Protective effects of tumor necrosis factor alpha inhibitors on methotrexate-induced pancreatic toxicity

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

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Conflict of interest
None declared

Abstract
Background. Methotrexate (MTX), a folate antagonist, is commonly used in the treatment of many different types of cancer and inflammatory diseases, including pancreatic cancer, although its side effects on the pancreas have not yet been researched. The mechanism of MTX-induced toxicity is not well known, and it has been reported in high-dose toxicity studies that the pancreas is sensitive to toxic effects.

Objectives. The aim of our study was to determine whether adalimumab (ADA) has a preventive effect on MTX-induced pancreas toxicity in rats.

Material and methods. The rats were equally and randomly divided into 3 groups (Group 1 comprised the healthy controls, Group 2 was the MTX group, and Group 3 was the MTX + ADA group). The rats in Groups 2 and 3 received an intraperitoneal (ip.) single-dose injection of MTX (20 mg/kg). A single dose of 5 mg/kg ADA (REMICADE®) was administered ip. to Group 3. All the rats were sacrificed under anesthesia 5 days after receiving the MTX injection.

Results. Significantly higher mean edema, necrotic cell, and inflammatory scores were recorded in Groups 2 and 3 compared to those recorded in Group 1. Significantly decreased edema, number of necrotic cells, and inflammatory scores were noted in Group 3 than in Group 2. A decrease in islets of Langerhans cell insulin and somatostatin-positive interneurons was demonstrated after the administration of MTX. An increase in insulin and somatostatin-positive cells in islets of Langerhans, as well as a remodeling of the structure of the pancreas, was shown following treatment with ADA.

Conclusions. Adalimumab was demonstrated to have a protective effect against MTX-induced pancreatic injury in this study.

Key words: adalimumab, methotrexate, pancreas, rat, toxicity
Introduction

Methotrexate (MTX) was discovered in 1948 by Farber as an effective treatment for acute leukemia and is an antimetabolite, antifolate, and chemotherapeutic drug.\(^1,2\) The effect of DNA synthesis in the "S" phase by inhibition and the affinity of dihydrofolate acid to inhibit the dihydrofolate reductase enzyme appear with the use of MTX. Thus, there is a drop in tetrahydrofolate acid levels and purine synthesis is inhibited.\(^3,4\) Methotrexate is used to treat many different types of cancer (colorectal, breast, and pancreatic cancers) and inflammatory diseases (rheumatoid arthritis – RA, psoriatic arthritis, systemic lupus erythematosus, and dermatomyositis).\(^1,2,5\) Nevertheless, its use is associated with side effects such as fibrosis affecting the liver, lung, and other organs.\(^1,2,5\) Acute intense MTX side effects are dose-dependent. Bone marrow suppression, gastrointestinal upsets or ulcers, and liver fibrosis are well-known acute or common side effects of MTX therapy. It has been proposed in recent studies that MTX has a cytotoxic effect in reducing intracellular tetrahydrofolate, but the mechanism of MTX-induced toxicity is not well known.\(^1,2,4–11\)

Methotrexate has been used in the treatment of pancreatic cancer, but there has been no research into its side effects on the pancreas, an organ which is relatively sensitive to various toxic substances and drugs. High-dose substances, causing oxidative stress, have been reported to have a toxic effect on the pancreas.\(^1,5–11\) However, the development of tolerance to MTX has been reported in patients over time. The tolerance mechanism of the metabolites in MTX is not known.\(^1,2,4,5,11\) Physicians increase the dosage of MTX to counter the development of tolerance. Accordingly, the risk of MTX toxicity increases. It was found in recent studies that an MTX overdose caused oxidative stress, leading to the release of proinflammatory cytokines and further tissue damage.\(^5,6,11\)

Tumor necrosis factor alpha (TNF-α) plays a key role in the mechanism of immune-mediated inflammatory diseases, such as RA, ankylosing spondylitis, Crohn’s disease, ulcerative colitis, psoriasis, and psoriatic arthritis. Inflammation induces the overproduction of inflammatory cytokines, such as TNF-α, interleukin: IL-1β, IL-6, IL-15, and IL-18, while TNF-α has been shown to increase the number of pancreatic cancer cells.\(^12\) It has been reported in the literature that TNF-α cytokines modulate the autocrine growth regulatory pathways in pancreatic cancer cells.\(^13\) Antagonists of TNF-α, such as adalimumab (ADA), infliximab, etanercept (ETN), golimumab, and certolizumab pegol, have been widely used in the treatment of RA. Inhibition of TNF results in the down-regulation of abnormal and progressive inflammatory processes, resulting in the prevention of target organ damage.\(^12,13\)

It has been found that a combination of TNF-α antagonists and MTX improves the symptoms and signs of inflammation and physical function in RA patients who do not respond to MTX alone.\(^10–13\) The combination of MTX and ADA has not been studied for its ability to counter toxicity in the pancreas. Irreversible tissue injury from edema and inflammatory and acinar necrosis has been reported in histopathological studies on pancreatic toxicity.\(^14–16\)

The aim of this study was to determine whether TNF-α inhibitors have a preventive or toxicity-enhancing effect on MTX-induced pancreatic toxicity.

Material and methods

Animal testing

Thirty male albino Sprague Dawley\(^\circledast\) rats, aged 3 months and weighing 250–300 g, were procured from the Animal Care and Research Unit (Rize, Turkey). All the rats were sacrificed under anesthesia with ketamine hydrochloride 50 mg/kg, intramuscularly (Ketalar, Parke-Davis, Istanbul, Turkey) 5 days after receiving the MTX injection.

Histological preparation

Pancreatic tissue samples were fixed in 10% formalin. After fixation, the specimens were dehydrated in an ascending series of alcohol, cleared in xylene, and embedded in paraffin. The paraffin blocks were cut 4–5 μm thick, using a microtome (Leica\(^\circledast\) RM2125 RTS), and the sections were stained with hematoxylin and eosin (Applichem GmbH, Darmstadt, Germany), according to the guidelines that govern conventional light microscopes.
Immunohistochemical preparation

Paraffin blocks of the pancreatic samples were cut into 1–2 µm semi-thin sections. The sections obtained for immunohistochemical staining were left in xylene for 10 min, twice, and after being passed through an alcohol series (70–99%) for 5 min, were kept in a 3% H₂O₂ solution for 10 min. After washing with phosphate buffered saline solution (PBS), they were heated 4 times in a citrate buffer for 5–10 min at 800 W of power, and allowed to stand for 20 min in a secondary blocking agent. Each preparation was allowed to stand for 60–75 min in various dilutions (1/50–1/200) of anti-laminin antibody staining (Polyclonal Guinea Pig Anti-Insulin® [clinical application for IS002], and Polyclonal Rabbit Anti-Human Somatostatin® [clinical application for A0566], both Dako Denmark A/S, Denmark). The 3,3’-diaminobenzidine (DAB) solution was used as a chromogenic dye, and stained with Mayer’s hematoxylin for contrast dye. Phosphate buffered saline solution was used for the negative controls instead of primary antibodies. The preparation was closed off with a suitable agent and photographed. According to the blind scoring evaluation performed by 2 histologists, the positivity was divided into the following 4 categories according to the percentage value: mild (+), moderate (++), severe (+++), and very severe (++++).

Pathological assessment of tissue

Parts of the tissue were fixed and embedded in paraffin for histological analysis. Blinded pathological scoring of the pancreas, by an experienced pathologist from Recep Tayyip Erdoğan University, Rize, Turkey, was performed with respect to edema, bleeding, inflammatory cell infiltration, and necrosis, according to Schmidt’s report (Table 1).¹⁴

Statistical analysis

Statistical analysis was performed using SPSS® v. 18 (IBM; Chicago, USA). Schmidt’s score was calculated.¹⁴ The data was expressed as mean ±standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance, followed by Tukey’s test for each paired experiment value. A p-value of p < 0.05 was considered to be significant. The sections obtained by immunohistochemical staining were evaluated and graded by 2 blinded histologists and the positivity was expressed in terms of percentage and divided into 4 grade categories. Data was expressed as median ±standard deviation (SD). The obtained values were statistically compared with the Kruskal-Wallis test (p < 0.05) to each other.

Results

Histological results

The morphologic structure of the tissues in the control group was observed to have a normal histology (Fig. 1A, 1B). The histopathologic features of the Group 2 samples were atypical islets of Langerhans, mild edema, necrosis, and inflammatory infiltration. Necrotic cells were observed in the islets of Langerhans and acinar cells. Necrotic acinar cells, with a pyknotic nucleus in the center, partial karyorrhexis, and a bright border due to cytoplasmatic shrinkage and cell degeneration, were observed. Connective tissue trabeculae were noted to increase considerably in volume with leukocytic infiltration (Fig. 1C–1E).

Complete destruction of islet of Langerhans cells, due to the toxicity of MTX, appeared in the Group II rats compared to Group 1 (Fig. 1A–1D).

Fig. 1. A, B − histology of a normal pancreas (L − islets of Langerhans, a − acinar); C, D, E − MTX group C − MTX group demonstrating necrotic acinar cells with a pyknotic nucleus (tailed arrow), and edema (E); D − there are numerous entirely destroyed lobules with extreme leukocytic infiltration (l) and frequently widespread hemorrhage (thick arrow), capillary congestion (c), atypical blood vessel (thin arrow); E − edema (asterisk) and mononuclear leukocyte infiltration were observed in islets of Langerhans (spiral arrow); F − MTX + ADA group (L − islet of Langerhans, a − acinar); H&E stains.
Decreased edema, necrosis, and inflammatory infiltration was demonstrated in the Group 3 samples. A decrease in necrotic cells was also observed in islets of Langerhans and acinar cells (Fig. 1F) after the administration of the ADA/MTX combination therapy.

**Immunohistochemical results**

The immunoreactivity rate for anti-insulin, together with islets of Langerhans, was found to be moderate (17%), severe (33%), and very severe (50%) in Group 1; mild (67%) and moderate (33%) in Group 2; and mild (17%), moderate (33%), severe (33%), and very severe (17%) in Group 3 (Fig. 2, 3).

Anti-somatostatin immunopositivity in islets of Langerhans was shown on immunohistochemical staining using the immunoperoxidase method, and was found to be mild (17%), moderate (17%), severe (33%), and very severe (33%) in Group 1; mild (83%) and moderate (17%) in Group 2; and mild (17%), moderate (33%), and very severe (50%) in Group 3 (Table 3).

**Stereological results**

All the study rats were scored by both investigators using Schmidt’s histopathological scoring method. There was no more than a 0.5-point deviation in their discernment. Schmidt’s histopathological scoring steps, shown in Table 2, were applied.

**Statistical results**

Significantly higher mean edema, necrotic cell, and inflammatory scores, compared to those obtained for Group 1, were recorded for Group 2 (p < 0.05). Significantly decreased edema, necrotic cell, and inflammatory scores were recorded for Group 3, when compared to Group 2 (p < 0.05) (Table 2).

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**Table 1. Schmidt’s histopathologic scoring criteria**

<table>
<thead>
<tr>
<th></th>
<th>Edema</th>
<th>Acinar necrosis</th>
<th>Hemorrhage and fat necrosis</th>
<th>Inflammation and perivascular infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Absent</td>
<td>0 Absent</td>
<td>0 Absent</td>
<td>0 0–1 intralobular or perivascular leukocytes/HPF</td>
</tr>
<tr>
<td></td>
<td>0.5 Focal expansion of interlobular septae</td>
<td>0.5 Focal occurrence of 1–4 necrotic cells/HPF</td>
<td>0.5 1 focus</td>
<td>0.5 2–5 intralobular or perivascular leukocytes/HPF</td>
</tr>
<tr>
<td></td>
<td>1 Diffuse expansion of interlobular septae</td>
<td>1 Diffuse occurrence of 1–4 necrotic cells/HPF</td>
<td>1 2 foci</td>
<td>1 6–10 intralobular or perivascular leukocytes/HPF</td>
</tr>
<tr>
<td></td>
<td>1.5 Same as 1 + focal expansion of interlobular septae</td>
<td>1.5 Same as 1 + focal occurrence of 5–10 necrotic cells/HPF</td>
<td>1.5 3 foci</td>
<td>1.5 11–15 intralobular or perivascular leukocytes/HPF</td>
</tr>
<tr>
<td></td>
<td>2 Same as 1 + diffuse expansion of interlobular septae</td>
<td>2 Diffuse occurrence of 5–10 necrotic cells/HPF</td>
<td>2 4 foci</td>
<td>2 16–20 intralobular or perivascular leukocytes/HPF</td>
</tr>
<tr>
<td></td>
<td>2.5 Same as 2 + focal expansion of interacinar septae</td>
<td>2.5 Same as 2 + focal occurrence of 11–16 necrotic cells/HPF</td>
<td>2.5 5 foci</td>
<td>2.5 21–25 intralobular or perivascular leukocytes/HPF</td>
</tr>
<tr>
<td></td>
<td>3 Same as 2 + diffuse expansion of interacinar septae</td>
<td>3 Diffuse occurrence of 11–16 necrotic cells/HPF (foci of confluent necrosis)</td>
<td>3 6 foci</td>
<td>3 26–30 intralobular or perivascular leukocytes/HPF</td>
</tr>
<tr>
<td></td>
<td>3.5 Same as 3 + focal occurrence of &gt;16 necrotic cells/HPF</td>
<td>3.5 Same as 3 + focal occurrence of &gt;16 necrotic cells/HPF (foci of confluent necrosis)</td>
<td>3.5 7 foci</td>
<td>3.5 &gt;30 leukocytes/HPF or focal microabscesses</td>
</tr>
<tr>
<td></td>
<td>4 Same as 3 + diffuse expansion of intercellular spaces</td>
<td>4 &gt;16 necrotic cells/HPF (extensive confluent necrosis)</td>
<td>4 8 foci</td>
<td>4 &gt;35 leukocytes/HPF or confluent microabscesses</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Histopathologic examination of liver tissue stained by anti-insulin IHC staining method by light microscopy

A – control group, insulin positive area; B – MTX group, insulin negative area; C – MTX + ADA group, insulin positive area.
Increased edema, acinar edema, and necrotic cells were observed in islets of Langerhans and acinar cells in Group 2, compared to those found in Group 1 (p < 0.05) (Table 2) in our study on MTX-induced pancreatic toxicity. It was also found that atypical islets of Langerhans and connective tissue trabeculae were observed to increase considerably in volume. A reduction in the amount of insulin and somatostatin was also found. An accumulation of mast cells in the connective tissue was observed.

According to the literature, increased oxidative stress leads to the release of proinflammatory cytokines, leading to further tissue damage. Methotrexate is used for the treatment of pancreatic cancer, but has been shown to destroy the beta cells of the pancreas, primarily through oxidative stress. In support of the data in the literature, we determined that in our study the number of insulin and somatostatin-positive cells significantly decreased in Group 2, when compared to that in Group 1 (Fig. 2B, 3B).

It has been proposed in previous studies that MTX increases lipid peroxidation and oxidative stress, leading to an increase in reactive oxygen species (ROS). Methotrexate is used for the treatment of pancreatic cancer, but has been shown to destroy the beta cells of the pancreas, primarily through oxidative stress. In addition, intense proinflammatory cytokine release, due to excessive ROS formation, has been reported during MTX treatment for cancer and autoimmune diseases. Accumulation of ROS leads to neutrophil infiltration and proinflammatory cytokine release, which triggers apoptosis. Methotrexate is used for the treatment of pancreatic cancer, but has been shown to destroy the beta cells of the pancreas, primarily through oxidative stress.

A decrease in islets of Langerhans insulin and somatostatin-immunopositive cells was shown in this study following the administration of MTX (Fig. 2B, 3B).

### Table 2. Statistical results

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Edema</th>
<th>Acinar necrosis</th>
<th>Inflammatory infiltration</th>
<th>Perivascular infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>0.19 ±0.09 b</td>
<td>0.13 ±0.08 b</td>
<td>0.06 ±0.06 b</td>
<td>0.06 ±0.06 b</td>
</tr>
<tr>
<td>MTX</td>
<td>8</td>
<td>1.75 ±0.66 a</td>
<td>1.8 ±0.16 a</td>
<td>1.88 ±0.08 a</td>
<td>0.44 ±0.11 a</td>
</tr>
<tr>
<td>MTX + ADA</td>
<td>8</td>
<td>0.5 ±0.09 b</td>
<td>0.56 ±0.6 b</td>
<td>0.05 ±0.09 b</td>
<td>0.13 ±0.08 b</td>
</tr>
</tbody>
</table>

Means in the same column by the same superscript letter are not statistically significantly different under the Tukey test (α = 0.05). Results are mean ±standard error of the mean. a vs control Group p < 0.05; b vs MTX Group p < 0.05. MTX – methotrexate; ADA – adalimumab.

### Table 3. Anti-insulin and anti-somatostatin shown in the pancreas tissues by the method of immunopersoxidase in immunohistochemical staining

<table>
<thead>
<tr>
<th>Group</th>
<th>Anti-insulin median ±SD</th>
<th>Anti-somatostatin median ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3 ±0.75</td>
<td>3 ±1.17</td>
</tr>
<tr>
<td>MTX</td>
<td>1 ±0.52 a</td>
<td>1 ±0.41 a</td>
</tr>
<tr>
<td>MTX + ADA</td>
<td>2.5 ±0.05 b,c</td>
<td>2.5 ±1.05</td>
</tr>
</tbody>
</table>

In the statistical evaluations performed, significant differences were observed between the control and MTX groups in terms of anti-insulin and anti-somatostatin immunopositivity according to the Kruskal-Wallis test (p < 0.05). In the statistical evaluations performed, significant differences were observed between the control and MTX + ADA groups in terms of anti-insulin and anti-somatostatin immunopositivity according to the Kruskal-Wallis test (p < 0.05). In the statistical evaluations performed, significant differences were observed between the MTX and MTX + ADA groups in terms of anti-insulin and anti-somatostatin immunopositivity according to the Kruskal-Wallis test (p < 0.05).

MTX – methotrexate, ADA – adalimumab.

### Discussion

The side effects of MTX on the pancreas have not been researched. However, high-dosage substances were reported to have a toxic effect on the pancreas. Irreversible tissue injury with edema, inflammation, and acinar necrosis was also found in histopathological studies on pancreatic toxicity. Increased edema, acinar edema, and necrotic cells were observed in islets of Langerhans and acinar cells in Group 2, compared to those found in Group 1 (p < 0.05) (Table 2) in our study on MTX-induced pancreatic toxicity. It was also found that atypical islets of Langerhans and connective tissue trabeculae were observed to increase considerably in volume. A reduction in the amount of insulin and somatostatin was also found. An accumulation of mast cells in the connective tissue was observed.
It has been suggested that TNF-α plays a key role in the mechanism of immune-mediated inflammation in recent studies. Inhibition of TNF results in the down-regulation of abnormal and progressive inflammatory processes, resulting in target organ damage.\textsuperscript{4,5,10,11,19,27} It has been experimentally proven that TNF inhibitors prohibit organ tissue damage by suppressing the formation of TNF-α, proinflammatory cytokines, and nitric oxide.\textsuperscript{5,26,27}

A decrease in edema, acinar cell edema, and necrotic cells was observed in Group 3 (p < 0.05) (Table 2) after the administration of ADA. An increase in islets of Langhans insulin and somatostatin-positive cells was also demonstrated following the application of ADA treatment to Group 3 (Fig. 2C, 3C).

As a result, ADA was shown to have a protective effect against MTX-induced pancreatic injury. It was shown in this study that ADA reduced inflammatory levels and provided better protection to the insulin and somatostatin cells than that provided by MTX. This was a pilot study. Thus, further studies are needed in this regard to confirm our findings.

References