The protective effect of infliximab against carbon tetrachloride-induced acute lung injury

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Abstract

Objectives: Carbon tetrachloride (CCl4) causes pulmonary toxicity. Infliximab (Ib) is a potent inhibitor of tumor necrosis factor-alpha (TNF-α). We aimed to investigate whether Ib has a protective effect on CCl4 induced lung injury.

Materials and Methods: Rats were divided into control, CCl4, and CCl4+Ib groups. A single dose of 2 ml/kg CCl4 was administered to CCl4 group and a single dose of 7 mg/kg Ib was given to CCl4+Ib group 24 hr before applying CCl4.

Results: TNF-α, malondialdehyde (MDA), nitric oxide (NO) and caspase-3 levels of the CCl4 group were markedly higher than both the control and CCl4+Ib groups. The CCl4+Ib group had lower histopathological injury than the CCl4 group.

Conclusion: Ib as a strong TNF-α blocker decreases the production of proinflammatory cytokines, MDA, and oxidative stress leading to a protective effect against CCl4 induced lung tissue injury.

Introduction

Carbon tetrachloride (CCl4) - an organic industrial solvent used in industry – is a vigorous carcinogenic agent that may create lung, liver, kidney and nervous system dysfunction (1, 2). After being absorbed by the gastrointestinal system, respiratory system, and skin, CCl4 is metabolized by cytochrome P-450 and exerts its toxic effects via its metabolites trichloromethyl free radical and trichloromethyl peroxyl radical (1-3). These free radicals interact with fatty acids of the lung cell membrane and increase lipid peroxidation and DNA fragmentation. Moreover, they suppress antioxidant enzymes including catalase, superoxide dismutase, glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase, and glutathione peroxide (1, 2, 4).

CCl4 has been shown to cause lung toxicity by intra-alveolar septal ruptures, interstitial cell degenerations, and fibrosis owing to accumulation of exaggerated neutrophils, fibroblasts, and macrophages in blood vessels (2). Increased reactive oxygen species (ROS) are responsible for lung carcinoma, pulmonary fibrosis, chronic bronchitis, emphysema, and pleural diseases (2). Also, the acute toxicity of CCl4 may bring about extreme release of proinflammatory cytokines like tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (5). CCl4 may induce excessive production of transforming growth factor beta1 (TGF-β1) (6), it leads to fibrosis of many tissues such as liver and lung (6, 7). CCl4 creates lung damage by promoting lipid peroxidation and increased malondialdehyde (MDA) levels (8). CCl4 increases apoptosis by activating caspases pathway thus leads to tissue injury (9).

Infliximab (Ib) is a TNF-α inhibitor agent that is used in various inflammatory diseases. In various in vitro studies that used human fibroblasts, endothelial cells, neutrophils, lymphocytes, and epithelial cells, Ib was reported to inhibit TNF-α functions (10). Also, it was shown to protect various tissues against ischemia-reperfusion injury (11, 12). TNF-α leads to tissue injury by increasing cytokine production, increasing ROS formation, and stimulating direct caspases pathway (13). Ib has been reported to

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prevent cytokine production and ROS formation induced by various drug toxicities in tissues such as lung, kidney, and liver via blocking TNF-α (11, 14, 15).

In literature, the chronic toxic effects of CCl₄ have been investigated. In this study, we aimed to investigate whether the high dosage of CCl₄ causes acute damage to lung tissue and to investigate its effect on cytokine production, oxidative stress, and apoptosis. Our second aim was to investigate whether Ib has a preventable effect on CCl₄ induced acute pulmonary toxicity.

Materials and Methods

Animals

We used 24 adult male Sprague-Dawley rats in our experimental study. The animals weighed 275–300 g and were aged 14–15 weeks. The animals were randomly divided into 3 groups: control (n = 8), CCl₄ (n=8), and CCl₄+Ib (n=8). The rats were maintained in light/dark cycles of 12/12 hr with a controlled temperature of 25±3 °C and were fed pelleted rat food and water ad libitum. All animals received care according to the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health and approved by the Local Ethical Committee.

Experimental design

Only isotonic saline solution was given to control group. The CCl₄ group was given a single 2 ml/kg CCl₄ (olive oil 1:1 v/v) intraperitoneal injection. A single dose of 7 mg/kg Ib (Remicade®, Schering-Plough (Brinny) Company, Innishannon, Ireland) was given intraperitoneally to the CCl₄+Ib group, after 24 hr of Ib administration, a single-dose 2 ml/kg CCl₄ was administered intraperitoneally to the CCl₄+Ib group. Rats in all groups were sacrificed after 2 days of CCl₄ administration (16). Before sacrificing the animals, they were anesthetized with ketamine hydrochloride (Ketalar®, 50 mg/kg, Parke-Davis Eczacıbasi, Istanbul, Turkey), which was administered intraperitoneally. The lung tissues of all groups were stored at -80°C before analysis.

Tissue homogenates and measurement of protein

Lung tissue samples were homogenized by phosphate-buffered saline (PBS; pH 7.4) and they were centrifuged for 20 min at 10,000 g. The supernatant was removed to be aliquoted to tubes and was kept frozen at -80°C. The parameters were studied within one month. The Lowry protocol was used to measure tissue homogenate protein levels (17). This method is based on both the Biuret reaction in which the peptide bonds of proteins react with copper under alkaline conditions to produce Cu++, which reacts with the Folin reagent and the Folin-Ciocalteu reaction (17). The protocols of protein measurement were as follows:

Tissue TNF-α

The concentration of TNF-α was measured using the enzyme-linked immunosorbent assay (ELISA) method which is commercially available as rat TNF-α ELISA kit (eBioscience, Vienna, Austria). The procedure for the ELISA protocol was performed according to the instructions provided by the manufacturer. Absorbance was measured at a wavelength of 450 nm using ELISA reader. The levels of TNF-α were presented as pg/ml and the intra- and interassay coefficients of variation were <5% and <10%, respectively. The limit of detection (LOD) for the TNF-α assay was 11 pg/ml. The final results were founded as ng/mg protein after dividing the obtained values by the protein values.

Tissue MDA

MDA levels were measured by the double heating method of Draper and Hadley. The principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA. The MDA levels were presented as µmol/l (18). Dividing the obtained values by the protein levels, the final results were obtained as nmol/mg protein.

Tissue nitric oxide (NO)

The concentration of NO was measured by using the colorimetric assay method. We used commercially available NO kit (Cayman Chemical Company, USA). The colorimetric method was performed according to the instructions provided by the manufacturer. Absorbance was measured at a wavelength of 540 nm using the reader. The levels of NO were presented as µM (µmol/l). The intra- and interassay coefficients of variation were 2.7% and 3.4%, respectively and the limit of detection (LOD) for the NO assay was 2.5 µM. The final results were founded as µmol/mg protein after dividing the obtained results by the protein levels.

Tissue GSH

The GSH levels were analyzed according to Ellman’s method. Tissue samples were homogenized and then deproteinized. The principle of this method is the spectrophotometric measurement of the absorbance of 412 nm of the formed colored complexes by the reaction of Ellman’s color reagent (DTNB 40% w/v, in 1% sodium citrate) with the supernatant. The GSH levels were presented as µmol/mg protein (19).
**Immunohistopathological evaluation**

After being allowed to stay for 48 hr in 10% neutral formaldehyde, lung tissues were washed in running water then let to stay in paraffin for 8 hr. After being deparaffinized in Xylene the sections were rehydrated in alcohol series and were cut into 4 micron thick sections for hematoxylin and eosin (H&E) and Masson’s trichrome staining and 3-micron sections for immunohistochemical (caspase 3) staining. The sections were incubated in 3% hydrogen peroxide to prevent endogenous peroxidase activity. Nonspecific binding sites of the antibodies were blocked by normal serum bovine (1:10 %). Primary antibody (Abcam 13847, Abcam, Cambridge, UK) for caspase 3 was diluted for 1/50. The results of tissue biochemical parameters such TNF-α, NO, MDA, tumor necrosis factor-alpha; GSH in the three groups. CCl4, Carbon tetrachloride; CCI4+Ib, Carbon tetrachloride + Infliximab.

**Table 1. Histopathological examination of lung tissue**

|                      | Control | CCl4 | CCI4+Ib |
|----------------------|---------|------|---------|
| Inflammation         | 0.1±0.52| 3.0±0.76* | 1.5±0.53**†† |
| Congestion           | 0.1±0.52| 1.5±0.53* | 1.0±0.52**†† |
| Edema                | 0.1±0.46| 3.0±0.52* | 2.0±0.71**†† |
| Vacuolization        | 0.1±0.46| 3.0±0.64* | 2.0±0.46**†† |
| Epithelial degeneration | 0.1±0.52| 3.5±0.53* | 2.0±0.46**†† |
| Alveolar macrophages | 1.0±0.52| 3.0±0.64* | 2.0±0.52**†† |
| Caspase 3            | 1.0±0.46| 3.0±0.52* | 2.0±0.52**†† |

CCI4, Carbon tetrachloride; CCI4+Ib, Carbon tetrachloride + Infliximab

*P<0.001, **P<0.05 vs. control group
†P<0.001, ††P<0.05 vs. CCl4 group

**Statistical analysis**

Statistical Package for Social Sciences for Windows, version 17 (SPSS Inc, Chicago, IL, USA) was used for data analysis and the results were presented as mean ± SD. Biochemical parameters such TNF-α, NO, MDA, and GSH were analyzed using ANOVA with Post hoc Bonferroni test. Mann–Whitney U-test was used to compare both groups according to the histopathological parameters. The statistical significance level was set at P<0.05.

**Results**

**The results of tissue biochemical parameters**

MDA level of the CCl4 group was excessively higher than the CCl4+Ib and control groups (respectively 46.6±11.6, 36.4±9.8, and 21.6±4.4 nmol/mg protein). MDA levels are shown in Figure 1. The tnf-α level of the CCl4 group was excessively higher than CCl4+Ib and control groups (respectively 3.9±1.3, 2.7±1.1, and 2.5±0.4 ng/mg protein). NO level of the CCl4 group was excessively higher than CCl4+Ib and the control groups (respectively 7.1±1.1, 6.0±0.9, and 4.8±0.4 nmol/mg protein).
Ib prevents CCl4-induced lung injury

\[ \text{GSH level of the CCl}_4 \text{ group was obviously lower than CCl}_4+\text{Ib and the control groups (respectively 0.7±0.07, 0.9±0.1, and 1.5±0.2 µmol/mg protein). The levels of NO, TNF-\( \alpha \), and GSH were shown in Figure 2.} \]

The histopathological investigation of the lung tissues of the control group rats showed them to be healthy, with no disruption of the tissue integrity, and to have a normal histology with morphologic appearance. In this group, inflammation and congestions have not been observed in the interstitial spaces of lung tissues. Thus, their scores were zero. Although there was mild epithelial cell shedding especially in the terminal bronchioles, connective tissues and cellular elements of the lamina propria were observed to have normal morphology. No histopathological changes were observed in the smooth muscles and endothelium of the vessels. Additionally, saccus alveolaris and the alveoli were observed to have a normal width and the number of the macrophages within them to be normal.

Edema, alveolar epithelial injury, shed epithelial cells polymorph nuclear leukocyte infiltration, and chronic inflammatory cell infiltrates were observed in the interstitial space of lung tissues in only the CCl4 applied group (Figures 3, 4). While the scores of inflammation and congestion in the interstitial space of lung tissues in the CCl4 group with abnormal histologic appearance were mostly 2 and 3, sometimes it was observed to be 4. While the inflammation scores of 2–3 were observed to be intense, the congestion rate was more intensive and within 3–4 (Table 1). In the CCl4 group, the congestion of the small vessels was low, while it was intensively seen in the large vessels. Intensive epithelial cell swellings and a cellular loss to shedding were observed in the bronchi epithelium of this group. The integrity of the tissue of lamina muscularis was observed to be impaired with scattered visualizations. The edematous areas were observed to be increased between lamina propria and basal membrane. There was an increase in the density of the interstitial tissue among alveoli and intensive infiltrations with mild fibrosis were observed in these regions.

**Figure 3.** Histopathological changes in lung tissues visualized by light microscopy. (A) Control group, (B) CCl4 group, and (C) CCl4+Ib group, s: saccus alveolaris, arrow: epithelial cell degeneration, e: edema, star: congestion, arrowhead: leukocyte infiltration, hematoxylin-eosin stain

**Figure 4.** Histopathological examination of lung tissue in all groups stained by Masson’s trichrome using light microscopy. (A) Control group, (B) CCl4 group, and (C) CCl4+Ib group, v: vacuolization, arrow: epithelial cell degeneration, e: edema, star: congestion

**Figure 5.** Anti-caspase 3 antibody immunohistochemical staining of lung tissues. (A) Control group, (B) CCl4 group, and (C) CCl4+Ib group, s: saccus alveolaris, arrowhead: strong positivity, arrow: weak positivity, immunoperoxidase stain
The infiltration of polymorphonuclear leukocytes (PMNL) and epithelial shedding were observed to be lower in the CCl4+Ib group. The degeneration of alveolar epithelial cells, cellular shedding and the increase in the interstitial connective tissue were observed to be lower especially in the CCl4 group. Although there were no changes in the intensity of fibrosis, there was a decrease in the edema intensity (Figure 4).

Caspase 3 enzyme activity of the CCl4 group was significantly higher than those of the CCl4+Ib (P<0.05) and control groups (P<0.001). Additionally, caspase 3 enzyme activity of the CCl4+Ib group was significantly higher than that of the control group (P<0.001) (Figure 5).

**Discussion**

In our study, TNF-α, MDA, and NO levels in the lung tissue of the CCl4 applied group were observed to be obviously higher than the control and Ib treated groups. GSH level was found to be obviously lower than the other two groups. The histopathological evaluation of lung tissues of the CCl4 group revealed intensive histopathological damage. In the histopathological evaluation of CCl4 group we observed alveolar macrophage accumulation, excessive lung tissue injury, and increased caspase 3 enzyme activities. On the other hand, the levels of TNF-α, MDA, and NO in the lung tissue of the CCl4+Ib group were found to be obviously lower than the CCl4 group and glutathione level to be preserved. In this group, there was lower histopathologic injury in the lung tissues, lower macrophage infiltration, and lower caspase 3 enzyme activities.

CCl4 is a toxic substance which is distributed and deposited in many tissues including the lung. CCl4 is metabolized by cytochrome P450 enzyme into two metabolites: trichloromethyl radical (•CCl3) and trichloromethyl peroxide radical (CCl3O2•). •CCl3 leads to reactive free radicals by rapid reaction with O2 (20). These radicals start lipid peroxidation by oxidation of polyunsaturated fatty acids of the membrane. MDA is one of the most important products of lipid peroxidation. Elevation of MDA level leads to severe damage of lung tissue and ROS formation (21). CCl4 increases ROS formation by stimulating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme system in lung tissues (2, 22). On the other hand, CCl4 increases lung tissue injury by inhibiting the antioxidant enzyme system. Glutathione peroxidase enzyme prevents lung tissue injury by oxidizing reduced GSH and the elimination of hydrogen peroxide. Reduced GSH protects the biological membranes against lipid peroxidation. In the presence of oxidative stress reduced glutathione decreases due to increased overuse. GSH has been reported to be decreased in CCl4 toxicity (2). Ib is a blocker of TNF-α that has been reported in many studies to decrease ROS formation and lipid peroxidation. Ib prevents the damage of biologic membranes and lipid membranes by preventing a decrease in the GSH level (23). In our study, while MDA level of CCl4 was extremely higher than the control and Ib groups, its GSH level was obviously lower than these two groups. It has been observed that MDA was lowered and GSH level was preserved in the Ib applied group. In the current study high MDA and low glutathione levels of the CCl4 group have shown that CCl4 leads to lipid peroxidation and ROS formation that end in lung tissue injury. The lower MDA level in Ib compared to the CCl4 group and the similar GSH level in the control group have shown Ib to prevent lung tissue injury by decreasing lipid peroxidation and ROS formation. TNF-α is one of the major regulatory cytokines of the immune system. Elevated TNF-α level increases the other proinflammatory cytokines and ROS formation.

Various lytic enzymes released from macrophages produce oxidant substances. The increase of alveolar macrophages leads to increased production of oxidant substances, damage of the epithelial cells and the bronchial membrane, mucus secretion and impairment of pulmonary functions (4). These macrophages also release cytokines such as TNF-α and interleukin 1 beta leading to acceleration of lung tissue injury. The administration of CCl4 is known to lead to tissue injury and fibrosis by stimulating the macrophages (24). TNF-α leads to tissue injury by increasing the migration of neutrophils to the damaged area, increases the production of proteolytic enzymes from neutrophils and leads to ROS formation (25). TNF-α also leads to apoptosis by direct stimulation of caspase pathway and tissue injury (26). It is known that TNF-α leads to lung toxicity during inflammation and the toxicity induced by drugs or organic substances. It is known that tissue NO increases after inflammation. Due to its high production NO reacts with superoxide radicals leading to the formation of peroxynitrite radicals. The resulting peroxynitrite radicals are highly toxic to the tissue (27). CCl4 leads to tissue injury in various tissues by increasing TNF-α and NO production (28). Studies conducted on Ib have shown it to suppress cytokine release and to decrease peroxynitrite radicals by increasing NO levels (11, 14). A previous study showed that Ib ameliorates methotrexate-induced pulmonary toxicity by diminished proinflammatory cytokine levels (15). Our study has shown that the intensive release of TNF-α and NO during acute CCl4 toxicity causes lung tissue injury by the formation of peroxynitrite radicals and ROS. Similar to the previous study (15), Ib may prevent pulmonary injury by reduced ROS formation through blocking TNF-α production.
**Conflict of interest**

Authors have no conflict of interest to declare.

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