

EXPERIMENTAL STUDY

Effects of resveratrol on methotrexate-induced intestinal injury

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ABSTRACT

BACKGROUND: Methotrexate (MTX) is an anticancer drug. Many studies have reported that MTX causes oxidative stress-associated damage in the small intestine. The purpose of this study was to investigate the possible protective effect of resveratrol (RES), an antioxidant, against MTX-induced damage in the small intestine. **MATERIALS AND METHODS:** Twenty-four Sprague Dawley rats were randomly divided into four groups; the control group, the RES group given 20 mg/kg RES for 10 days, the MTX group given single dose 30 mg/kg MTX, MTX+RES group given 20 mg/kg RES i.p. for 7 days and 30 mg/kg MTX i.p. on the 7th day, RES being maintained for 3 further days. All rats were sacrificed on the 10th day, and small intestinal tissue was removed for histopathological and biochemical analysis. Additionally, mucosal apoptosis was analyzed using the TUNEL method. **RESULTS:** Histopathologically, villar fusion, atrophic villus epithelium, cystic expansion in crypts, hemorrhage and inflammatory cell infiltration were seen in the small intestine in the MTX group. In the MTX+RES group this histopathological damage decreased significantly. Apoptotic score was significantly higher in the MTX group and significantly lower in the MTX+RES group. Tissue malondialdehyde (MDA) level was significantly higher in the MTX group. Superoxide dismutase (SOD) activity was significantly decreased in the MTX group. The MDA level in the MTX+RES group decreased while SOD and catalase (CAT) activities rose, this was not statistically significant.

CONCLUSIONS: RES treatment may ameliorate MTX induced small intestine damage especially at histopathological level (Tab. 2, Fig. 2, Ref. 41). Text in PDF www.elis.sk.

KEY WORDS: methotrexate, resveratrol, rat, small intestine, oxidative stress.

Introduction

Methotrexate (MTX) is a cytotoxic chemotherapeutic agent widely used in leukemia and other malignancies. It is also used in non-oncological diseases, such as rheumatoid arthritis and psoriasis (1, 2). MTX is a folic acid antagonist and inhibits the enzyme dihydrofolate reductase (the essential enzyme in DNA and RNA synthesis) (3). The cytotoxic effects of MTX are not limited to cancer cells alone. It has side-effects on rapidly proliferating cells, such as crypts of gastrointestinal mucosa (1, 4). MTX damages the intestinal mucosa and causes intestinal flora to enter the circulation. It affects the entire gastrointestinal system and leads to conditions that may result in nausea, vomiting, diarrhea, ulceration and hemorrhage (1, 5). This reduces quality of life in chemotherapy treated patients.

The mechanism causing these side-effects of MTX has not yet been fully clarified. However, oxidative stress and neutrophil infil-

tration have recently been reported to play a role in the intestinal damage caused by MTX (6). MTX has been shown to significantly reduce antioxidant protein and enzyme levels and to increase the levels of oxidant markers such as malondialdehyde (MDA) (7, 8). Therefore researchers have studied various antioxidant agents in order to reduce the side-effects of MTX to a minimum (9, 10).

Resveratrol (trans-3,4',5-trihydroxy-stilben) (RES) is a polyphenol phytoalexin. It is found in various different plants, and particularly in grape, peanut and mulberry (11, 12). Research into the effects of RES has increased in recent years. Studies have emphasized its antioxidant, anti-inflammatory, antiplatelet, anti-atherogenic and cancer inhibiting effects (13). RES scavenges reactive oxygen species (ROS), prevents damage to DNA and lipid peroxidation in the cell membrane (14).

The purpose of this study was to investigate the role of oxidative stress in MTX-associated small intestine injury and also to show the probable protective effects of RES, a known antioxidant, in small intestinal injury developing in association with MTX.

Materials and methods**Animals**

Twenty-four healthy Sprague Dawley male rats (8 weeks old, weighing 200–250 g) were utilized in this study. Rats were kept in standard, controlled environments at a stable temperature (22 ± 2 °C) in a 12:12-h light/dark cycle with access to standard rat

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chow and tap water ad libitum. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health. The study was approved by the Institutional Animal Ethical Committee of the Karadeniz Technical University, Trabzon, Turkey.

Study groups and Experimental design

Rats were randomly divided into four groups and each group contained six rats. These were the control group, the RES group given 20 mg/kg of resveratrol (Resveratrol, R5010–500 mg, Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally (i.p.) over 10 days, the methotrexate (Methotrexate, Kocak Farma, Tekirdag, Turkey) group given 30 mg/kg i.p. on the 7th day of the experiment, and the MTX+RES group given 20 mg/kg RES i.p. for 7 days and 30 mg/kg MTX i.p. on the 7th day and then 20 mg/kg RES for another 3 days. At the end of the 10th day laparotomy was performed in all rats under ketamine hydrochloride (Ketalar, Pfizer, Istanbul, Turkey) anesthesia. The abdominal cavity was opened, and a 5-cm intestinal segment from a point 5 cm proximal to the ileocecal ligament was rapidly dissected. Half the tissue was fixed in 10% formalin for histopathological evaluation, while the other half was placed in an eppendorf tube and stored at -80°C for biochemical investigation. Immediately afterwards, 3 cc blood specimens were placed in an EDTA containing tube, centrifuged and stored at -80°C for biochemical investigation.

Biochemical analysis

Determination of MDA

A piece of ileum tissue was used to measure malondialdehyde (MDA) levels. The sample was minced and homogenized in an ice-cold 1.15% KCl solution containing 0.50 mL/L Triton X-100 using an Ultra-Turrax T25 homogenizer. MDA levels in testis samples were determined as MDA concentration by the method of Mihara and Uchiyama (15). Briefly, 0.5 mL of homogenate was mixed with 3 mL of 1% H_3PO_4 . After adding 1 mL of 0.67% thiobarbituric acid, the mixture was heated in boiling water for 45 min. The formed color was extracted into n-butanol. The mixture was centrifuged at 4000 rpm for 10 min at room temperature. Absorbance of the organic layer was read at 532 nm. Tetramethoxypropane was used as a standard, and MDA levels were calculated as nanomoles per gram of wet tissue.

Determination of SOD and CAT Activity

Superoxide dismutase (SOD) and catalase (CAT) activities were determined in the remaining part of the ileum tissue. The sample was minced and homogenized in an ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing 0.50 mL/L Triton X-100. SOD activities were measured by degree of the inhibition rate of nitroblue tetrazolium reduction in the xanthine-xanthineoxidase system (16). Enzyme activity leading to 50% inhibition was accepted as one unit and results were expressed as U/g tissue protein. CAT activity was determined by the method of Aebi (17) and results are expressed as k/g protein (k, rate constant). Protein concentrations were determined according to Bradford's method (18).

Histopathological evaluation

Ileum tissues were dehydrated, and embedded in paraffin for light microscopic evaluation. Sections 5 μm in thickness were taken and stained with hematoxylin-eosin (H&E). The sections were analyzed under a light microscope (Olympus BX-51; Olympus, Tokyo, Japan). Five different fields were evaluated at a magnification of X200 in ileum slides from all groups. Each section was scored semi-quantitatively from 0 to 3 (0: none; 1: mild; 2: moderate and 3: severe) according to defined criteria: Degeneration of the apical surface epithelium, villar fusion, hemorrhage and inflammatory cell infiltration. The mean histopathologic score was calculated for each group (19).

TUNEL assay

We evaluated the levels of apoptosis in ileum using a standard terminal deoxynucleotidyl transferase (TdT) deoxyuridine triphosphate nick end labeling assay (TUNEL) method. TUNEL staining of sections was performed using an in situ cell death detection kit, AP (Roche, Mannheim, Germany), in accordance with the manufacturer's instructions. The sections were incubated in a humidified chamber with the TUNEL reaction mixture. The color was then developed with 3,3'-diaminobenzidine (DAB) including kit (DAB, Sigma, St. Louis, MO, USA). The TUNEL staining cells appear as brown and other cells nuclei appear as blue stains (20). TUNEL-stained histologic slides were examined by a blinded histologist, a score on the scale of 0–4 was determined. Under that classification, score 0 represented only a few apoptotic TUNEL positive nuclei observed in the villous epithelium, score 1 observed apoptotic nucleus clusters at the villous tips, score 2 observed apoptotic nuclei in all villi but not in crypts, score 3 observed apoptotic nuclei in villi and crypts, score 4 represented apoptotic nuclei in all layers (21).

Statistical analysis

All statistical analyses were performed using the SPSS version 13.0 Med Calc 12.3 statistical software. All data were presented as mean (\pm) standard deviation (SD). Kruskal Wallis analysis of variance was used to compare differences between group parameters. Dual comparisons between groups exhibiting significant values were evaluated with a Mann-Whitney U-test with corrected Bonferroni test. Statistical significance was accepted for all tests at $p < 0.05$.

Results

Clinical signs

Rats in all groups survived during the experiment. Diarrhea was observed in all rats in the MTX group following MTX administration. While diarrhea was not observed in the control and RES groups, it was seen in two rats in the MTX+RES group. However, this was not as severe as that in the MTX group. Body weight (194.90 g) decreased in the MTX group compared to control and RES group (227.26 g and 221.40 g, respectively), body weight was greater in the MTX+RES group (198.8 g) compared to the MTX group, but the differences were not statistically significant ($p > 0.05$).

Biochemical analysis results

Ileal MDA level, SOD and CAT activities are shown in Table 1. Ileal MDA levels were significantly higher in the MTX group compared to the control group ($p < 0.05$). MDA levels in the MTX+RES group were decreased compared to the MTX group, but there was no statistical significance. Ileal SOD activity was significantly lower in the MTX group compared to the control group ($p < 0.05$). Ileal CAT activity was lower in the MTX group compared to the control group, but there was no statistical significance. Although SOD and CAT activities increased in the MTX+RES group compared to the MTX group, that increase was not statistically significant ($p > 0.05$).

Histopathological findings

Ileal damage score values on the basis of parameters analysis are shown in Table 2. The small intestine was evaluated macroscopically. No color change was observed in the control, RES and MTX+RES groups. In the MTX group, however, a dark, blue/purple color was seen. At evaluation under light microscopy, control and RES groups ileal tissue exhibited normal histological findings (Figs 1a, b). Examination of the MTX group revealed vacuolization and shedding in ileal epithelial cells and shortening, blunting and fusion in villi. Hemorrhage was present in all layers, particularly in the lamina propria. A decrease in crypts, epithelial loss and a cystic appearance were observed. Loss of goblet cells and wide-spread inflammatory cell infiltration in mucosa were observed (Fig. 1c). Ileal damage score values on the basis of parameters analysis were significantly increased in MTX group compared to the control group ($p < 0.05$). In MTX+RES group, villous architecture was close to normal, and villus epithelial cells exhibited a regular arrangement. Goblet cells were present among the epithelial cells (Fig. 1d). Degeneration of the apical surface epithelium and hemorrhage were significantly decreased compared to the MTX group ($p < 0.05$). Moderate inflammatory cell infiltration was observed in the mucosa.

Tab. 1. Biochemical analysis of ileum tissues for each group.

Groups	Parameters		
	MDA(nmol/g)	SOD (U/g tissue)	CAT (k/g)
Control	114.65±23.20	279.65±29.99	0.65±0.09
RES	110.65±26.90	284.09±77.77	0.61±0.08
MTX	182.31±21.82*	199.24±19.19*	0.47±0.05
MTX+RES	139.79±23.41	226.17±44.26	0.53±0.12

MDA – malondialdehyde, SOD – superoxide dismutase, CAT – catalase, RES – Resveratrol, MTX – Methotrexate, * $p < 0.05$ vs Control

Tab. 2. The histological damage score on the basis of analysis parameters and apoptotic score in control, RES, MTX, MTX+RES groups.

Groups	Parameters				
	Inflammatory cell infiltration	Villar fusion	Degeneration of the apical surface epithelium	Hemorrhage	Apoptotic score
Control	0.33±0.51	0.16±0.4	0.16±0.4	0.16±0.4	0.16±0.4
RES	0.50±0.54	0.50±0.54	0.16±0.4	0.33±0.51	0.3±0.51
MTX	2.5±0.54*	2.5±0.54*	2.66±0.51*	2.33±0.51*	3.66±0.51*
MTX+RES	1.66±0.51*	1.33±0.51*	1.33±0.51**	1.16±0.4**	1.83±0.75**

RES – resveratrol, MTX – methotrexate, * $p < 0.05$ vs Control, ** $p < 0.05$ vs MTX group

Evaluation of TUNEL staining

Apoptotic score values of the study groups' ileal tissues are shown in Table 2. A few apoptotic cells were observed in the villus tips in the control and RES groups (Figs 2a, b). Examination of the MTX group revealed wide spread apoptotic cells in all ileal layers (Fig. 2c). Apoptotic score was significantly higher in the MTX group compared to the control group ($p < 0.05$). In the MTX+RES group, however, apoptotic cells were restricted to the villi (Fig. 2d). In the MTX+RES group, apoptotic score was significantly lower compared to the MTX group ($p < 0.05$).

Discussion

MTX is one of the most frequently used anti-metabolic agents in clinical oncology. Clinical use is frequently accompanied by diarrhea and gastrointestinal symptoms (22, 23). Ileal injury developing with the administration of MTX has been identified in the literature (10, 23, 24, 25). Previous studies have shown that a single dose of MTX is sufficient in the development of ileal damage (24), together with villous atrophy, flattening of the epithelium, crypt loss and restructuring of the crypts (9, 26). Damage evaluation in intestinal mucosal revealed damage the 72nd h following MTX administration (22). In this study we evaluated ileal damage histopathologically and biochemically at the end of the 3rd day after MTX administration.

MTX-induced organ toxicity mechanism has not been completely clarified. Recent studies show that the side-effects of antitumor drugs may be associated with free oxygen radicals and hydrogen peroxide (9, 27). Damage induced by the administration of MTX is characterized by inflammatory cell response (9, 22). Inflammatory cells activated by pro-cytokines lead to excessive free oxygen radical production, and that increases MDA, a product of lipid peroxidation (28). In our study, lipid peroxidation level in the MTX group exhibited a pronounced increase compared with the control and RES groups. The rise in MDA level may be attributed to tissue damage and oxygen defense mechanisms being insufficient in the prevention of free radical formation (6, 9). In the MTX+RES group, we observed a decrease in lipid peroxidation levels compared to the MTX group. This may be due to oxidation mechanisms becoming activated and binding free radicals.

Various enzymes play a role in oxidative stress and are effective in the protective mechanism against oxidative damage. These include enzymes such as SOD, CAT and glutathione peroxidase. These convert free oxygen radicals and reactive oxygen surfaces into non-radical products (29). SOD is a powerful antioxidant en-

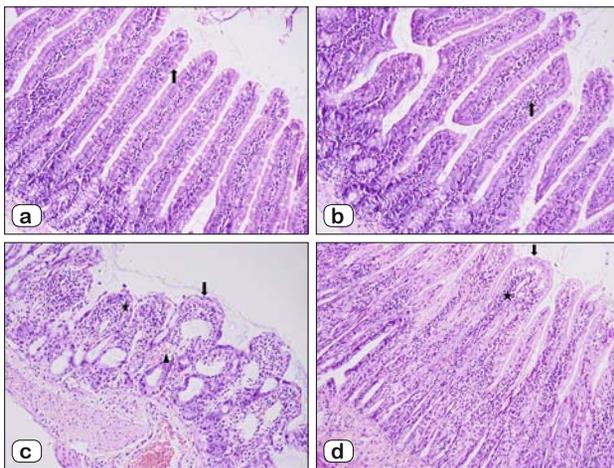


Fig. 1. Light microscopic sections of ileum tissues in different groups. **a, b** Normal villous architecture (*thick arrow*) was observed in control group and RES groups. **c** In MTX group, villus epithelial degeneration (*thick arrow*), a cystic appearance in the crypts, severe inflammatory cell infiltration (*star*) and hemorrhage (*arrow head*) were observed. **d** In MTX+RES group, regeneration in epithelial cells in villi (*thick arrow*), crypts and presence of goblet cells, moderate inflammatory cell infiltration (*star*) and slight hemorrhage were observed (H&E x200).

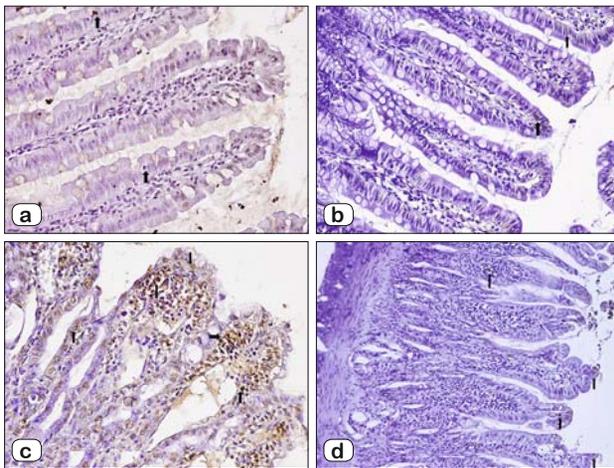


Fig. 2. TUNEL staining of ileum tissues in different groups. **a, b** In Control and RES group, respectively, a few TUNEL positive apoptotic cells were observed in villus tips (*thick arrow*), **c** In MTX group, apoptotic cells (*thick arrow*) were present in all layers of ileal tissues, **d** In MTX+RES group, apoptotic cells (*thick arrow*) were only present in villous architecture (TUNEL x200).

zyme that protects cells against reactive oxygen radicals. It does this by catalyzing the conversion of superoxide radicals to hydrogen peroxide (30). CAT is an antioxidant enzyme that consumes peroxisome hydrogen peroxide and protects the cells against oxidative damage (31). Cetin et al (32) observed an increase in MDA levels in hepatic oxidative damage induced by MTX, and also a decrease in SOD and CAT levels. Vardi et al (10) reported similar findings in MTX administration-associated small intestine damage. In our study, SOD and CAT levels were lower in the MTX group compared to the control group. The decrease in SOD and

CAT levels may be due to a decreased or increased consumption in the synthesis of antioxidant enzymes. There was a rise in SOD and CAT levels in the MTX+RES. With these results, we think that RES can regulate enzymatic activity in small intestinal damage.

In our MTX group we determined atrophy in the villi and crypts. We also observed that crypts have been replaced by cystic-like structures and there was a decrease in goblet cells. Histopathological findings in the MTX group were compatible with findings from previous studies (10, 33, 34). In the MTX+RES group there was a pronounced improvement in villus structure compared to the MTX group; the appearance of the villous epithelium and goblet cells was close to normal, crypts were clearly visible, the cystic structure had disappeared and there was a pronounced decrease in hemorrhage. In terms of the pathology of villous degeneration, Gao et al (35) reported that MTX inhibits de novo purine and thymidine kinase synthesis, and that this effect also suppresses cell proliferation in the intestinal epithelial cells and crypts. MTX inhibits the dihydrofolate reductase enzyme. Dihydrofolate reductase is an enzyme essential for DNA synthesis. In this way, DNA synthesis is impaired (3) and apoptosis takes place in the cells (3, 36). While elevated Bax protein has been determined in MTX administrations, the level of Bcl 2 protein decreases (26). Bax protein stimulates leakage of cytochrome c from the mitochondria into cytoplasm, and this process activates apoptotic proteases. Additionally, production of reactive oxygen radicals such as superoxide radical gives rise to apoptotic cell death (9). Also, due to its antioxidant property, like vitamins C and E, RES also prevents cell death induced by oxidized lipoproteins (37). In our study, while widespread apoptosis was observed in all ileal layers in the MTX group, apoptotic cells were restricted to the villi in the RES treatment group. RES therapy was accompanied by a significant improvement in ileal morphology and a significant decrease in apoptosis compared to the MTX group. Since RES is both a free radical scavenger and also activates antioxidant enzymes, it has been described as a potent antioxidant (38, 39). RES inhibits the peroxidation of membrane lipids (37). Previous studies have shown positive effects of RES therapy in a small intestine model with induced ischemia-reperfusion damage (40, 41).

In conclusion, our study showed that oxidative stress plays an important role in the pathogenesis of MTX-induced intestinal damage. The powerful antioxidant RES given before MTX treatment has a protective effect on MTX-induced intestinal damage and apoptotic cell death in rats. This beneficial effect of RES might be due to its anti-oxidant properties.

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Received April 3, 2013.

Accepted March 20, 2015.