The effects of lycopene on intestinal injury due to methotrexate in rats

Yusuf Yucel¹, Suzan Tabur², Orhan Gozeneli¹, Sezen Kocarslan³, Ahmet Seker¹, Hasan Buyukaslan⁴, Emin Şavik⁵, Alpay Aktumen⁶, Abdullah Ozgonul¹, Ali Uzunkoy¹, Nurten Aksoy⁵

¹Department of General Surgery, Medical Faculty, Harran University, 63100 Sanliurfa, Turkey, ²Division of Endocrinology, Department of Internal Medicine, Gaziantep University Faculty of Medicine, Turkey, ³Department of Pathology, Medical Faculty, Harran University, 63100 Sanliurfa, Turkey, ⁴Emergency Department, Medical Faculty, Harran University, 63100 Sanliurfa, Turkey, ⁵Department of Clinical Biochemistry, Faculty of Medicine, Harran University, 63100 Sanliurfa, Turkey, ⁶Department of Pathology, Medical Faculty, Karabuk University, Karabuk, Turkey

Objective: The aim of this study was to investigate the effects of lycopene (Lyc) on methotrexate (Mtx)-induced intestinal damage in rats.

Method: Twenty-eight male Sprague Dawley rats were divided into four equal groups: control, Mtx, Lyc, and Mtx-L.

Control group: Rats were given only the vehicle.

Lyc group: Rats were given Lyc (10 mg/kg) with corn oil by oral gavage for 10 days.

Mtx group: Rats were injected intraperitoneally with a single dose of 20 mg/kg of Mtx and given corn oil by oral gavage.

Mtx-L group: Rats were treated with Lyc (10 mg/kg) for 10 days after a single dose of Mtx (20 mg/kg). All of the rats were euthanized using terminal anesthesia, and the intestinal tissues were removed for histological examination and for pro-inflammatory cytokine measurement (tumor necrosis factor alpha (TNF- α), interleukin-1 beta (IL-1 β)), total oxidative status (TOS), total antioxidant capacity (TAC), and oxidative stress index (OSI).

Results: Mtx administration increased histopathological damage and increased TNF- α , IL-1 β , TOS, TAC, and OSI levels in the small intestine tissues. Lyc therapy applied to the Mtx-L group provided significant improvement in all parameters of histopathological damage to the small intestine and significantly reduced the levels of IL-1 β , TOS, and OSI in the intestinal tissues.

Conclusions: The results of this study indicate that Lyc might be useful for protecting intestinal damage induced by Mtx in rats by reducing the increased oxidative stress and pro-inflammatory cytokine (IL-1 β) levels.

Keywords: Lycopene, Intestinal injury, Methotrexate, TNF- α , IL-1 β , Oxidative stress

Introduction

Methotrexate (Mtx), an antineoplastic and immunosuppressive agent commonly used in the treatment of malignancies and various inflammatory diseases, is toxic not only to cancer cells but also to normal cells. Its cytotoxic structure causes life-threatening side effects, such as intestinal injury, and as a result of this, use of this agent is often limited.^{1–3} While the mechanism of Mtx-induced intestinal injury has not yet been fully elucidated, many studies have

shown that reactive oxygen species play an important role in its pathogenesis. This mechanism is probably related to the increase in oxidative stress.^{4–6} Mtx administration induces oxidative stress and significantly reduces antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase in the intestinal mucosa of rats.⁷ Antioxidant agents that have been shown to have beneficial effects on Mtx-induced intestinal damage are ascorbic acid, melatonin, *N*-acetylcysteine, and vitamin A and these findings support the theory of oxidative stress.^{4,8–10} It is also currently believed that pro-inflammatory cytokines, such as tumor necrosis factor alpha

Correspondence to: Yusuf Yucel, Department of General Surgery, Medical Faculty, Harran University, 63300 Sanliurfa, Turkey.
Email: dryusufyucel@yahoo.com

(TNF- α), interleukin-1 beta (IL-1 β), and interleukin-6, may also play a role in Mtx-induced intestinal damage. Human and animal experiments have shown that TNF- α inhibitors repair mucositis. It has been suggested that inflammation might have a role in the pathogenesis of Mtx-induced nephrotoxicity, intestinal damage, and pneumonitis.^{1,11–14} These cytokines have all been demonstrated in the mucosa and peripheral blood of patients undergoing cancer treatment.^{11,12}

Lycopene (Lyc) is a carotenoid pigment produced by vegetables and fruits, such as tomatoes. It is one of the most powerful antioxidant, anticancer, and anti-inflammatory agents among all the dietary carotenoids,^{15–18} and it has been shown to be effective in reducing organ toxicity, e.g., pancreas, testis, and kidneys.^{19–22} Recent studies have indicated that Lyc plays an important role in the prevention of the gastrointestinal tract cancer. It protects mammalian cells from lipid peroxidation and oxidative DNA damage and inhibits the proliferation of cancer cells while accelerating the intestinal mucosal cell proliferation.^{23,24}

Fruits and vegetables including Lyc have a very long history of use in the diet of humans without any problems. Additionally, the studies investigating the safety of Lyc support this. The deposition of Lyc in plasma, liver, and other tissues had no adverse effects and no teratogenic effects were observed in rat studies.^{25–29} The antioxidant, anticancer, and anti-inflammatory properties of Lyc are thought to be primarily responsible for its beneficial effects.^{24,30–32}

Several antioxidant agents have been shown to be effective experimentally, by reducing the increased oxidative stress and pro-inflammatory cytokine levels in intestinal injury caused by Mtx. However, no study has been performed yet to evaluate the effects of Lyc on intestinal injury by Mtx in rats. Therefore, the aim of this study was to investigate the effects of Lyc on intestinal injury caused by Mtx in rats.

Materials and methods

Chemicals

Mtx was purchased from a local pharmacy (Koçak Farma, Turkey). Lyc (Redivivo® 10% CWS/S-TG, DSM, Basel, Switzerland) was provided by DSM Nutritional Products, Istanbul, Turkey. TNF- α and IL-1 β enzyme-linked immunosorbent assay (ELISA) kits and oxidative stress kits used for biochemical

analysis were obtained from RayBiotech, Diaclone, and Rel Assay Diagnostics, respectively.

Animals

Twenty-eight healthy, adult male Sprague Dawley rats weighing 200–275 g were used in this study. The animals were obtained from Dollvet Animal Laboratory (Sanliurfa, Turkey), where the experimental protocol was performed. The animals were allowed to acclimate under standard laboratory conditions (12 hours light, 12 hours dark) in a room with controlled temperature ($24 \pm 3^\circ\text{C}$) for 1 week prior to the experimental study. The animals had free access to water and were fed a standard commercial pellet diet *ad libitum*. The Dollvet Animal Care and Use Committee approved the study (approval number: 2014/35). All experimental procedures were conducted in accordance with the *Guide to the Care and Use of Laboratory Animals*.

Experimental protocol

After 7 days of acclimatization, the rats were divided into four groups of seven rats each, as follows (Table 1):

Control Group: The rats in this group served as the controls and were administered the vehicle only.

Lyc Group: The rats were given Lyc (10 mg/kg) with corn oil by oral gavage for 10 days.

Mtx Group: The rats were injected intraperitoneally (i.p.) with a single dose of Mtx (20 mg/kg) (1, 2) and given corn oil by oral gavage.

Mtx-L Group: The rats were treated with Lyc (10 mg/kg) for 10 days after being administered a single dose of Mtx (20 mg/kg).

All of the rats were euthanized by terminal anesthesia (ketamine – 75 mg/kg i.p. and xylazine – 8 mg/kg i.p.), and the small intestines were removed immediately and divided into two equivalent sections. One of the sections was fixed with 10% buffered formalin solution at room temperature for histopathological evaluation and the other part was stored at -80°C for biochemical analysis.

Histopathological examination

The small intestine tissues were removed aseptically from all of the rats, cut into small sample pieces, and fixed in 10% formaldehyde solution. The tissue sections (5 μm) were mounted on glass slides and

Table 1 Experimental study protocol

Groups	1*	2*	3*	4*	5*	6*	7*	8*	9*	10*	11*
Control	a,b	b	b	b	b	b	b	b	b	b	e
Lyc	b,c	b,c	b,c	b,c	b,c	b,c	b,c	b,c	b,c	b,c	e
Mtx	d										e
Mtx-L	b,c,d	b,c	b,c	b,c	b,c	b,c	b,c	b,c	b,c	b,c	e

*Days, a:0.9% NaCl(i.p.), b:Corne Oil (o.g.), c:Lycopene (10 mg/kg)(o.g.), d:Mtx (20 mg/kg) (i.p.), e:Euthanized.

stained with hematoxylin–eosin to evaluate the intestinal structure. Each stained section was semiquantitatively evaluated under a light microscope (Olympus BX51 microscope with a magnification of $\times 200$) by a histologist who did not know which treatment group each sample was from. The histologist used the scoring system devised by Duman *et al.* Scores were assigned for each criterion 0 (none), 1 (mild), 2 (moderate), and 3 (severe) using the following semiquantitative scale: (1) degeneration of surface and crypt epithelium, (2) degeneration of villus structure, and (3) inflammatory cell infiltration. Five pathological sections were prepared from each rat. The microscopic score of the ileum was calculated as the sum of the scores given to each criterion, and at least five microscopic areas were examined to score each specimen. A maximum score of nine indicated the most severe damage of the small intestine.²

Biochemical analysis

Tissue preparation and homogenization

Before the biochemical assays, small intestine tissues were weighed, broken down into very small pieces, and placed in empty glass tubes. A portion (1 ml) of 140 mM potassium chloride (KCl) solution per gram of tissue was added to each tube, and then all the tissues were homogenized in a motorized homogenizer. The homogenate was centrifuged at $2800 \times g$ for 10 minutes at 4°C .³³ The resulting supernatant was used to obtain the levels of total antioxidant capacity (TAC), total oxidative status (TOS), TNF- α , and IL-1 β .

Determination of oxidative stress biomarkers

Measurement of TAC

The TAC of the fractions of the supernatant was determined using a novel automated measurement method developed by Erel.^{34,35} Using this method, the antioxidative effect of the sample against the potent free radical reactions initiated by the hydroxyl radical produced was measured. The results are expressed as mmol Trolox equivalent/g protein.

Measurement of TOS

The TOS of the supernatant fractions was determined using a novel automated measurement method developed by Erel.^{34,35} The assay results are expressed as μmol hydrogen peroxide (H_2O_2) equivalent/g protein.

Oxidative stress index

The percentage ratio of TOS level to TAC level was accepted as the oxidative stress index (OSI). The OSI value was calculated according to the following formula:

$\text{OSI (arbitrary unit)} = \text{TOS } (\mu\text{mol H}_2\text{O}_2 \text{ equivalent/g protein}) / \text{TAC (mmol Trolox equivalent/g protein)}$.

Determination of pro-inflammatory cytokines

TNF- α and IL-1 β levels were measured using ELISA kits according to the manufacturer's instructions. TNF- α and IL-1 β levels are expressed as pg/g protein.

Statistical analysis

All data are expressed as mean $\pm$ standard deviation. The use of the Kruskal–Wallis analysis of variance method was followed by the application of the non-parametric Mann–Whitney *U* test, with a Bonferroni correction for binary comparisons to evaluate the differences between the experimental groups. Probability values less than 0.05 were considered to be statistically significant. All the data were processed using the SPSS 16.0 for Windows Statistical Software Package (SPSS, Inc., Chicago, IL, USA).

Results

Histopathological results

In the Mtx group, Mtx caused damage to the bowel tissue across all histopathological parameters (degeneration of surface and crypt epithelium, degeneration of villus structure, and inflammatory cell infiltration) (Fig. 1-IIa–IIc). The differences in intestinal tissue damage between the Mtx group and the control group were statistically significant in all histopathological parameters. The group with the most tissue damage was the Mtx group, and the group with the least tissue damage was the control group (Table 2). There were no statistically significant histopathological differences between the Lyc group and the control group (Table 2). There was significant improvement in all histological parameters in the Mtx-L group compared with the Mtx group (Fig. 1-III; Table 2).

Biochemical results

Biochemical examination of the small bowel tissue samples showed improvements in all parameters in the Mtx group compared with the control group. However, only the increases in TNF- α and TOS were significant (Table 3). The Mtx-L group exhibited significant improvement in IL-1 β , TOS, and OSI values (Table 3). There were no statistically significant differences in biochemical parameters between the Lyc and control groups.

Discussion

The results of this experimental study revealed that Lyc reduced pathological damage, oxidative stress, and pro-inflammatory cytokine levels in rat small intestine tissues. Lyc therapy applied to the Mtx-L group provided significant improvement in all histopathological parameters and in IL-1 β , TOS, and OSI levels.

Several studies have indicated that increased oxidative stress and pro-inflammatory cytokine levels

Figure 1 Sections of the small intestine were stained with hematoxylin–eosin, scale bar = 200 μ m. (I) Normal histological appearance of the small intestine tissues in the control rats. (II) Histopathological effects of high dose methotrexate on the small intestine structure. (a) Degeneration of surface and crypt epithelium. (b) Degeneration of villus structure. (c) Inflammatory cell infiltration. (III) Section indicating that lycopene treatment in the Mtx-L group is providing significant histological improvement.

might be effective in Mtx-induced intestinal damage.^{14,36–38} In the present pathological examination of intestinal tissue damage and tissue biochemical parameters, the rats in the Mtx group had the highest levels. However, only the TOS and TNF- α levels of the Mtx group were significantly higher than those of the control group, suggesting that both oxidative stress and increased levels of pro-inflammatory cytokines might be involved in the intestinal damage caused by Mtx.

The microscopic examination of tissue damage showed that in the Mtx-L group there was significant improvement in all of the histopathological

parameters. This result can be interpreted to mean that the dose and length of time of Lyc administration were effective in treating the small bowel damage caused by Mtx.

Antioxidants inhibit damage to cells by binding the free oxygen radicals. Antioxidants such as amifostine, ascorbic acid, melatonin, *N*-acetylcysteine, and resveratrol have been used in Mtx-induced hepatotoxicity, and beneficial effects on oxidative stress have been demonstrated. Lyc, a strong antioxidant, anti-inflammatory, and anticancer agent, has been used in nephrotoxicity caused by cisplatin and has been found to reduce oxidative stress.^{10,18,20,39} It has also been

Table 2 Histopathologic examination results in rat small intestine tissues

Histopatologic parameters	Groups			
	Control ^a	Lyc ^a	Mtx ^a	Mtx-L ^a
Degeneration of surface and crypt epithelium	0.00 (0.0–1.0)	0.00 (0.0–1.0)	1.00 (1.0–2.0) ^b	0.50 (0.0–1.0) ^c
Degeneration of villus structure	0.00 (0.0–1.0)	0.00 (0.0–1.0)	1.00 (1.0–2.0) ^b	1.00 (0.0–1.0) ^c
Inflammatory cell infiltration	0.00 (0.0–0.0)	0.00 (0.0–0.0)	1.00 (1.0–2.0) ^b	0.00 (0.0–1.0) ^c

^aData were expressed as median (min–max).

^bSignificance of Mtx compared with control.

^cSignificance of Mtx-L compared with Mtx.

P < 0.01 is significant.

Table 3 Biochemical assessment results in rat small intestine tissues

	Groups			
	Control ^a (n = 7)	Lyc ^a (n = 7)	Mtx ^a (n = 7)	Mtx-L ^a (n = 7)
TNF- α (pg/g protein)	1030 $\pm$ 176	1091 $\pm$ 100	1253 $\pm$ 114 ^b	1123 $\pm$ 120
IL-1 β (pg/g protein)	11.91 $\pm$ 3.09	11.92 $\pm$ 3.15	14.18 $\pm$ 2.73	10.64 $\pm$ 2.78 ^c
TAC (mmol Trolox Eq/g protein)	0.43 $\pm$ 0.06	0.46 $\pm$ 0.07	0.45 $\pm$ 0.08	0.46 $\pm$ 0.07
TOS (μ mol H ₂ O ₂ Eq/g protein)	6.93 $\pm$ 0.58	7.57 $\pm$ 2.25	8.84 $\pm$ 1.54 ^b	7.20 $\pm$ 1.33 ^c
OSI (arbitrary units)	1.63 $\pm$ 0.24	1.66 $\pm$ 0.59	2.18 $\pm$ 0.82	1.62 $\pm$ 0.65 ^c

TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 beta; TAC, total antioxidant capacity; TOS, total oxidative status; OSI, oxidative stress index.

^aData were expressed as mean $\pm$ standard deviation.

^bSignificance Mtx compared with control

^cSignificance Mtx-L compared with Mtx.

P < 0.01.

shown to be effective in reducing organ toxicity in the pancreas, testes, and kidneys.^{3–6} Because of this, the effects of Lyc on Mtx-induced small bowel injury were investigated, and the results of this research showed that OSI and TOS levels decreased significantly after Lyc treatment. This result was interpreted to mean that Lyc might be effective by reducing oxidative stress in the damage of the small intestine caused by Mtx.

Cytokines are molecules secreted by inflammatory cells against antigens that regulate immune and inflammatory responses and two of these cytokines, TNF- α and IL-1 β , play active roles in inflammation. Mtx is an agent that causes an increase in inflammatory cytokines.³⁹ Several studies have shown that Lyc treatment decreases elevated TNF- α levels.^{36–38,40} However, to the best of our knowledge, no previous studies have shown the effect of Lyc on IL-1 β levels in intestinal injury caused by Mtx in rats. This study is the first to demonstrate that Lyc might be effective by reducing IL-1 β levels. In this study, IL-1 β levels decreased significantly in the Mtx-L group compared to the Mtx group.

In our study, all biochemical parameters in the intestinal tissues of the rats in the Mtx group were high when compared with those of the control group, but the only significant differences were in TNF- α and TOS. These results suggest that Mtx created an effective bowel injury. IL-1 β only improved significantly in the Mtx-L group. This result was interpreted as an indication that the effectiveness of Lyc in treating the Mtx-induced intestinal damage might be because of the reduced IL-1 β levels.

Finally, Lyc might be useful for protecting intestinal damage from Mtx in rats by reducing the increased IL-1 β and oxidative stress levels. It is thought that new studies with Lyc should be conducted to reveal the role of Lyc in Mtx-induced intestinal injury.

Disclaimer statements

Contributors All authors contributed equally.

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Conflicts of interest All the authors have no conflict of interest to declare. This work was carried out at Harran University School of Medicine, Department of General Surgery.

Ethics approval Dollvet Animal Care and Use Committee has been approved the study (approval number: 2014/35).

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