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ORIGINAL ARTICLE

Protective effects of melatonin and quercetin on experimental lung injury induced by carbon tetrachloride in rats

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ABSTRACT

Introduction: Exposure to carbon tetrachloride (CCl_4), a well-known toxicant, causes tissue damage by inducing oxidative stress via formation of free radicals. The fundamental structure of the organs of rats and humans is similar, so administration of CCl_4 to rats is an accepted experimental model to produce oxidative damage to various tissues including pulmonary tissue. In this study, we evaluated the protective capacity of melatonin and quercetin against CCl_4-induced oxidative lung damage in rats. *Material-Metods*: Rats were divided into five groups each containing seven rats as follows: Control group, Olive oil group CCl_4 group, CCl_4+Melatonin, and CCl_4+Quercetin group. The tissue samples were processed by routine histological and biochemical procedures. Sections were stained with Hematoxylin-eosin and Masson's trichrome. Histopathologic damage score was calculated. Malondialdehyde (MDA) and glutathione (GSH) levels and catalase (CAT) activities were assayed. *Results*: The lung sections of control groups showed normal histological characteristics. Fibrosis, interstitial hemorrhage, epithelial desquamation in bronchiole and alveoli, intra-alveolar edema, leukocyte, and macrophage infiltration were observed in lung sections of rats exposed to CCl_4 alone. The findings were reduced in the treatments groups. The MDA level in the CCl_4 group were significantly higher than in the other groups ($p < .001$), and the CAT and GSH levels in the CCl_4+Mel and CCl_4+Quer groups were significantly higher than in the CCl_4 group ($p < .05$). *Conclusion*: In conclusion, we suggest that agents with antioxidant properties such as melatonin and quercetin may have positive effects in the treatment of pulmonary diseases characterized by especially edema, inflammation, and fibrosis.

KEYWORDS carbon tetrachloride, lung, melatonin, quercetin, rat

INTRODUCTION

Exposure to carbon tetrachloride (CCl_4), a well-known toxicant, causes tissue damage by inducing oxidative stress via formation of free radicals [1–3]. Chemical injury induced by CCl_4 is mediated by metabolites that react with antioxidant enzymes, such as glutathione (GSH) and catalase (CAT) [4] increase the level of inflammatory cytokines. It is known that CCl_4 particularly induce liver damage; however, it has been shown that it also leads to oxidative injury in kidney, brain, lungs, testicles, and muscles [5–10]. Trichloromethyl (CCl_3) radical, produced via biotransformation of carbon tetrachloride in liver, is responsible from the toxic effect of CCl_4. CCl_3 radical forms the trichloromethyl peroxyl (CCl_3O_2) radical by binding to oxygen. Peroxyl radical is a potent lipid peroxidation inducer that involves in the cellular injury as a primary mechanism via disruption of cell membrane [7]. The fundamental structure of the organs of rats and humans is similar, therefore administration of CCl_4 to rats is an accepted experimental model to produce oxidative damage to various tissues including pulmonary tissue.

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Administration of antioxidants could protect the human body from the effects of free radicals and ROS and of lipid peroxidation and thereby retard the progress of many chronic diseases. Animal research resulted in improvement of oxidative stress-related organ damage (e.g., liver, pancreas, skin, brain, cerebellum, kidney) by administration antioxidant agents such as melatonin, caffeic acid phenethyl ester, ascorbic acid, N-acetyl cysteine, etc. in several disease models and aging [11–15].

Melatonin (N-acetyl-5-methoxytryptamine), most important indolamine derived from tryptophan, is produced by pineal gland, ovaries, retina, and gastrointestinal system. Melatonin is a direct scavenger of free radicals and the most powerful known antioxidant [16]. It exerts its protective effects by increasing antioxidant enzyme levels/activities [11, 12, 15, 17] or inhibiting pro-oxidative enzymes via its action on melatonin receptor [16, 18]. The quercetin (3,5,7,3′,4′-pentahydroxiflavon), one of the well-recognized flavonoids, is also a potent antioxidant [19]. Flavonoids are primarily found in vegetables, fruits, red wine, green tea, and onion [20, 21].

The benefit of certain substances containing antioxidants can be evaluated by assessing their protective potency against the CCl_4-induced alterations in lipid peroxide, and antioxidant status as well as histopathological picture of organs. In this study, we evaluated the protective capacity of melatonin and quercetin against CCl_4-induced oxidative lung damage in rats.

MATERIALS AND METHODS

Thirty-five female Wistar albino rats (3–4 months old) weighing 220–240 g obtained from the Experimental Animal Research Center of Inonu University were used in the present study. The animals were housed in individual cages for 10 days in a well ventilated room with a 12: 12-hour light/dark cycle at 21°C. Animals were fed with standard rat chow and tap water *ad libitum*. The experiments were performed in accordance with the Guidelines for Animal Research from National Institute of Health and were approved by the Committee on Animal Research at Inonu University, Malatya, Turkey.

Experimental Design

Rats were divided into five groups each containing seven rats as follows: Control group: (administered by 5% ethanol, 1 mL/day/ip), Olive oil group (administered by olive oil, 0.5 mL/every other day/ip), CCl_4 group (administered by 0.5 mL/kg CCl_4 dissolved in 0.5 mL/kg olive oil, for 10 days/every other day/ip), and CCl_4+Melatonin (CCl_4+Mel) (administered by 10 mg/kg/day/ip. melatonin dissolved in 5% ethanol, injected 24 hours after administration of CCl_4), CCl_4+Quercetin (CCl_4+Quer) group (administered by 25 mg/kg/day/ip. quercetin dissolved in 5% ethanol, injected 24 hours after administration of CCl_4). At the end of the study, the rats were sacrificed by ketamine anesthesia and by midline incision lungs were removed and divided into two portions. One part of the samples were used for histopathological examination whereas the others were used for evaluation of oxidative stress parameters by biochemical methods.

Histological Evaluation

The lung tissues fixed in 10% formalin for 24 hour were embedded in paraffin. Tissue sections cut at 5 μm stained with hematoxylin-eosin (H-E) and Masson's trichrome methods. Samples were examined using a Leica DFC280 light microscope and a Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK). Assessment of tissue alterations in 20 different fields for each section was conducted by an experienced histologist who was unaware of the treatment. Under 40X magnification, sections were examined for the alterations including epithelial desquamation, edema, hemorrhage, cell infiltration, and fibrosis indicating pulmonary injury. Each alterations was scored as follows: 0 = normal, 1 = mild, 2 = moderate, and 3 = severe, with a maximum score of 15.

Biochemical Evaluation

Preparation of tissue homogenates

Tissues were homogenized (PCV Kinematica Status Homogenizator) in ice-cold phosphate buffered saline (pH 7.4). The homogenate was sonified with an ultrasonifier (Bronson sonifier 450) by 3 cycles (20-s sonications and 40-s pause on ice). The homogenate was centrifuged (15,000 x*g*, 10 min, 4°C) and cell-free supernatant was subjected to enzyme assay immediately.

Determination of protein

Protein levels of the tissue samples were measured by the Bradford method [22]. The absorbance measurement was taken at 595 nm using a UV-VIS spectrophotometer. Bovine serum albumin (BSA) was used as protein standard.

Determination of malondialdehyde (MDA) levels
The analysis of lipid peroxidation was carried out as described [23] with a minor modification. The reaction mixture was prepared by adding 250 μL homogenate into 2 mL reaction solution (15% trichloroacetic acid: 0.375% thiobarbituric acid: 0.25 N HCl, 1:1:1, w/v) and heated at 100°C for 15 minute. The mixture was cooled to room temperature, centrifuged (10.000 g for 10 min) and the absorbance of the supernatant was recorded at 532 nm. 1,1,3,3-tetramethoxypropane was used as MDA standard. MDA results were expressed as nmol/mg protein in the homogenate.

Determination of GSH levels
The formation of 5-thio-2-nitrobenzoate (TNB) is followed spectrophotometrically at 412 nm [24]. The amount of GSH in the extract was determined as nmol/mg protein utilizing a commercial GSH as the standard.

Measurement of catalase (CAT) activity
CAT activity was measured at 37°C by following the rate of disappearance of hydrogen peroxide (H_2O_2) at 240 nm ($\varepsilon_{240} = 40\ M^{-1}\ cm^{-1}$) [25]. One unit of catalase activity is defined as the amount of enzyme catalyzing the degradation of 1 μmol of H_2O_2 per min at 37°C and specific activity corresponding to transformation of substrate (in μmol) (H_2O_2) per min per mg protein.

Statistical Evaluations

Statistical analysis was carried out using the SPSS for Windows version 13.0 (SPSS Inc., Chicago, III., USA) program. All data are expressed as arithmetic mean ± Standard error (SE). Normality for continued variables in groups were determined by the Shapiro Wilk test. Since the variables didn't show normal distribution ($p < .05$), Kruskal–Wallis and Mann Whitney *U* tests were used for comparison of variables among the studied groups. $p < .05$ was regarded as significant.

RESULTS

Histopathological Findings

Lung specimens of control and olive oil groups were normal in histological appearance. (Figure 1A-B, and C-D; respectively). In the CCI_4 group, epithelial desquamation, edema, congestion, hemorrhage, cell infiltration, and fibrosis were prominent (Figure 2A–E). The alveolar architecture was destroyed. Alveolar wall containing inflammatory cells was thickened (Figure 2A, B, and D). Tissue edema (Figure 2B, C, and E), congestion of vessels and hemorrhage were observed (Figures 2A and C). Massive

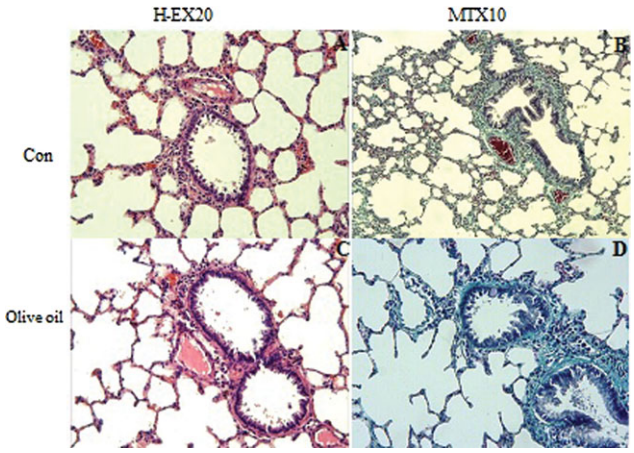

FIGURE 1. A and B. Micrographs of lung specimens of control; H-E; X 20, Masson's trichrom; X 10; respectively, C and D. of olive oil groups; H-E; X 20, Masson's trichrom; X 10; respectively.

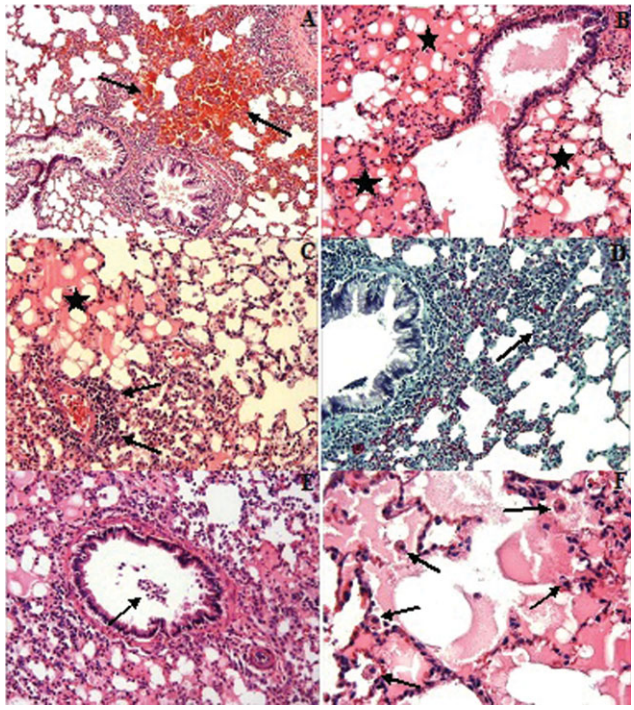

FIGURE 2. Micrographs of lung specimen of CCI_4 group. A. Hemorrhage within alveolar wall, (arrows) H-E; X20, B. Alveolar edema (asterisks), H-E; X 20, C. Inflammatory cell infiltration (arrows) and edema (asterisk), H-E X 20, D. Thickening of alveolar wall, Masson's trichrom; X 20, E. Epithelial desquamation (arrow), H-E; X 20, F. Alveolar, and septal macrophages (arrows) H-E; X 20.

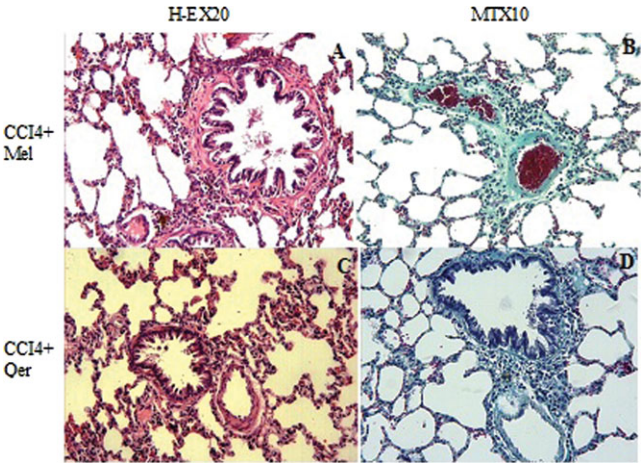

FIGURE 3. Micrograph of lung specimens of A, B. CCl_4+Mel group, C, D. CCl_4+Quer groups. Histopathological picture seems nearly normal.

cell infiltration was observed at the alveolar wall (Figure 2C and D). Numerous alveolar and septal macrophages were also detected (Figure 2E). The histopathological damage was apparently proved in CCl_4+Mel and CCl4+Quer groups (Figure 3A-B, 3C, and D; respectively). Mean histopathological damage scores (MHDSs) were 0.42 ± 0.53 in control, 12.14 ± 1.67 in CCl_4, 3.14 ± 1.46 in CCl_4+Mel, and 4.00 ± 1.63 in CCl_4+Quer groups. MHDS of CCl_4+Mel and CCl_4+Quer groups were significantly lower than that of CCl_4 group, but significantly higher than that of control and oil groups ($p < .001$). The severity of tissue damage in CCl_4+Quercetin group was higher than CCl_4+Mel group suggesting that melatonin provided a higher protection than quercetin. MHDSs of all groups are summarized in Table 1.

Biochemical Findings

In CCl_4 group, mean tissue MDA level was increased ($p < .001$) whereas CAT, GSH level, and SOD activitiy were decreased significantly ($p < .001$) in comparison with control group. However, MDA level was decreased in the CCl_4+Quercetin and CCl_4+Mel groups when compared with the CCl_4 group ($p < .001$). Also, the MDA level was returned to control values by their treatments Mean tissue GSH level of CCl_4+Mel group versus CCl_4 group was significantly increased ($p < .001$). But quercetin administration had no statistically significant effect on GSH levels ($p > .05$). Mean tissue CAT activities of CCl_4+Mel and CCl_4+Quer groups were significantly increased when compared with that of CCl_4 group ($p < .001$) These results indicate that melatonin and quercetin are able to inhibit lipid peroxidation and the production of MDA, and stimulate the production of antioxidant enzymes. Mean tissue MDA and GSH levels and CAT activities of all groups are summarized in Table 2.

DISCUSSION

CCl_4 causes tissue damage in several organs, mainly in liver and kidneys [26, 27]. It has been suggested that the underlying mechanism of tissue injury is oxidative stress caused by lipid peroxidation that is promoted by the biotransformation of CCl_4 to highly toxic CCl_3 and CCl_3O_2 via P450 enzyme [28, 29].

Several authors reported CCl4-induced lung injury in rats [30–32]. Paakko et al. [30, 31] showed that intraperitoneal CCl_4 administration resulted in alveolar degeneration, interstitial fibrosis, hemorrhage, and edema. Miyamoto et al. reported pneumonia development due to fibrosis [32] whereas Frukawa et al. [33] observed lymphocyte infiltration. In the present study, we detected desquamation in the epithelium of bronchioles, hemorrhage, and polymorphonuclear leukocytes, lymphocyte, and macrophage infiltration. Alveolar macrophages induce recruitment of inflammatory cells by releasing cytokines such as interleukin-8, tumor necrosis factor-alpha, and interleukin-1, while they promote fibrogenesis by releasing growth factors. Fibroblast activation occurs as a result of inflammatory and immunological process and type I and II collagen accumulation occur thereafter [34]. Miziguchi et al. [35] reported CCl_4-induced pulmonary fibrosis. In the present study, we clearly observed fibrosis at alveolar wall.

MDA is considered as one of the most important marker of lipid peroxidation; therefore, oxidative stress [36]. Free oxygen radicals promote oxidation

TABLE 1. The Results of Semiquantitative Histological Assessment

Parameter	Control	Olive oil	CCl_4	CCl_4+Mel	CCl_4+Quer
Histopathologic damage score	0.42 ± 0.53	0.28 ± 0.48	12.14 ± 1.67^a	3.14 ± 1.46^b	4.00 ± 1.63^b

[a]Significant increase ($p < .001$), vs. Control group.
[b]Significant decrease ($p < .001$), vs. CCl_4 group.

TABLE 2. The Levels of Biochemical Parameters of All Groups (mean ± SE)

Parameters	Control	Olive oil	CCI4	CCl_4+Mel	CCl_4+Quer
MDA (nmol/mg prot)	0.17 ± 0.20	0.18 ± 0.01	0.26 ± 0.01^{a}	0.17 ± 0.01^{b}	0.16 ± 0.01^{b}
CAT (U/mg prot)	114.66 ± 3.56	102.05 ± 6.11	95.06 ± 6.50^{c}	156.26 ± 13.46^{d}	124.12 ± 9.58^{d}
GSH (nmol/mg prot)	9.52 ± 0.50	9.83 ± 0.84	7.37 ± 0.31^{e}	10.27 ± 0.89^{f}	8.12 ± 0.67

[a]Significant increase ($p < .001$), vs. Control group.
[b]Significant decrease ($p < .001$), vs. CCI4 group.
[c]Significant deccrease ($p < .001$), vs. Control group.
[d]Significant increase ($p < .01$), vs. CCI4 group.
[e]Significant decrease ($p < .05$), vs. Control group.
[f]Significant increase ($p < .05$), vs. CCI4 group.

reaction that starts by removal of a hydrogen from methylene groups of fatty acids at cell membrane. Then, lipid peroxides are formed by binding of oxygen to these groups. At early steps of lipid peroxidation, bioactive aldehydes are produced by degradation of lipid peroxides. It was shown that CCl_4 administration results in increases in tissue MDA levels [37, 38]. In the present study, mean tissue MDA level of CCl_4 group was significantly higher than that of control group.

Within the cell, reactive oxygen species (ROS) are regulated by antioxidant defense system consisting of nonenzymatic (e.g., GSH) and enzymatic (e.g., CAT) antioxidants [39]. We found significant decreases in mean tissue CAT activity and GSH level in CCl_4 group compared to controls. Gaine et al. [37] reported a marked decrease in GSH levels in rats in which pulmonary injury was induced by CCl_4 GSH is one of the most important intracellular mechanisms that protect healthy cells from oxidative injury by acting as a free radical scavenger and a substrate GSH redox cycle [40]. CAT, which act as a preventive antioxidant, play an important role in protection against to the deleterious effect of lipid peroxidation [41]. Ganie et al. reported significant decreases in tissue CAT activities in pulmonary injury induced by CCl_4 [37]. In our study, we also observed that mean tissue CAT activity of CCl_4 group was significantly decreased in comparison with that of control group.

Melatonin hormone, released by pineal gland in dark and by circadian rhythm, has several functions including regulation of endocrine system, enhancement of immune function, regulation of smooth muscle tone, and suppression of gonad function [42]. Melatonin can reach all organelles of the cells including nucleus, as it is soluble in both lipids and water. This feature brings superiority to melatonin particularly in the protection against oxidative injury of DNA [43]. Melatonin is a potent antioxidant, which has been reported to prevent oxidative injury resulted from lipid peroxidation [44]. In the experimental studies, it has been shown that melatonin hormone protects lung against pulmonary fibrosis [45]. In agreement with literature, we found that histological injury observed in lungs was significantly decreased in CCl_4+Mel group. Mean tissue MDA level was decreased but mean tissue GSH level and CAT activity were increased in CCl_4+Mel group. The histopathological and biochemical results support the potency of melatonin against oxidative damage. On the other hand, quercetin has been shown to be effective in prevention against injury of cell membrane caused by free radicals [46]. The protective activity of quercetin has been shown in distinct toxicity models of lungs [47]. In the study, Park HK et al. [48] quercetin found to be effective in reducing pulmonary damage induced by paraquat, In the studies on CCl_4-induced pulmonary injury, it has been reported that increased lipid peroxidation can be reduced by giving melatonin [49] and quercetin [50]. In the previous studies, it was shown that GSH levels and CAT activities, which consists antioxidant defense mechanism, were increased by using quercetin [51]. In the present study, tissue MDA and GSH levels and CAT activity recovered by melatonin and quercetin treatment.

The present study shows that melatonin and quercetin have healing effects on pulmonary injury induced by CCl_4. In conclusion, we suggest that agents with antioxidant properties such as melatonin and quercetin may have positive effects in the treatment of pulmonary diseases characterized by especially edema, inflammation, and fibrosis.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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