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EXPERIMENTAL STUDY

The effects of lycopene on methotrexate-induced liver injury in rats

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ABSTRACT
BACKGROUND: The aim of this study was to investigate the effects of lycopene (Lyc) on methotrexate (Mtx) induced liver toxicity in rats.
METHODS: Twenty-eight male Sprague-Dawley rats were divided into four equal groups: control, Lyc, Mtx 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 ten 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 post-treated with Lyc (10 mg/kg) for ten days after a single dose of Mtx (20 mg/kg).
RESULTS: Mtx administration increased histopathological damage, TNF-α, IL-1β, TOS, TAS and OSI levels in tissues; AST, ALT levels in the blood. Sinusoidal dilatation, inflammatory cell infiltration and congestion were significantly improved in the Mtx-L aon histopathologic examination of the rats.
In Mtx-L group that were treated at the Lyc, TNF-α and IL-1β levels of liver tissue were decreased significantly compared to Mtx group whereas the decrease in OSI was not significant. Lyc treatment improved the AST and ALT values in Mtx-L group. But only AST improvement was significant.
CONCLUSIONS: The results of this study revealed that Lyc might be useful in protecting the liver from injury due to Mtx in rats by reducing the increased proinflammatory cytokine levels *(Tab. 4, Fig. 1, Ref. 44)*. Text in PDF *www.elis.sk.*
KEY WORDS: lycopene, liver injury, methotrexate, TNF-α, IL-1β, oxidative stresss.

Introduction

Methotrexate (Mtx) is an anti-neoplastic and immunosuppressive agent widely used in the treatment of various malignancies and autoimmune diseases (1, 2). It is toxic not only to cancer cells but also to normal cells. Its cytotoxic characteristic causes some life-threatening adverse effects. One of the most serious side effect is hepatotoxicity, limiting the use of this agent (1–5). The mechanism of Mtx-induced hepatotoxicity has not been fully understood yet (6, 7). However, some mechanisms that could explain the toxicity have been suggested. The mechanism of its action in creating damage can be ascribed to the production of free radicals and proinflammatory cytokines in consequence of using Mtx (8–10). Mtx administration induces oxidative stress and significantly reduces anti-oxidant enzymes such as superoxide dismutase in the liver and intestinal mucosa tissues of rats. Therefore, several anti-oxidant agents have been used to reduce its side effects (10). Akbulut et al. demonstrated the cytoprotective effects of amifostine, ascorbic acid and N-acetylcysteine against Mtx-induced hepatotoxicity in rats (4). In addition, it has been reported that proinflammatory cytokines are involved in the pathogenesis of Mtx-induced nephrotoxicity and pneumonitis, suggesting that inflammation may also have a possible role in Mtx-induced hepatotoxicity (11–16).

Lycopene (Lyc) is a carotenoid pigment involved in vegetables and fruits, especially in tomato. It is a powerful anti-oxidant, anticancer and anti-inflammatory agent among all dietary carotenoids (17–20). Anti-oxidant and anti-inflammatory properties of Lyc are thought to be primarily responsible for its beneficial effects (21, 22). The fruits and vegetables including lycopene, have a very long history of use in the diet of humans without any safety problems. Additionally, the studies investigating the safety of lycopene support this. The deposition of lycopene in plasma, liver, and other tissues had no adverse effects and no teratogenic effects were observed in rat studies (23–27).

Several anti-oxidant agents have been showed to be effective experimentally by reducing the increased oxidative stress and proinflammatory cytokine levels in liver injury caused by Mtx. However, no study has been performed to evaluate effects of Lyc on hepatic injury by Mtx in rats yet. Therefore, this study

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was carried out to investigate the effects of Lyc on Mtx-induced hepatotoxicity in rats.

Material and methods

Chemicals

Mtx was purchased from a local pharmacy (Koçak™ Farma, Turkey). Lyc (Redivivo Lyc 10 %; CWS/-TG, Basel, Switzerland) is provided by DSM Nutritional Products, Istanbul, Turkey. TNF-α, IL-1β Elisa-kits and oxidative stress kits used for biochemical analysis were obtained from RayBio®, Diaclona® and Rel Assay®, respectively.

Animals

Twenty-eight healthy adult male Sprague-Dawley rats (weighing between 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-h light/12-h dark) in a room with controlled temperature (24 ± 3 °C) 1 week prior to the experimental study. The animals had free access to water and were fed a standard commercial pellet diet *ad libitum*. Dollvet Animal Care and Use Committee has approved the study (approval number: 2014/33). All experimental procedures were conducted in accordance with the Guide to the Care and Use of Laboratory Animals.

Experimental protocol

After seven days of acclimatization, rats were divided into four groups. Each group consists of seven rats according to the following protocol (Table 1):

Control group; Vehicle was administrated to the rats in this group and served as control.

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

Mtx group; Rats were injected intraperitoneally (i.p.) with a single dose of of Mtx (20 mg/kg) (14, 28) and given corn oil by oral gavage.

Mtx-L group; Rats were post-treated with Lyc (10 mg/kg) for ten days after a single dose of Mtx (20 mg/kg).

All rats were sacrificed by terminal anesthesia (ketamine 75 mg/kg (i.p.), xylazine 8 mg/kg (i.p.)) and blood samples were collected at the end of the experiment. Serum samples were separated by centrifugation at 2000 x g for 10 minutes at 25 °C. Livers 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 one was stored at −80 °C for biochemical analysis.

Histopathological examination

Liver tissues removed aseptically from all the groups were cut into small pieces and samples were fixed in 10 % formaldehyde solution. The tissue sections (5 μm) were mounted on glass slides and stained with hematoxylin-eosin (HE) for evaluating the liver structure. Five pieces of pathological sections were prepared from each rat. Each stained section was semi-quantitatively evaluated under light microscope (Olympus BX51 microscope with a magnification of x 200) by a histologist blinded to the treatment group. Previously the scoring system by Akbulut et al. was used to assess the severity of hepatic injury: 1) sinusoidal dilatation; 2) inflammatory cell infiltration; 3) congestion, and 4) hydropic degeneration (cytoplasmic vacuolization/swelling of hepatocyte), with features scored as 0 (normal), 1 (mild), 2 (moderate), and 3 (severe). The maximum score of 12 indicated the most severe hepatic injury (4).

Biochemical analysis

Tissue preparation and homogenization

Before biochemical assays, hepatic tissues were weighed, broken down into very small pieces, and placed in empty glass tubes. 1 ml of 140 mMKCl solution per gram of tissue was added to each tube, and then all tissues were homogenized in a motor-driven homogenizer. The homogenate was centrifuged at 2,800×g for 10 min at 4 °C (29). The resulting supernatant was used for the levels of TAC, TOS, TNF-α and IL-1β.

Alanine aminotransferase (ALT), aspartate aminotransferase (AST)) determination:

Plasma was used to measure AST and ALT as indicative parameters of hepatic function. The plasma activities of AST and

Tab. 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:Corn Oil (o.g.), c:Lycopene (10 mg/kg)(o.g.), d:Mtx (20 mg/kg) (i.p.), e:Euthanized

Tab. 2. Histopathologic examination results in rat liver tissues.

Histopatalogic parameters	Groups			
	Control[x]	Lyc[x]	Mtx[x]	Mtx-L[x]
Sinusoidal dilatation	1.00 (0.0-1.0)	0.00 (0.0-0.0)	2.00 (1.0-3.0)[a]	0.00 (0.0-1.0)[b]
İnflammatory cell infiltration	0.00 (0.0-0.0)	0.00 (0.0-0.0)	1.00 (0.0-1.0)[a]	0.00(0.0-1.0)[b]
Congestion	0.00 (0.0-1.0)	0.00 (0.0-1.0)	1.00 (0.0-2.0)[a]	0.00 (0.0-1.0)[b]
Hydropic degeneration	0.00 (0.0-1.0)	0.00 (0.0-1.0)	1.00 (0.0-3.0)[a]	1.00 (0.0-1.0)

[x]Data were expressed as Median (min-max), [a]Significance of Mtx compared with control, [b]Significance of Mtx-L compared with Mtx, $p < 0.01$ is significant

Tab. 3. Biochemical assessment results in rat liver tissues.

	Groups			
	Control[x] (n=7)	Lyc[x] (n=7)	Mtx[x] (n=7)	Mtx-L[x] (n=7)
TNF-α (pg/g protein)	1820±102	1607±35.9	2412±175[a]	1682±152[b]
IL-1 β (pg/g protein)	31.58±5.59	37.73±5.87	50.88±7.12[a]	35.93±4.27[b]
TAC (mmol TroloxEq/g protein)	0.29±0.02	0.29±0.04	0.37±0.04[a]	0.30±0.04[b]
TOS (μmol H_2O_2 Eq/g protein)	14.69±0.94	13.86±1.77	15.09±2.18	14.83±2.03
OSI(ArbitraryUnits)	5.08±0.52	5.76±1.11	6.06±0.74[a]	4.92±1.12

TNF-α: Tumor Necrosis Factor-alpha, IL-1β: Interleukin-1 beta, TAC (nmolTroloxEqv./mg protein): Total antioxidant capacity, TOS (nmol H_2O_2Eqv./mg protein): Total oxidative status, OSI (Arbitrary Unite): Oxidative stress index, [x]Data represent as Mean± SD, [a]Significance of Mtx compared with control, [b]Significance of Mtx-L compared with Mtx, $p < 0.01$ is significant

ALT were measured by commercially available kits using an autoanalyser (Abbott c16000 ®Abbott Laboratories)

Determination of oxidative stress biomarkers

Measurement of TOS and TAC levels

TOS and TAC levels were calculated by Erel's methods, which are automated and colorimetric (30–32). The percentage of TOS level to TAC level was regarded as oxidative stress index (OSI) (33, 34). The unit of hepatic tissue TOS and TAC was μmole H_2O_2 Equiv./gram protein and millimole TroloxEquiv./gram protein, respectively (34). The hepatic tissue OSI value was calculated as follows: OSI = [(TOS, μmole H_2O_2 Equiv./gram protein)/(TAC, millimole Trolox Equiv./gram protein)×100]. The results were expressed as arbitrary units (AU) (35).

Determination of proinflammatory cytokines

TNF-α (RayBio®) and IL-1β (Diaclone trademark) levels were measured using ELISA kits according to the manufacturer's instructions. TNF-α and IL-1β levels are expressed pg/g protein.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). The Kruskal–Wallis analysis of variance method, followed by the application of the non-parametric Mann–Whitney U-test, with Bonferroni correction for binary comparisons to evaluate differences between the experimental groups. Probability values (P) less than 0.05 were considered statistically significant. All data were processed using the SPSS 16.0 for Windows statistical software package (SPSS Inc., Chicago, IL).

Results

Histopathological results

In group of Mtx, tissue damage was revealed in all histopathological parameters (sinusoidal dilatation, inflammatory cell infiltration, congestion and hydropic degeneration) in liver tissue (Fig.1 IIa, b, c, d).

Liver tissue damage of rats in this group, compared with the liver tissue of those in the control group was statistically significant in all histopathological parameters.

Lyc group compared with control group was not statistically significant in all histopathological parameters (Tab. 1).

The severest tissue damage was in Mtx group, minimal tissue damage was in Lyc group (Tab. 2).

Sinusoidal dilatation, inflammatory cell infiltration and congestion improved significantly in the Mtx-L group compared with Mtx (Fig.1 III, Tab. 2).

Biochemical results

All the biochemical parameters of liver tissue samples except TOS was increased significantly in Mtx group compared with the control group (Tab. 3).

Lyc group compared with control group was not statistically significant in all the biochemical parameters (Tab. 3).

Mtx-L group was Lyc treatment group and Lyc, in this group, provided a significant reduction in proinflammatory cytokines (Tab. 3).

Biochemical results of tissue samples were statistically significant in compared with the treatment results of Mtx-L group (Tab. 3).

In the Mtx group, AST and ALT levels were increased significantly compared with the control group. The treatment that was applied in Mtx-L group reduced AST and ALT values. However, in the ML group, the treatment provided a significant improvement only in AST levels (Tab. 4).

Discussion

The results of this experimental study showed that Lyc reduced the pathological damage, oxidative stress and proinflammatory cytokine levels in rat liver tissues as well as AST and ALT levels in blood serum of rats.

Sinusoidal dilation, inflammatory cell infiltration and congestion improvement was observed significantly in Mtx-L group on histopathologic examination of the rats. In Mtx-L group treated with Lyc, TNF-α and IL-1β levels in liver tissue were decreased significantly compared to Mtx group whereas the decrease in OSI was not significant. Lyc treatment improved the AST and ALT values in Mtx-L group but only the decrease of AST was significant.

Tab. 4. Biochemical assessment results in serum.

	Groups			
	Control[x]	Lyc[x]	Mtx[x]	Mtx-L[x]
AST	64.40±7.92	89.40±30.04	162.80±42.72[a]	134.40±47.70[b]
ALT	39.20±6.31	43.60±15.37	48.40±8.64[a]	34.20±4.38

Hepatic serum aspartatetransaminase (AST), alaninetransaminase (ALT), [x]Data represent as Mean± SD, [a]Significance of Mtx compared with control, [b]Significance of Mtx-L compared with Mtx, $p < 0.01$ is significant

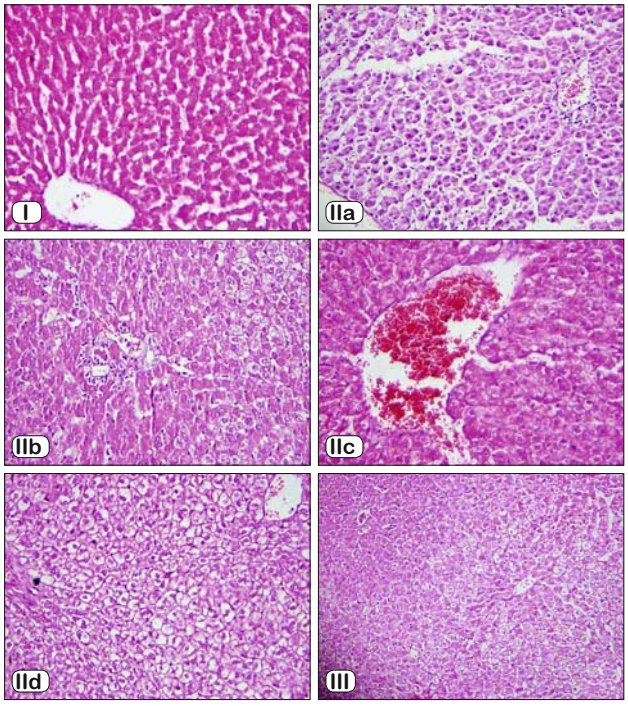

Fig. 1. I: Normal histological appearance of HE-stained control liver tissues. II: Histopathological effects of high-dose methotrexate on liver structure. a) sinusoidal dilatation, b) inflammatory cell infiltration, c) congestion, d) hydropic degeneration. III: The section is showing a significant improvement at sinusoidal dilatation, inflammatory cell infiltration and congestion in the liver tissue of rats in Mtx-L group.

Low dose long-term use and high dose short-term use of Mtx causes the elevation in hepatic serum transaminase levels. More disquieting is its association with changes in liver histopathology, especially with fibrosis and cirrhosis. These indicate liver cellular injury. We measured liver transaminase levels in serum and oxidative stress and pro-inflammatory cytokine levels in liver tissue to determine the hepatotoxicity due to MTx administration. There are several studies indicating that increased oxidative stress and proinflammatory cytokine levels may be effective in the Mtx-induced hepatotoxicity (15, 36, 37).

The pathological examination of the liver of rats in the Mtx group, tissue damage was the highest in comparison to the other groups. The tissue OSI, TNF-α and IL-1β levels and serum AST and ALT levels were significantly higher in this group. The elevation of these parameters suggests that both oxidative stress and increase in proinflammatory cytokines may be involved in the hepatotoxicity caused by Mtx.

Mtx-L were the treatment group in our study. The microscopic examination showed that sinusoidal dilatation, inflammatory cell infiltration and congestion were significantly improved in the Mtx-L group. These findings suggest that Lyc might be effective in the treatment of Mtx-induced hepatotoxicity.

Anti-oxidants have a reducing effect on DNA, protein, lipid and cell membrane damage caused by oxidative stress. Therefore, melatonin, amifostine, ascorbic acid, N-acetylcysteine and resveratrol as antioxidants have been used in hepatotoxicity caused by Mtx and beneficial effects have been identified. These effects were realized by reducing the oxidative stress.

Lyc, a powerful anti-oxidant, has been showed to be effective in reducing the nephrotoxicity caused by cisplatin, via decreasing oxidative stress (4, 38–40). Lyc's also has anti-inflammatory and anti-cancer properties.The studies showed that it may be effective in reducing organ toxicity such as pancreas, testis and kidney (39, 41–43). In this study, we investigated the effects of Lyc on MTX-induced hepatotoxicity. OSI parameters of the liver tissue of the rats were reduced by lyc treatment, but it was not significant. This result make us think that lycopen is not effective in preventing oxidative stress in the Mtx hepatotoxicity at a dose of 10 mg/kg, used in this study.

Cytokines are polypeptide structure molecules secreting against microorganisms and other antigens to regulate immune and inflammatory responses. TNF-α and IL-1β play an active role in the inflammation. TNF-α is produced mainly by activated macrophages. A cytokine involved in systemic inflammation, mediates acute inflammation. Mtx causes an increase in TNF-α40. There are many studies showing that Lyc treatment reduces the increase in TNF-α, one of the proinflammatory cytokines (15, 36, 37, 44). However, to the best of our knowledge there is not any study showing the effect of Lyc on IL-1β levels in liver injury caused by Mtx in rats. This study is the first study demonstrating that Lyc may be effective in reducing IL-1β Levels. In our study, both TNF-α and IL-1β levels were decreased significantly in Mtx-L group compared to Mtx-administrated group.

Conclusion

These results suggest that Lyc may be effective in reducing hepatotoxicity induced by Mtx via decreasing the levels of proinflammatory cytokines, but not OSI, at a dose of 10 mg/kg, used in this study. We think that new studies with different doses of Lyc should be made to reveal the role of Lyc in Mtx-induced hepatotoxicity.

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