Abstract. - OBJECTIVES: In addition to its antimicrobial effects, inhibitory effects of minocycline have been demonstrated, including against inflammation, apoptosis, proteolysis, angiogenesis, and tumor metastasis. In this study, we aimed to determine the beneficial effects of minocycline on lung histology and its antioxidant activity in a murine model of pulmonary fibrosis.

MATERIALS AND METHODS: Twenty-eight Swiss albino mice were randomly allocated into four groups of seven animals per group. Group I (control group) received intraperitoneal injection of saline. Group II (methotrexate group) received methotrexate orally 3 mg/kg for 28 days. Group III (minocycline group) received methotrexate orally 3 mg/kg and 15 mg/kg of intraperitoneally injected minocycline for 28 days. Group IV (minocycline group) received 15 mg/kg of intraperitoneally injected minocycline for 28 days. Twenty-eight days later, the animals were euthanized. Thereafter, lung tissue samples were harvested. Histological findings of airways were evaluated by light microscopy. The levels of malondialdehyde (MDA), the product of reactive oxygen in lung tissue, and catalase, an antioxidant enzyme, were also determined.

RESULTS: In the light microscopic examination, the lung tissues of the control group showed normal histological features. In the methotrexate group, the degree of lung damage (grade 3 fibrosis) was higher than the control and other groups (p: 0.001). In the minocycline-treated group, improvement in lung tissue was noted (median fibrosis score: 3 (MTX group) vs 1 (MTX plus minocycline group); p: 0.001). Only the minocycline group showed normal histological features. Although minocycline reduced the MDA levels in lung tissue, an increase in catalase activity was detected (p: 0.018 and p: 0.014, respectively).

CONCLUSIONS: The administration of minocycline may be effective in MTX-induced lung fibrosis in mice. However, further studies with high-dose and long-term treatments are needed.

Key Words: Methotrexate, Pulmonary fibrosis, Minocycline.

Introduction

Methotrexate (MTX) is an analog of the vitamin folic acid. It inhibits cellular proliferation by inducing acute intracellular deficiency of certain folate coenzymes. Many pharmacological mechanisms of MTX action have been suggested, including inhibition of purine synthesis, promotion of adenosine release, inhibition of production of proinflammatory cytokines, suppression of lymphocyte proliferation, neutrophil chemotaxis and adherence, and reduction of serum immunoglobulin 1. MTX has been widely used to treat many types of cancer, rheumatoid arthritis (RA), psoriasis, immunological abnormalities, and systemic inflammation. However, MTX causes serious ad-
verse effects, resulting in discontinuation of treatment; these adverse effects are usually dose dependent. One of the most important adverse effects of MTX is lung toxicity manifesting as pneumonia, interstitial lung disease, and pulmonary fibrosis. MTX stimulates components of the p38 MAPK signaling cascade (TAK1 → MKK3/ MKK6 → p38 MAPK → MAPKAPK2 → HSP27) and promotes the release of interleukin (IL)-1 and IL-8. Pulmonary toxicity occurs through this mechanism. To date, drug-induced pulmonary fibrosis has been demonstrated in most animal models, and bleomycin was used in most of these studies. However, few studies have addressed this issue using MTX in animal models.

Minocycline is a second-generation and semisynthetic tetracycline analog. It exhibits efficacy against both gram-positive and -negative bacteria and has been approved by the United States Food and Drug Administration (FDA). In addition to its antimicrobial effects, minocycline has anti-inflammatory, -apoptotic, and -proteolysis activities; the drug also inhibits angiogenesis and tumor metastasis. In addition, minocycline has been shown to have a significant effect on asthma in human studies and animal models. The present study was undertaken to evaluate the potential protective effects of minocycline against the development of MTX-induced pulmonary fibrosis.

Materials and Methods

Ethics Statement

All experimental procedures complied with the requirements of the Animal Care and Ethics Committee of the Adnan Menderes University.

Materials

MTX and minocycline were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Experimental Animals

Specific pathogen-free, 6-8-week-old, Swiss albino mice, weighing 25-30 g, were maintained in a pathogen-free laboratory at Adnan Menderes University. They were kept in hygienic macrolene cages in air-conditioned rooms on a 12-h light/12-h dark cycle and were allowed food and water ad libitum.

Experimental Design

Twenty-eight Swiss albino mice (from the Animal Center of the Adnan Menderes University) were randomly allocated into four groups containing seven animals per group. Group I (control group) received intragastric and intraperitoneal injection of saline. Group II (MTX group) received MTX orally 3 mg/kg for 28 days. Group III (MTX+minocycline group) received MTX orally 3 mg/kg and 15 mg/kg of intraperitoneally injected minocycline for 28 days. Group IV (minocycline group) received 15 mg/kg of intraperitoneally injected minocycline for 28 days. The doses and administration route of MTX and minocycline were based on previous studies and our preliminary experiments. Twenty-eight days later, the animals were euthanized. Thereafter, lung tissue samples were harvested.

Histological Analysis

To examine the effect of minocycline in mice with pulmonary fibrosis caused by MTX, lung tissues taken at the end of the experiment were placed in 10% formaldehyde, embedded in paraffin blocks, and then subjected to histological analysis. Next, serial sections of 3.5-µm thickness were prepared on the lam from these blocks. These sections were stained with hematoxylin and eosin (HE) and closed with a coverslip. The mice in each group were evaluated using the Ashcroft scoring system. The mice were photographed using an Olympus BX20 microscope (Tokyo, Japan).

Preparation of Tissue Specimens

The lipid peroxidation product and catalase levels were defined biochemically in lung tissue specimens of mice. Mouse lung tissues were weighed using a precision scale, mixed with 50 mM phosphate tamponate (pH 7.4) at an appropriate ratio to achieve 10 times the weight of the lung tissue. Thus, 10-fold dilutions were obtained. Thereafter, each specimen was homogenized for 3 min at 2000 turnovers/minute using a homogenizer. The homogenization was enhanced by treatment with a sonicator after homogenization. All procedures were carried out on ice to achieve continuity of the cold environment and preserve important parameters unchanged. The supernatant was obtained by centrifugation for 15 min at 4°C at 1800 rpm. Before retrieving the supernatant, tissue protein amount that was 1/10 diluted with distilled water was worked at autoanalyzer as mg/dl. Next, malondialdehyde (MDA), an indicator of lipid peroxidation, was assayed, followed by measurement of catalase activity.
Determination of Lipid Peroxidation

Levels of MDA, the final product of polyunsaturated fatty acid peroxidation, were determined using the thiobarbituric acid method, which is based on the Draper and Hadley method. MDA is present in biological materials in various covalently bonded forms as well as a somewhat free form. The covalent bond structure is disrupted when treated with acid or alkali in a hot medium. Measurement of the MDA levels is based on monitoring spectrophotometrically the colored complexes formed by reaction with thiobarbituric acid. Trichloroacetic acid (TCA) solution (10%): 10 g of TCA were dissolved in bidistilled water to a final volume of 100 ml. Thiobarbituric acid (TBA) solution (0.67%): 0.67 g of TBA were dissolved in bidistilled water to a final volume of 100 ml.

Next, 2.5 ml of the 10% TCA solution were added to 0.5 ml of each supernatant in reaction tubes. The tubes were then mixed by vortexing, incubated for 15 min in boiling water, and then immediately cooled on ice. Thereafter, the supernatant/TCA mixtures were centrifuged at 5000 rpm for 10 min, and 2 ml of each mixture were transferred to another tube. Each 2 ml sample was then overlayed with 1 ml of the 0.67% TBA solution and vortexed. The samples were then incubated in boiling water for 15 min and cooled on ice, and their absorbances at 532 nm were recorded. The level of MDA was calculated using the absorbance values at 532 nm, which is the highest absorbance observed for the formed MDA-TBA complex:

\[
A = \varepsilon \times l \times c \Rightarrow c = \frac{A}{\varepsilon \times l}
\]

where \( A \) is the absorbance, \( l \) is the light way in cm, \( \varepsilon \) is the molar absorption coefficient, and \( c \) is the concentration. The MDA values were expressed as nmol/mg protein.

Determination of Tissue Catalase Levels

The catalase assay was based on the Aebi method that relies on kinetic measurement. Catalase catalyzes the decomposition of hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) by adding water and molecular oxygen.

Two \( \text{H}_2\text{O}_2 \) molecules are converted to two \( \text{H}_2\text{O} + \text{O}_2 \) molecules by catalase. In the present study, catalase activity was determined by observing the decrease in \( \text{H}_2\text{O}_2 \) concentration per unit time spectrophotometrically at 240 nm.

Supernatants of prepared tissue homogenates were diluted with 50 mM phosphate tamponate (pH 7.00) at a proportion of 1:5. The absorbance changes at 240 nm were recorded at 15-s intervals after homogenization by adding 1.0 ml of \( \text{H}_2\text{O}_2 \) (30 mM) to 2.0 ml of the supernatant/phosphate tamponate mixture. Similar procedures were repeated in blind trials involving 2.0 ml supernatant and 1.0-ml phosphate tamponate. Decomposition of \( \text{H}_2\text{O}_2 \) is initially (approximately the initial 30 s) conforming to a first-order kinetic reaction. Thus, the activity calculation was performed according to the following formula considering the initial and 30-s absorbances: \( k = \frac{(2.303/\Delta t)(\log A_1/\log A_2)}{\text{catal/mg protein}} \).

Statistical Analysis

Kruskall-Wallis analysis of variance was used to evaluate the statistical significance of differences. Post hoc multiple comparisons were performed using the Mann-Whitney U-test with Bonferroni correction. \( p \) values less than 0.05 were deemed to indicate significance. The results were presented as medians (min–max).

Results

Upon light microscopic examination, lung tissues of the control group showed normal histological features (Figure a). In the MTX group, lung damage (Figure b, c, d) was higher than that in the control and other groups (Table I; \( p < 0.001 \)). In the minocycline-treated group, improvement in lung tissue was noted (Figure e, g, h). Only the minocycline group showed normal histological features (Figure f; grade 0 fibrosis).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Fibrosis</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>0 (0-1)*</td>
</tr>
<tr>
<td>MinoMetho</td>
<td>7</td>
<td>1 (1-3)*</td>
</tr>
<tr>
<td>Metho</td>
<td>7</td>
<td>3 (2-3)*</td>
</tr>
<tr>
<td>Mino</td>
<td>7</td>
<td>0 (0-2)*</td>
</tr>
<tr>
<td>( p ) values*</td>
<td>( &lt; 0.001 )</td>
<td></td>
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</tbody>
</table>

Data are medians (min–max) of seven mice per group. *\( p \) values obtained from Kruskall-Wallis analysis of variance. When the post hoc Mann-Whitney U-test was performed with Bonferroni correction, the \( p \) value between the MinoMetho and Metho groups was found to be 0.001.

*Medians with different superscripts within the same column for different parameters are significantly different.
Minocycline against pulmonary fibrosis in mice

The groups were statistically significant ($p: 0.001$). The catalase levels in the MTX+minocycline group were significantly increased compared with those in the MTX group ($p: 0.014$). The differences in the MDA levels were also significant ($p < 0.001$). Similarly, treatment with MTX+minocycline resulted in a significant decrease in MDA levels compared with treatment with MTX only ($p: 0.018$). The MDA levels were significantly higher in the MTX group than in the control group (Table II; $p < 0.001$), and the catalase levels were significantly lower in the MTX group than in the control group (Table II; $p: 0.01$).

Catalase and MDA results are shown in Table II. The differences in the catalase levels between

<table>
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<tr>
<th>Groups</th>
<th>Catalase*</th>
<th>MDA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.000231</td>
<td>1.740</td>
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<tr>
<td>Minocycline</td>
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<td>1.02</td>
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<td>MTX</td>
<td>0.00008978</td>
<td>3.918</td>
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<tr>
<td>MTX plus Minocycline</td>
<td>0.0003695</td>
<td>1.950</td>
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</table>

*Median levels (nmol/mg protein).
*Median levels (k/mg protein).

Table II. Catalase and MDA results according to group.

Figure 1. A, Lungs with a normal appearance in control group mice (grade 0) (HE, ×100). B, A view of grade 2 lung fibrosis. The intensively damaged area is marked by an arrow (HE, ×100). C, A view of grade 3 lung fibrosis. Significant damage was observed in the methotrexate group (HE, ×100). D, Wall thickening (arrow) in Grade 3 lung fibrosis (HE, ×100). E, Correction findings in the group of grade 1 lung fibrosis group treated with methotrexate and minocycline (HE, ×100). F, Lung tissue in the minocycline group (grade 0) (HE, ×100). G, The effects of minocycline administered to mice in which pulmonary fibrosis was induced by means of methotrexate treatment of lung tissue (grade 1) (HE, ×200). H, Grade 2 pulmonary fibrosis in the methotrexate and minocycline groups (HE, ×200). I, View of bronchioles and alveoli in the control group (grade 0) (HE, ×200).
Discussion

We investigated the protective properties of minocycline against the fibrosis-inducing effect of MTX in mice. The present study is the first to show the preventative effect of minocycline against the development of lung fibrosis related to MTX. In animal experiments, lung fibrosis models are often created using bleomycin and C57BL/6J mice. These mice were shown to be more sensitive than BALB/c mice in terms of the development of lung fibrosis. In addition, in the bleomycin-induced lung fibrosis models, successful lung fibrosis development also occurred using Swiss albino mice. Information concerning lung fibrosis models using MTX in mice is limited. In our study, we used Swiss albino mice and could successfully generate fibrosis induced by MTX in these mice (Table I; Figure b, c, d). However, we believe that further studies of the development of lung fibrosis induced by MTX in other mouse backgrounds should be performed.

MTX stimulates the components of the p38 MAPK signaling cascade (TAK1 → MKK3/MKK6 → p38 MAPK → MAPKAPK2 → HSP27) and promoted the release of IL-1 and IL-8. Pulmonary toxicity occurs through this mechanism. In addition, MTX was determined to increase the levels of proinflammatory and inflammatory cytokines such as IL-8, MCP-1, IL-1, and TNF-α in airway epithelial cells in pneumonia induced by MTX. MTX administration resulted in increased production of reactive oxygen species (ROS), leading to damage of macromolecules and triggering many pathological processes. MTX was shown to increase the levels of MDA and reduce the levels of catalase. Similar to the literature, in MTX-treated mice, we showed that MTX significantly increased the MDA levels in lung tissue and reduced the catalase levels (Table II; respectively; p < 0.001; p: 0.01).

Minocycline, a second-generation semisynthetic tetracycline derivative, has biological effects that are different from its antimicrobial action. In addition to its antimicrobial effects, inhibitory effects of minocycline have been demonstrated, including against inflammation, apoptosis, proteolysis, angiogenesis, and tumor metastasis. Administering minocycline to diabetic mice reduces IL-1β and TNF-α levels, lipid peroxidation, and nitrite levels. In addition, minocycline improves the antioxidant defense system. Furthermore, its anti-inflammatory effects are mediated by reducing levels of inflammatory cytokines. Another study showed that minocycline significantly inhibited nitric oxide and prostaglandin E2 production and inducible nitric oxide synthase and cyclooxygenase-2 expression in BV2 microglial cells.

In a rodent model of hemorrhagic shock/resuscitation plus abdominal compartment syndrome, in minocycline-treated mice, the levels of polymorphonuclear leukocyte infiltration and concentrations of inflammatory molecules (e.g., chemokines, cytokines, and prostaglandin E2) were found to be lower than in control mice. Recently, accumulating evidence has indicated that minocycline is neuroprotective in the models of cerebral ischemia spinal cord injury. Minocycline was determined to exert this neuroprotective effect in spinal microglia by inhibition of p38 MAPK. Additionally, minocycline demonstrated significant effects on asthma in both human studies and animal models. Minocycline-mediated decreases in IgE responses are associated with suppression of phosphorylated p38 MAP kinase by T lymphocytes in allergic asthmatic humans.

Based on the abovementioned literature, inflammatory, proinflammatory, and oxidant effects, as well as activation of the p38 MAPK signaling cascade, can be considered lung toxicity effects of MTX. By contrast, minocycline demonstrates anti-inflammatory, p38 MAPK inhibitory, and anti-oxidant effects. It was also shown that Minocycline penetrated into the lung tissue by oral or intravenous using. Therefore, we speculated that minocycline could prevent lung fibrosis related to MTX in mice. According to our results, minocycline significantly reduced lung fibrosis related to MTX (median fibrosis score of 3 for the MTX group vs. 1 for the MTX plus minocycline group; p: 0.001). Additionally, minocycline significantly prevented the reduction in catalase levels, which have antioxidant effect in lung tissue related to the use of MTX (p: 0.014) as well as significantly reduce the MDA levels, which represent the oxidant products (p: 0.018).

Additionally, induction of pneumonitis by minocycline has been reported; however, the underlying mechanism remains unknown. In our experiment model, the dosage used was lower than that in the other animal experiment models (15 mg/kg). Generally, the minocycline dosage in mouse models varies between 45 and 120
mg/kg. According to our study, use of a low minocycline dosage resulted in preventative effects against fibrosis. This dual effect of minocycline was thought to be related to the dosage. Thus, dosage studies are warranted.

**Conclusions**

The administration of minocycline may be effective against MTX-induced lung fibrosis in mice. However, further studies using high-dose and long-term treatments are needed.

**Conflict of Interest**

The Authors declare that there are no conflicts of interest.

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