO, USA), Carl Roth (Karlsruhe, Germany) or Richter Pharma AG (Wels, Austria). On the day of the experiment, melatonin was dissolved in absolute ethanol and further diluted with a sterile saline solution (0.9 % NaCl) prior to the administration at a dose of 50 mg/kg (10).

**Animals and housing**

Adult male disease-free Wistar rats (n = 24) weighing 250–300 g were maintained under standard laboratory conditions: 22 ± 2 °C, 60 % humidity, and 12/12 light/dark cycle. Food and water were available ad libitum throughout the experiment. All experimental procedures were conducted in compliance with The European Council Directive (EU Directive of 2010; 2010/63/EU) and were approved by the local Ethics Committee.

**Experimental design**

The animal model of acute organ damage, in this case lung tissue damaged by CCl₄, was performed according to previous publications (2, 9). Groups of animals were treated as follows:

- **Group I**: Animals treated once with saline containing 8 % of ethanol (10 ml/kg).
- **Group II**: Animals treated once with MLT (50 mg/kg).
- **Group III**: Animals received a single CCl₄ dose (1 ml/kg).
- **Group IV**: Animals treated once with MLT (50 mg/kg) 1h after a single CCl₄ dose.

Twenty-four hours after the last treatment all animals were sacrificed with an overdose of general anaesthetic, namely ketamine, and their body weight was measured. After that, their thoracic cavity was opened, lung tissue dissected, weighted and stored in Eppendorf tubes at –80 °C until homogenization.

**Tissue biochemical parameters**

**Tissue homogenization**

After scarification, the lung tissue was isolated from the thoracic cavity, washed and homogenized in cold distilled water (10 %, w/v). The obtained homogenate was centrifuged at 12,000 rpm for 10 min at 4 °C in order to obtain a clear supernatant which was further used for determining the tissue biochemical parameters. The protein content in lung tissue homogenate was determined by using the Lowry’s method (16), and the amount of proteins was calculated based on the bovine serum albumin standard curve. All measured biochemical parameters were calculated based on the amounts of proteins in tissue homogenates.

**Determination of tissue antioxidant capacities**

Lung tissue antioxidant capacity (TAC) and vitamin C were determined by using colorimetric assay kits (Abcam65329 and ab65656). The amount of GSH in lung tissue homogenates was determined using standard Ellman method which is based on the reaction of non-protein thiols with DTNB reagent (17).

The activity of GPx was determined by using a previously described spectrophotometric method that utilises GSH, H₂O₂ and DTNB reagent (5), while the catalase activity was measured spectrophotometrically after the reaction of the enzyme with H₂O₂ as a substrate and ammonium molybdate at 405 nm (10).

**Inflammatory parameter determination**

The activity of MPO was measured through the amount of oxidized o-phenylenediamine in a reaction which contained lung tissue homogenate supernatant and H₂O₂ (18).

**Determination of biochemical parameters related to arginine metabolism**

The concentration of nitrates/nitrites present in lung tissue homogenates was determined in a mixture consisting of tissue homogenate and Griess reagent (19). The amount of citrulline in lung tissue homogenate was determined according to the standard method that is based on the reaction with diacetyl monoime and thiosemicarbazide (20). The arginase activity in tissue was determined using a colorimetric method which is based on the reaction between ornithine and ninhydrin (21). Quantitative determination of rat lung tissue iNOS was performed using CUSBIO (CSB-08325r) sandwich enzyme immunoassay kit following manufacturers’ instructions.

**Lipid and protein damage determination**

The amount of lipid peroxidation was estimated through the levels of MDA that was measured in reaction with thiobarbituric
protein carbonyl content was used for quantifying the oxidatively modified proteins and their content was determined spectrophotometrically using 2,4-dinitrophenylhydrazine as a colour reagent (22).

Statistical analysis
The obtained variables are presented as means ± SD and were compared using one-way ANOVA and appropriate post-hoc tests (GraphPad Prism, ver. 5.03; San Diego, CA). Probability values were taken to be statistically significant if the p value was lower or equal to 0.05.

Results
Although the exposure of animals to CCl₄ led to a decrease in relative lung tissue mass, this decrease was not found to be statistically significant when compared to group I (control group). Interestingly, the mean value for the relative lung tissue weight in group IV was almost identical to that in one of the control groups. Nevertheless, no significant difference was observed (data not shown).

Total antioxidant capacity in lung tissue was significantly increased in healthy animals treated with MLT, i.e. in group II (Tab. 1), while a single dose of CCl₄ led to a decrease in TAC (group III). Such a dramatic decrease in TAC was prevented by the treatment with MLT in group IV (Tab. 1).

Compared to untreated animals, the acute application of CCl₄ significantly affected all studied participating species of arginine metabolism (enzymes and their products) (Tab. 1). The metabolism of arginine in animals treated with MLT after CCl₄ was only slightly disturbed, and in majority of parameters, MLT’s effect appeared to be significant compared to the parameters obtained from animals treated with CCl₄ only (Tab. 1).

When compared to the untreated animals from group I, the applied CCl₄ produced a significant increase in MDA and lung tissue concentrations of oxidized proteins (p < 0.001) (Figs 2A and B). The treatment with MLT taking place 1 h after CCl₄ administration prevented such a significant increase in these two parameters. However, its potential was limited since the detected concentrations of MDA and carbonylated proteins were still higher than those in group I (Figs 2A and B).

Discussion
Free radicals generated in the liver as well as in other tissues after the application of CCl₄ (4) react with polyunsaturated fatty acids present in cell and organelle membranes. Such enhancement in lipid peroxidation leads to the formation of products that can be identified by HPLC and mass spectrometry.

Table 1. Lung tissue non-enzymatic and enzymatic antioxidant capacities determined for different experimental groups.

<table>
<thead>
<tr>
<th>Biochemical parameter/Group</th>
<th>I (Vehicle control)</th>
<th>II (MLT control)</th>
<th>III (CCl₄ control)</th>
<th>IV (CCl₄ + MLT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total antioxidant capacity (% change)</td>
<td>100±1.9</td>
<td>112.9±6.6**</td>
<td>88.4±0.6*</td>
<td>96.1±2.1*</td>
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<tr>
<td>Non-enzymatic oxidative capacities</td>
<td></td>
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<tr>
<td>GSH (nmol/mg of proteins)</td>
<td>0.25±0.007</td>
<td>0.27±0.004*</td>
<td>0.065±0.004*</td>
<td>0.140±0.003**</td>
</tr>
<tr>
<td>Vitamin C (nmol/g of proteins)</td>
<td>32.4±4.2</td>
<td>37.9±2.5</td>
<td>6.1±1.8*</td>
<td>27.6±14.4*</td>
</tr>
<tr>
<td>Enzymatic oxidative capacities</td>
<td></td>
<td></td>
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<tr>
<td>GPx (U/mg of proteins)</td>
<td>1.12±0.11</td>
<td>1.14±0.20**</td>
<td>0.82±0.19*</td>
<td>0.93±0.05</td>
</tr>
<tr>
<td>CAT (U/g of proteins)</td>
<td>113.6±9.9</td>
<td>109.7±8.6</td>
<td>82.7±5.1*</td>
<td>99.6±2.3**</td>
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<tr>
<td>Arginine metabolism parameters</td>
<td></td>
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<tr>
<td>Nitrite/nitrate (nmol/mg of proteins)</td>
<td>35.7±3.4</td>
<td>38.4±1.1</td>
<td>45.2±2.8*</td>
<td>39.1±0.8**</td>
</tr>
<tr>
<td>Citrulline (nmol/g of proteins)</td>
<td>73.1±9.4</td>
<td>67.4±5.3</td>
<td>89.2±4.2**</td>
<td>77.5±6.5**</td>
</tr>
<tr>
<td>Arginase (μmol/g of proteins)</td>
<td>24.7±1.5</td>
<td>26.5±0.2</td>
<td>10.7±6**</td>
<td>26.2±13.5</td>
</tr>
<tr>
<td>iNOS (IU/mg of proteins)</td>
<td>0.079±0.003</td>
<td>0.065±0.010</td>
<td>0.098±0.011*</td>
<td>0.081±0.013**</td>
</tr>
</tbody>
</table>

Values are given as means ± SD, n = 6. One-way ANOVA followed by Tukey’s test was used to compare the groups. * p < 0.001, ** p < 0.05 vs Control (group I), # p < 0.001, ## p < 0.05 vs CCl₄-treated animals (group III).
After CCl₄ application, TAC is shown to be decreased. Due to excessive ROS (such as hydroxy-2-nonenal) causes enzyme inhibition (GPx and others) and, in consequence, decreases protein synthesis (24). Generally, due to oxidative stress, disturbances in tissue oxidative capacities, CCl₄ is known to cause tissue inflammatory response, and the lungs are not an exception (8). In the present work, we estimated lung tissue inflammation based on MPO activity and NO concentrations (Fig. 1 and Tab. 1), where a significant increase in inflammatory parameters in rat lungs was observed after CCl₄ application. As in the case of studied antioxidant capacities, exogenous MLT partially ameliorated the inflammatory reaction in rat lung tissue (Fig. 1 and Tab. 1). Previous studies showed that MLT application prevents lung tissue neutrophil infiltration estimated based on MPO activity in mice with pulmonary fibrosis induced by bleomycin (11). This partial discrepancy in the findings might be attributed to different models of lung tissue damage, as well as to a different treatment regime, which in the mentioned study was sub-chronic while in ours, it was acute. Released MPO was found to be acting as a pro-inflammatory cytokine on alveolar and bronchial epithelial cells and leading to an increase in haemoxigenase-1 expression, thus causing lung tissue oxidative damage (29). Since the effect of MLT on MPO activity was slight, it leaves a “window” for this neutrophil/macrophage-borne enzyme to act and cause cell oxidative damage.

In the pathophysiological response of lungs to CCl₄, macrophages seem to play an important role since they release numerous different lytic enzymes (MPO) and inflammatory signalling molecules (NO, TNF-α and IL-1 β) (8). An increase in these signalling molecules is known to be tightly connected to the increase in calcium-independent iNOS activity and NO which increases tissue blood flow by causing profound vasodilation (30, 31). An application of MLT in rats with damaged lung tissue effectively inhibited NO production and iNOS activity which is in accordance with a previously observed decrease in NO production by inhibition of iNOS expression via inhibition p300 histone acetyltransferase in macrophages (32). The hereby obtained results of the lung tissue arginine activity in rats treated with CCl₄ and MLT are in accordance with previous studies, where possibly due to an increase in arginine concentrations following iNOS inhibition, the substrate upregulates the activity of the enzyme (arginase) by itself (33).

The activity of MLT in CCl₄-mediated lung tissue damage model can occur in consequence of several factors. Having in mind that MLT was administered after intoxication with CCl₄ in a single dose of 50 mg/kg, we can conclude that MLT most certainly possesses a significant protective potential. Since the application of CCl₄ is causing multiorgan damage via a very similar, one may say identical mechanism, it is unrealistic to expect that a single dose of MLT would completely prevent multiorgan damage.
speaking, MLT predominantly improved tissue antioxidant capacities, or prevented its decline, and decreased oxidative tissue damage that was estimated through the levels of MDA and carbonyl-modified proteins. Also, MLT inhibited the inflammatory response by mitigating the increase in MPO activity produced by the application of CCl₄.

References


7. Agency for toxic substances and disease registry (ATSDR) Toxico-


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