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The Effect of Chlorogenic Acid on Methotrexate-Induced Oxidative Stress and Inflammation in Lung Tissue of Rats

Sıçanların Akciğer Dokusunda Metotreksat ile Oluşturulan Oksidatif Stres ve İnflamasyon Üzerine Klorojenik Asitin Etkisi

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ABSTRACT

Although methotrexate (MTX) is a widely used chemotherapeutic agent, lung toxicity remains a significant problem, limiting its use. The molecular mechanism of MTX-related lung toxicity is not fully understood. However, increased reactive oxygen species-induced oxidative stress (OS) and inflammation play an important role in lung injury. Chlorogenic acid (CHA) is a natural phenolic compound that has been shown in recent years to have beneficial effects in many pathologies associated with OS and inflammation. This study focused on investigating for the first time, the potential therapeutic effects of CHA in the lung tissue of rats exposed to MTX. After lung toxicity was induced in rats by MTX (20 mg/kg) injection on the first day, two different doses of CHA (1.5 and 3 mg/kg) were used for treatment for 3 days. The results showed that CHA treatment reduced the level of pulmonary lipid peroxidation, inflammation and apoptosis and promoted the pulmonary antioxidant system in rats subjected to MTX. Taken together, the antioxidant and anti-inflammatory properties of CHA may play a central role in attenuating MTX-induced lung injury, but the exact mechanism needs to be investigated in more extensive preclinical studies.

Keywords: Apoptosis, Chlorogenic acid, Inflammation, Lung toxicity, Methotrexate, Oxidative stress

ÖZET

Metotreksat (MTX) yaygın olarak kullanılan bir kemoterapötik ajan olmasına rağmen, akciğer toksisitesi önemli bir sorun olmaya devam etmekte ve ilacın kullanımını sınırlamaktadır. MTX'e bağlı akciğer toksisitesinin moleküler mekanizması tam olarak anlaşılamamıştır. Ancak artan reaktif oksijen türlerinin neden olduğu oksidatif stres (OS) ve inflamasyon, akciğer hasarında önemli bir rol oynamaktadır. Klorojenik asit (CHA), son yıllarda OS ve inflamasyon ile ilişkili birçok patolojide yararlı etkilere sahip olduğu gösterilen doğal bir fenolik bileşiktir. Bu çalışma, MTX'e maruz bırakılan sıçanların akciğer dokusunda CHA'nın potansiyel terapötik etkilerinin ilk kez araştırılmasına odaklandı. Sıçanlarda ilk gün MTX (20 mg/kg) enjeksiyonu ile akciğer toksisitesi oluşturulduktan sonra, 3 gün boyunca iki farklı dozda CHA (1.5 ve 3 mg/kg) ile tedavi uygulandı. Sonuçlar, CHA tedavisinin, MTX'e maruz bırakılan sıçanlarda pulmoner lipid peroksidasyon, inflamasyon ve apoptoz seviyesini azalttığını ve pulmoner antioksidan sistemi desteklediğini gösterdi. Birlikte ele alındığında, CHA'nın antioksidan ve anti-inflamatuar özellikleri MTX'in neden olduğu akciğer hasarını hafifletmede merkezi bir rol oynayabilir, ancak kesin mekanizmanın daha kapsamlı klinik öncesi çalışmalarla araştırılması gerekmektedir.

Anahtar Kelimeler: Akciğer toksisitesi, Apoptoz, İnflamasyon, Klorojenik asit, Metotreksat, Oksidatif stres

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INTRODUCTION

Chemotherapy is a method that aims to eliminate cancer cells while causing minimal damage to healthy cells and used to treat many cancers today.[1] Methotrexate (MTX) is a folate inhibitor that is employed in the treatment of ectopic pregnancy and rheumatoid diseases at low doses and in cancer chemotherapy at high doses.[2] The anticancer effect of MTX is attributed to its capacity to inhibit thymidylate synthesis by suppressing the dihydrofolate reductase enzyme.[3] Nevertheless, the advent of adverse effects, including nephro-, haematological- and lung toxicity, particularly in the context of cancer therapy, has constrained the clinical utilisation of this treatment.[2,4] A significant proportion of patients undergoing MTX therapy experience respiratory complications, including cough, wheezing, shortness of breath and other respiratory problems.[5] Long-term MTX use can result in the development of lung toxicity, which can manifest as fibrosis, interstitial pneumonia and even serious alveolar damage.[2,6] The aetiology of MTX-induced tissue damage is underpinned by the presence of oxidative stress (OS) and inflammation, which are caused by an increased amount of reactive oxygen species (ROS).[7-9] Glutathione (GSH) is the most important cellular antioxidant molecule, and the reducing power of NADPH is indispensable in the reduction of oxidised GSH.[9] MTX inhibits the pentose phosphate pathway, which is responsible for the production of NADPH, resulting in a depletion of the cell's GSH pool.[10] MTX also induces the production of ROS.[11] MTX exposure results in OS through the concurrent elevation of ROS production and inhibition of the antioxidant system regeneration. Consequently, OS results in the damage of lipids, proteins and DNA.[9] In general, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are considered to be pro-inflammatory cytokines that regulate inflammatory responses.[12] MTX is a known cause of inflammation, with a particular effect of increasing TNF-α concentration.[13] Chronic inflammation has been shown to further exacerbate OS and apoptosis over time.[14] Therefore, it is of paramount importance to identify molecules that can prevent the lung injury induced by MTX.[15-17]

Epidemiological studies have shown that a diet rich in natural products promotes good health and helps in the prevention and treatment of a variety of diseases.[18] Chlorogenic acid (CHA) is a dietary polyphenol found in many natural products, including coffee, apple, pear, strawberry and grape.[19,20] A series of experimental and clinical studies have demonstrated that CHA can exert beneficial biological effects on a number of different systems, including the nervous, cardiovascular, gastrointestinal, respiratory and reproductive systems.[20,21] CHA exhibits a multitude of functions, including neuroprotective, antidiabetic, anti-inflammatory, antioxidant, antimicrobial, cardioprotective and antitumor activities.[22] The lack of adverse effects on healthy tissues and the favourable tolerability profile in experimental animals and humans have led to an increase in the number of studies investigating the beneficial activities of CHA on an annual basis.[22,23] Prior investigations have substantiated the therapeutic and/or protective efficacy of CHA against lung injury resulting from lipopolysaccharide (LPS)[24], paraquat[25] and LPS+polyinosinic/polycytidylic acid[26] exposure. Nevertheless, to the best of our knowledge, there are no reports that evaluate the potential beneficial effects of CHA against MTX-associated lung injury. This study represents the first evaluation of the therapeutic effectiveness of CHA in an acute model of MTX-related lung toxicity in rats.

METHODS

Animals

This study was conducted on 9-week-old female Sprague-Dawley rats that were housed in an air-conditioned room (temperature 23±2ºC), maintained on a 12-h light/dark cycle and in laboratory cages, with unrestricted access to pellet feed and tap water *ad libitum*.

Experimental design and treatments

The protocol was approved by the Local Animal Ethics Committee of Karadeniz Technical University (Protocol Number: 2023/08). Following a five-day acclimation period, 30 animals were divided into five groups (six subjects in each group). The groups were designated as follows: Control, MTX, MTX+CHA (1.5 and 3 mg/kg) and CHA only (3 mg/kg). Lung toxicity was induced by a MTX injection in first day of experiment. During the subsequent three days, the MTX group received saline injections. In order to investigate the effectiveness of CHA, two different doses of CHA (1.5 and 3 mg/kg) were administered intraperitoneally to the rats for three days. The doses of CHA[27-29] and MTX[30-32] used in the study were determined based on previous experimental

studies. On the 5th day, all rats were sacrificed by cervical dislocation and the removed lung tissues were stored at -80ºC for biochemical analysis.

Biochemical assessment

Following homogenisation of the lung tissues in ice-cold phosphate buffered saline and centrifugation, protein determination of the resulting supernatants was conducted via the bicinchoninic acid method.[33] Lipid peroxidation levels were quantified in accordance with the previously described methodology for the measurement of malondialdehyde (MDA).[34] Briefly, the supernatants were mixed with 3 mL of 1% phosphoric acid and 1 mL of 0.672% thiobarbituric acid, then placed in a boiling water bath for 1 hour. Following this period, the tubes were cooled and centrifuged at 1800xg for 10 minutes. Two hundred microlitres of each supernatant was transferred to a 96-well plate and the absorbances were read at 532 nm using a microplate reader (Versamax, Molecular Devices, CA, USA). The standard employed was 1,1,3,3-tetramethoxypropane, with the resulting tissue MDA levels expressed as nmol/mg protein.[35]

The total antioxidant status (TAS) and the total oxidant status (TOS) of lung tissue were quantified using commercially available kits (Rel Assay Kit Diagnostics, Gaziantep, Turkey). Briefly, in the TOS measurement, firstly 45 µL of supernatant was mixed with 300 µL of Reagent 1 (25 mM H_2SO_4) and after 30 seconds the initial absorbance was measured at 530 nm using a spectrophotometer. Then, 15 µL of Reagent 2, which contains o-dianisidine and ferrous ion, were added to the mixture. After 5 minutes of incubation at 37ºC, the final absorbance was measured at 530 nm. 10 mmol/L H_2O_2 was used as the standard. The TOS levels of the samples were calculated using the following formula:

$$\text{The TOS level of sample} = (\Delta\text{Abs sample} / (\Delta\text{Abs standard}) \times 10$$

Briefly, in the TAS measurement, firstly 18 µL of supernatant was mixed with 300 µL of Reagent 1 (0.4 mol/L acetate buffer) and after 30 seconds the initial absorbance was measured at 660 nm using a spectrophotometer. Then, 45 µL of Reagent 2, which contains 30 mmol/L ABTS, were added to the mixture. After 5 minutes of incubation at 37ºC, the final absorbance was measured at 660 nm. 1 mmol/L trolox was used as the standard. Distilled water was used as the blank. The TAS levels of the samples were calculated using the following formula:

$$\text{The TAS level of sample} = (\Delta\text{Abs blank} - \Delta\text{Abs sample}) / (\Delta\text{Abs blank} - \Delta\text{Abs standard})$$

The OS index (OSI) was calculated using the following formula, based on the TOS and TAS values obtained for the samples:[36]

$$\text{OSI} = (\text{TOS/TAS}) \times 100$$

In the supernatants, the levels of superoxide dismutase (SOD) as an antioxidant enzyme[37], TNF-α as an inflammatory cytokine[38] and caspase-3 (CASP3) as an apoptosis marker[39] were determined using commercial enzyme-linked immunosorbent assay kits (BT LAB, Zhejiang, China) in accordance with the manufacturer's recommendations.

Statistical analysis

Each result is expressed as the mean±SEM. The statistical significance of the observed differences between the groups was evaluated by one-way ANOVA and Tukey's post-hoc test. A p-value of less than 0.05 was considered to be statistically significant.

RESULTS

The effects of CHA on OS biomarkers

The changes of tissue OS parameters were presented in Table 1. The levels of tissue MDA, TOS and OSI were increased in the MTX group compared with control group, whereas CHA treatments demonstrated a dose-dependent reduction in MDA, TOS and OSI levels.

The antioxidant capacity of lung tissue in rats treated with MTX was evaluated using TAS and SOD parameters. MTX diminished a significant reduction in the levels of lung TAS and SOD. In contrast, the administration of CHA exhibited a dose-dependent improvement in TAS and SOD levels.

The effects of CHA on inflammation and apoptosis biomarkers

The levels of inflammation and apoptosis in the lung tissue were evaluated using two distinct parameters: TNF-α and CASP3, respectively (Figure 1). MTX triggered a significant elevation in the levels of lung TNF-α and CASP3. Conversely, CHA treatment (in particular at the dose of 3 mg/kg) largely abolished inflammatory and apoptotic processes.

Table 1. Effects of CHA on the levels of OS markers in the lungs of rats treated with MTX

	Control	MTX (20 mg/kg)	MTX+CHA (1.5 mg/kg)	MTX+CHA (3 mg/kg)	CHA (3 mg/kg)
MDA (nmol/mg protein)	10.55±0.70	26.04±3.05***	13.73±1.28$^{\#\#\#}$	10.17±0.33$^{\#\#\#}$	10.37±0.61
TOS (µmol H_2O_2 equivalent/L)	19.48±0.52	37.23±5.02**	31.83±3.65*	22.14±0.65$^{\#\#}$	20.79±1.30
TAS (mmol trolox equivalent/L)	7.81±0.64	2.55±0.41***	4.85±0.39$^{**,\ \#\#}$	7.84±0.33$^{\#\#\#,+++}$	7.93±0.33
OSI (arbitrary unit)	0.26±0.02	1.58±0.31***	0.66±0.06$^{\#\#}$	0.28±0.01$^{\#\#\#}$	0.27±0.02
SOD (ng/mg protein)	0.45±0.04	0.20±0.02*	0.32±0.04	0.46±0.02$^{\#}$	0.59±0.09

MTX: methotrexate, CHA: chlorogenic acid, MDA: malondialdehyde, TOS: total oxidant status, TAS: total antioxidant status, OSI: oxidative stress index, SOD: superoxide dismutase.

P-values according to one-way ANOVA test, post-hoc Tukey test. Data were expressed as mean±SEM.

Compared with control group *p<0.05, **p<0.01 and ***p<0.001.

Compared with MTX group $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001.

Compared with MTX+CHA (1.5 mg/kg) group $^{+++}$p<0.001.

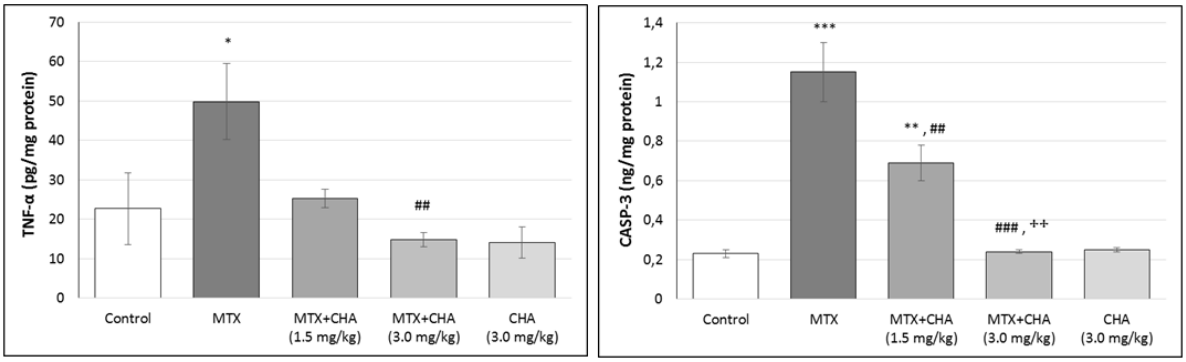

Figure 1. Effects of CHA on the levels of inflammatory and apoptosis markers in the lungs of rats treated with MTX

MTX: methotrexate, CHA: chlorogenic acid, TNF-α: tumor necrosis factor-alpha, CASP3: caspase-3.
P-values according to one-way ANOVA test, post-hoc Tukey test. Data were expressed as mean±SEM.
Compared with control group *p<0.05, **p<0.01 and ***p<0.001.
Compared with MTX group $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001.
Compared with MTX+CHA (1.5 mg/kg) group $^{++}$p<0.01.

DISCUSSION

The current study aimed to determine for the first time the therapeutic effect of CHA on MTX-associated lung injury. MTX was administered intraperitoneally at a dose of 20 mg/kg in a single injection, which is a frequently used dose in the literature to induce lung toxicity.[30-32] Subsequently, the rats were administered two distinct doses of CHA for a period of three days.[29,40] The results demonstrated that MTX induced OS, inflammation, and apoptosis in lung tissue, while CHA treatment mitigated this damage.

Although the precise mechanism by which MTX induces tissue toxicity remains elusive, one of the most compelling theories is that it results from increased OS, characterised by elevated levels of ROS and a deficiency in the antioxidant system.[1,8] As a consequence of elevated ROS attack, membrane phospholipids are predominantly oxidised, resulting in elevated MDA levels, a by-product of this oxidation.[1,29] The TOS, TAS and OSI have been employed with considerable frequency in recent years as straightforward and useful parameters for evaluating the overall OS degree in a biological sample.[41] SOD is an important cytoprotective enzyme that prevents the conversion of superoxide radicals to hydroxyl radicals.[37,42] Consistent with previous reports[43-45], it was determined that only MTX administration increased lipid peroxidation in the lung tissue of rats and caused OS by suppressing the antioxidant system. Conversely, the administration of CHA following MTX application has been observed to enhance the antioxidant system and to reduce OS levels dose-dependently. In a similar vein with our results, Vardi *et al.* have reported that CHA has the potential to exert a protective effect on the

cerebellum by suppressing MDA levels while promoting the levels of catalase (CAT), SOD and GSH in an experimental model of MTX-induced cerebellar injury.[18] In an another experimental approach, Ali *et al.* reported that CHA can suppress levels of lipid peroxidation and protect liver tissue from apoptosis by supporting antioxidant system elements, including glutathione reductase, glutathione peroxidase, SOD, CAT and GSH, in an MTX-induced liver injury model.[1] In addition to the aforementioned findings, it has been demonstrated that CHA can also mitigate oxidative tissue damage in experimental models of triptolide-induced hepatotoxicity[46], 5-fluorouracil-induced ovotoxicity[40] and testicular ischemia/reperfusion injury[47]. The powerful antioxidant activity of CHA is due to its hydroxyl, carboxyl and *o*-diphenol hydroxyl groups, which help it to donate electrons and quench free radical.[22] The reduction in OS levels following the administration of MTX and concurrent treatment with CHA may be attributed to the *in vivo* antioxidant activity of CHA.

In addition, the mechanisms of inflammation and inflammation-induced apoptosis have been proposed as other important mechanisms for MTX-associated tissue damage.[45,48] Although inflammation is an acute adaptive response of the body to an invading attack, in the process of chronic inflammation, the sustained production of TNF-α increases tissue destruction, resulting in the activation of CASP3.[49] CASP3 activation is the main indicator that a cell has entered the irreversible apoptotic process.[50] In addition, high levels of MDA and TNF-α can induce apoptosis by activating CASP3.[45] Consistent with previous reports[15,50], it was determined that only MTX administration increased inflammation and apoptosis levels in the lung tissue. Conversely, the administration of CHA following MTX has been observed to suppress the inflammation and apoptosis levels dose-dependently. In parallel with our findings, Xu *et al.* have demonstrated that CHA exhibit a hepatoprotective effect by inhibiting the nuclear factor-kappa B (NF-κB) pathway and reducing the levels of toll like receptor 4 (TLR4), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and TNF-α in an experimental liver injury model induced by LPS.[51] In another experimental study, Ji *et al.* demonstrated that CHA can suppress inflammation-induced liver apoptosis by inhibiting the extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (p38 MAPK) pathways in acetaminophen-induced liver injury.[52] The reduction in the levels of inflammation and inflammation-induced apoptosis observed following the administration of MTX in conjunction with CHA treatment may be attributed to the *in vivo* anti-inflammatory activity of CHA. In support of this hypothesis, the anti-inflammatory activity of CHA is attributed to its capacity to inhibit the synthesis and secretion of pro-inflammatory mediators, including TNF-α and IL-6.[22] A various of studies have indicated that CHA exerts a suppressive effect on a multitude of inflammatory signalling pathways, including the Janus kinase/signal transduction and transcription activation (JAK/STAT), NF-κB and NLR family pyrin domain containing 3 (NLRP3) inflammasome pathways. Furthermore, it has been demonstrated that CHA have the capacity to activate nuclear factor-erythroid 2 related factor 2 (Nrf2) signalling, which plays a pivotal role in regulating antioxidant and anti-inflammatory mechanisms.[20,22] The most significant limitation of this study is that the signalling pathways pertaining to OS and inflammation could not be incorporated into the research design. It would be beneficial for future studies to elucidate the therapeutic effect of CHA against MTX-induced lung injury through the inclusion of more comprehensive molecular studies.

CONCLUSION

This study revealed for the first time the therapeutic effects of CHA in MTX-induced lung toxicity. The therapeutic activity of CHA may be attributed to a combination of its antioxidant and anti-inflammatory properties. Nevertheless, further molecular studies will be beneficial in elucidating the precise mechanism underlying the lung-protective effects of CHA.

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Authorship contribution statement

Consept and desing: AM and SD.
Acquisition of data: AM, SD, NTA and EAD.
Analysis and interpretation of data: AM, SD, NTA, EAD and YA.
Drafting of the manuscript: AM and SD.
Critical revision of the manuscript for important intellectual content: YA.
Statistical analysis: AM.

Declaration of competing interest

None of the authors have potential conflicts of interest to be disclosed.

Ethical approval

This study was approved by the Local Animal Research Ethics Committee of Karadeniz Technical University (Protocol no: 2023/08) and performed according to the animal research reporting of *in vivo* experiments (ARRIVE) guidelines.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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