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Gallic Acid Alleviates Methotrexate-Induced Oxidative Ovarian Damage in Rats

Gallik Asit Sıçanlarda Metotreksatın Neden Olduğu Oksidatif Yumurtalık Hasarını Azaltır

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ABSTRACT

Although methotrexate (MTX) is an effective chemotherapeutic agent in the treatment of cancer, its use is limited due to the occurrence of systemic tissue toxicity, including those affecting the reproductive system. Gallic acid (GAL) is a phenolic compound that has been demonstrated to exert beneficial effects in a number of pathological conditions associated with oxidative stress (OS) in recent years. This study was designed to investigate the potential therapeutic benefits of GAL in the treatment of MTX-induced ovarian damage, for the first time. Adult female rats (n=30) were randomly allocated to five groups: control, MTX, MTX+GAL (2.5 and 5 mg/kg) and high-dose GAL only (5 mg/kg). A single intraperitoneal injection of MTX (20 mg/kg) was administered to induce ovarian toxicity. The treatment groups were administered 2.5 and 5 mg/kg of GAL intraperitoneally for a period of three consecutive days. The levels of OS, inflammation and apoptosis were determined in ovarian tissue samples collected on the fifth day of the study using spectrophotometric methods. The results showed that GAL treatment reduced the level of ovarian lipid peroxidation, inflammation, and apoptosis and promoted the ovarian antioxidant system in rats subjected to MTX. The results of this study indicate that GAL may have the potential to ameliorate MTX-associated oxidative and inflammatory ovarian damage. The ovarian protective effect of GAL requires further confirmation through more extensive preclinical studies.

Keywords: Apoptosis, Gallic acid, Inflammation, Methotrexate, Ovarian damage, Oxidative stress, Rat model

ÖZET

Metotreksat (MTX) kanser tedavisinde etkili bir kemoterapötik ajan olmasına rağmen, üreme sistemi de dahil olmak üzere sistemik doku toksisitesinin ortaya çıkması nedeniyle kullanımı sınırlanmaktadır. Gallik asit (GAL), son yıllarda oksidatif stres (OS) ile ilişkili çeşitli patolojik durumlara karşı faydalı etkiler gösterdiği kanıtlanmış bir fenolik bileşiktir. Bu çalışma, MTX'in neden olduğu yumurtalık hasarının tedavisinde GAL'ın terapötik potansiyelini ilk kez araştırmak için tasarlandı. Yetişkin dişi sıçanlar (n=30) rastgele beş gruba ayrıldı: kontrol, MTX, MTX+GAL (2,5 ve 5 mg/kg) ve yalnızca yüksek doz GAL (5 mg/kg). Yumurtalık toksisitesi tek doz intraperitoneal MTX (20 mg/kg) enjeksiyonu ile oluşturuldu. Tedavi gruplarına ise 2,5 ve 5 mg/kg dozundaki GAL ardışık üç gün boyunca intraperitoneal yoldan uygulandı. Çalışmanın beşinci gününde toplanan yumurtalık doku örneklerinde OS, inflamasyon ve apoptoz seviyeleri spektrofotometrik yöntemler kullanılarak belirlendi. Bulgular, GAL tedavisinin, MTX'e maruz bırakılan sıçanlarda yumurtalık lipid peroksidasyon, inflamasyon ve apoptoz seviyesini azalttığını ve yumurtalık antioksidan sistemini desteklediğini gösterdi. Bu çalışmanın sonuçları, GAL'ın MTX ile ilişkili oksidatif ve inflamatuar yumurtalık hasarını iyileştirme potansiyeline sahip olabileceğini göstermektedir. GAL'ın bu yumurtalık koruyucu etkisinin daha kapsamlı klinik öncesi çalışmalarla doğrulanması gerekmektedir.

Anahtar Kelimeler: Apoptoz, Gallik asit, İnflamasyon, Metotreksat, Oksidatif stres, Rat modeli, Yumurtalık hasarı

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INTRODUCTION

Methotrexate (MTX) is an anti-folic acid compound employed for the treatment of ectopic pregnancy, autoimmune and malignant diseases, due to its inhibitory activity against dihydrofolate reductase.[1,2] This inhibitory feature affects one-carbon metabolism, which in turn disrupts nucleotide acid synthesis and amino acid metabolism, thereby exerting an anticancer effect.[3,4] MTX is employed in high doses in the treatment of cancer, and has the capacity to negatively affect rapidly dividing cells, including trophoblast and gonadal cells.[5] The toxicity of systemic MTX administration to healthy tissues, including ovaries, represents a significant limitation to its use and a source of concern in young female patients.[3,6] MTX has been documented to exert gonadotoxic effects on ovarian tissue, resulting in a reduction in the number of ovarian follicles.[7-9] It is well documented that high doses of MTX used in cancer treatment can cause early menopause[10], and one study of breast cancer patients undergoing chemotherapy even found that 68% of those treated with MTX experienced menopause.[11] It is proposed that the elevation in reactive oxygen species (ROS) and pro-inflammatory molecules may be a significant contributing factor in the development of MTX-induced tissue toxicity.[4,12-15] Nicotinamide adenine dinucleotide phosphate (NADPH), produced by the pentose phosphate pathway (PPP) and malic enzyme, plays a pivotal role in anabolic reactions and the regeneration of oxidized glutathione (GSSG) back to reduced glutathione (GSH).[16] It is well established that MTX inhibits glucose-6-phosphate dehydrogenase (G6PD), the control enzyme of the PPP. This results in a reduction in the quantity of NADPH, which possesses reducing power, and thus a depletion of GSH, the most significant endogenous antioxidant molecule.[7,17,18] MTX induces oxidative stress (OS) in cells by two mechanisms: firstly by depleting the GSH pool, and secondly by directly increasing the amount of ROS.[19] Consequently, OS results in the damage of lipids, proteins and DNA.[20] In general, tumor necrosis factor-alpha (TNF-α) is considered to be one of the pro-inflammatory cytokines that regulate inflammatory responses.[21] MTX is a known cause of inflammation, with a particular effect of increasing TNF-α concentration.[14,15] Chronic inflammation has been shown to further exacerbate OS and apoptosis over time.[22] Consequently, it is a reasonable approach to assess the efficacy of antioxidant molecules in counteracting the toxicity of MTX, which is exacerbated by elevated OS and inflammation.[6,14]

Gallic acid (GAL) is a trihydroxybenzoic acid with hydroxy groups in the 3, 4 and 5 positions. It is currently employed to a significant extent in the cosmetic, food and dyeing industries.[23] It can be found in a multitude of commonly consumed plants and fruits.[24] A series of experimental and clinical investigations have revealed that GAL can exert advantageous biological effects on a number of disparate physiological systems, including the cardiovascular, central nervous, gastrointestinal and reproductive systems.[23,24] Previous research has shown that GAL has therapeutic and/or protective effects against ovarian damage caused by exposure to cisplatin, letrozole and doxorubicin.[20,25,26] However, to date, there have been no reports that have evaluated the potential beneficial effects of GAL in the context of MTX-associated ovarian damage. This experimental study aimed to elucidate the effects of GAL treatment on MTX-induced ovarian OS, inflammation and apoptosis, for the first time.

METHODS

Animals

A total of 30 healthy adult female Sprague-Dawley rats (weighing approximately 195±5 g) were utilised in this study. The animals were housed under standard laboratory conditions (12 h light/dark cycle and 22±2°C) with *ad libitum* access to pellet feed and tap water.

Experimental protocol

The protocol was approved by the Local Animal Ethics Committee of Karadeniz Technical University (Protocol Number: 2023/07). Vaginal smears were conducted on a daily basis, and following three consecutive cycles, rats exhibiting normal estrous cycles of 4-5 days were included in the experiments.[7] The 30 animals were divided into five groups (six subjects in each group): Control, MTX, MTX+GAL (2.5 and 5 mg/kg) and GAL only (5 mg/kg). The control group was administered an intraperitoneal injection of physiological saline solution for a period of four days. The MTX group was administered an intraperitoneal MTX (20 mg/kg) injection on the first day, followed by an intraperitoneal physiological saline solution injection for the subsequent three days. The GAL treatment groups were administered an intraperitoneal MTX injection at a dose of 20 mg/kg on the first day. This was followed by two different doses of GAL (2.5 and 5 mg/kg)

intraperitoneal injection over the subsequent three days. The GAL *per se* group was administered an intraperitoneal physiological saline solution injection on the first day, followed by an intraperitoneal high-dose GAL (5 mg/kg) injection for the subsequent three days. GAL (Cat no: G7384, purity ≥98.5%) and MTX (Cat no: 454126, purity ≥98%) were purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA) and dissolved in physiological saline solution and administered to rats. The doses of GAL[20,27] and MTX[3,6,15] were determined based on previous experimental studies. On the morning of the fifth day of the study, cervical dislocation and subsequent oophorectomy were performed on the rats under general anesthesia. The excised tissues were subsequently stored at -80ºC for subsequent biochemical analyses.

Biochemical analysis

The ovary samples were homogenised in phosphate buffer solution (1:10 w/v, pH 7.4) using a homogeniser (IKA, T25 Ultra-Turrax, Staufen, Germany) with cooled tubes within ice. The resulting homogenates were then subjected to centrifugation at 1800xg for a period of 15 min at a temperature of 4°C, after which the supernatants were separated for further analysis. The protein content of supernatants was quantified using the bicinchoninic acid assay[28], and the supernatants were subsequently employed in subsequent analyses. The degree of tissue lipid peroxidation (LPO) was determined by the previously described spectrophotometric measurement of malondialdehyde (MDA), a LPO end product.[29] The absorbance of the pink complex formed by MDA and thiobarbituric acid, which is indicative of LPO, was measured at 532 nm. The standard employed was 1,1,3,3-tetramethoxypropane, with the results expressed in nanomolar per milligram of protein.[30]

The total antioxidant status (TAS) and the total oxidant status in ovarian tissue were quantified using commercially available kits (Rel Assay Kit Diagnostics, Gaziantep, Turkey) and the OS index (OSI) was calculated.[31] In the supernatants, the levels of superoxide dismutase (SOD) as an antioxidant enzyme[21], TNF-α as an inflammatory cytokine[13] and caspase-3 (CASP3) as an apoptosis marker[20] were determined using commercial assay kits (BT LAB, Zhejiang, China). The measurements were conducted in accordance with the manufacturer's instructions, and the intra-assay CV% values were found to be less than 8% in all three analyses.

Statistical analysis

The data are presented as the arithmetic mean ± standard error of the mean (SEM). The statistical analysis of the data was conducted using ANOVA, with Tukey's test employed for comparisons between groups. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Effects of GAL treatments on ovarian OS levels induced by MTX

As illustrated in Table 1, treatment with MTX resulted in a significant elevation of MDA, TOS and OSI levels and a reduction of SOD and TAS levels in comparison to the control group. Nevertheless, three-day treatments with GAL following MTX demonstrated a dose-dependent improvement in OS parameters, with a concomitant strengthening of the antioxidant system.

Table 1. Effects of GAL on OS biomarkers in MTX-induced ovarian injury

	Control	MTX (20 mg/kg)	MTX+GAL (2.5 mg/kg)	MTX+GAL (5 mg/kg)	GAL (5 mg/kg)
MDA (nmol/mg protein)	47.50±7.63	95.04±6.28***	64.11±6.29$^{\#}$	50.28±2.45$^{\#\#\#}$	54.73±7.15
TOS (μM H_2O_2 equivalent/L)	16.60±3.47	30.99±2.04**	25.00±2.83	19.54±1.68$^{\#}$	14.32±1.47
TAS (mM trolox equivalent/L)	2.52±0.09	0.71±0.12***	1.69±0.16$^{**,\#\#\#}$	2.04±0.17$^{\#\#\#}$	2.43±0.14
OSI (arbitrary unit)	0.66±0.13	5.75±1.81**	1.59±0.28$^{\#}$	1.00±0.15$^{\#\#}$	0.61±0.08
SOD (ng/mg protein)	1.82±0.38	0.84±0.05*	1.20±0.16	1.51±0.11$^{\#}$	2.11±0.31

MTX: methotrexate, GAL: gallic 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.

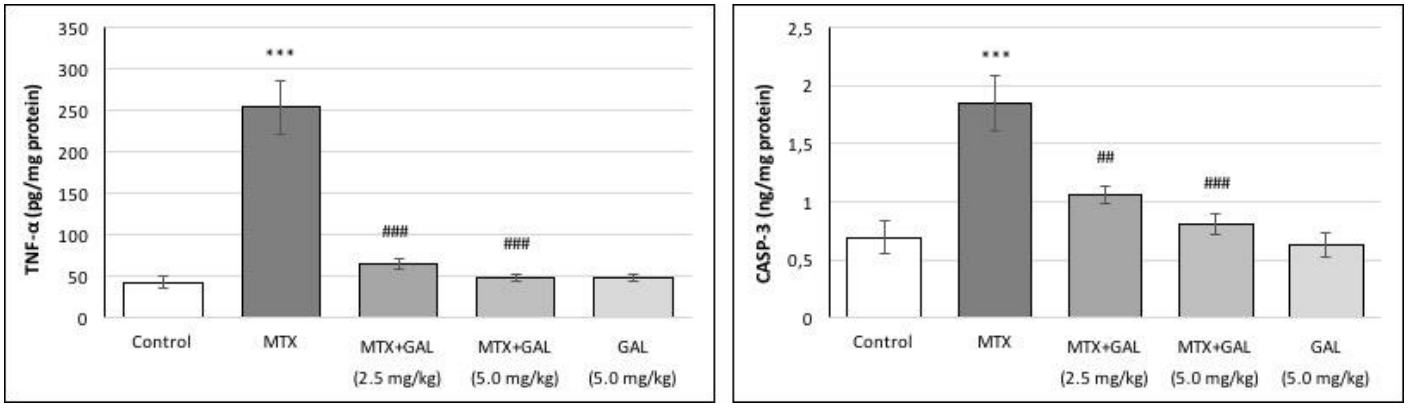

Figure 1. Effects of GAL on inflammatory and apoptosis biomarkers in MTX-induced ovarian injury
MTX: methotrexate, GAL: gallic acid, TNF-α: tumor necrosis factor-alpha, CASP3: caspase-3.
P-values according to one-way ANOVA test, post-hoc Tukey test. Data were expressed as mean±SEM.
Compared with control group ***p<0.001,
Compared with MTX group ##p<0.01 and ###p<0.001.

Effects of GAL treatments on ovarian inflammation and apoptosis levels induced by MTX

As illustrated in Figure 1, MTX treatment led to a notable elevation in TNF-α and CASP3 levels in ovarian tissue in comparison to the control group. Nevertheless, the administration of GAL following MTX administration resulted in a significant reduction in TNF-α and CASP3 levels in a dose-dependent manner.

DISCUSSION

Although MTX is one of several chemotherapeutics that can be employed alone or in combination with other drugs in clinical practice, the primary concern of young female patients undergoing MTX therapy is the potential impact of treatment on future fertility.[6] Although low-dose MTX is not considered to be relatively gonadotoxic in the context of autoimmune disorders[32,33], experimental studies have demonstrated that high-dose MTX used in chemotherapy harms ovarian tissue.[4,7-9] This study was therefore conducted to investigate the therapeutic effects of GAL on MTX-induced ovarian damage for the first time. In order to fulfill these objectives, a model was devised for the study of ovarian toxicity. In it, MTX was administered in a dose of 20 mg/kg[3,6,15], and the levels of biochemical biomarkers were subsequently determined in tissue samples taken three days after the commencement of the GAL treatments.[20] The results of the study demonstrated that the established hypothesis was indeed correct. Specifically, the findings disclosed that MTX administration led to an increase in OS, inflammation and apoptosis levels. Furthermore, the findings also disclosed that GAL treatments significantly alleviated MTX toxicity.

Although the precise molecular mechanism of MTX-induced toxicity remains unclear, OS and chronic inflammation resulting from the depletion of the antioxidant system are identified as the primary drivers of tissue damage.[4,12,14,15] The OS is defined as an imbalance between the amount of oxidants and the antioxidant system capacity.[15] Increased ROS attacks membrane lipids, resulting in LPO. Consequently, the level of reactive aldehyde derivatives, such as MDA, is elevated as a consequence of the chain reactions of LPO.[34] MDA is a highly reactive molecule that not only inhibits enzymes but also damages membrane integrity.[35] 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.[14] It has been demonstrated that elevated OS can precipitate infertility by inducing aberrations in oocyte development.[8,14] Our study, which was in accordance with previous experimental literature, demonstrated that MTX application led to an increase in OS, which was attributed to a depletion of the antioxidants.[4,6,12,14,15,36] It is established that MTX reduces the intracellular concentration of NADPH by inhibiting G6PD and malic enzyme.[37] As a consequence of the reduction in intracellular NADPH, the regeneration of GSH is impaired, thereby increasing the levels of OS within the cells. It is highly probable that the elevated MDA, TOS and OSI levels and the reduced TAS level observed in the MTX-treated group were a consequence of GSH depletion in the tissue.[14,18] Nevertheless, the administration of GAL treatments subsequent to MTX was found to alleviate ovarian OS by supporting the levels of TAS and SOD. The reduction in OS levels following the administration of MTX and concurrent treatment with GAL may be attributed to the *in vivo* antioxidant activity of GAL. In accordance with our findings, it has been demonstrated that GAL can exert protective and/or therapeutic effects by supporting the

antioxidant system and quenching LPO in a range of experimental models.[38-41]

In addition to the previously discussed mechanisms, inflammation and apoptosis have been proposed as other potential mechanisms for MTX-induced tissue damage.[14,15,18] MTX stimulates neutrophils, resulting in an increase in the amount of hydrogen peroxide, which in turn leads to an elevation in the level of ROS. This process ultimately culminates in the induction of cellular damage.[42] Furthermore, MTX also increases the concentration of pro-inflammatory cytokines, including TNF-α, by activating the nuclear factor kappa B (NF-κB) pathway.[12] TNF-α is a crucial pro-inflammatory cytokine that plays a pivotal role in numerous inflammatory processes.[14] 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.[43] In accordance with previous experimental literature, our findings demonstrated that MTX application resulted in elevated levels of inflammation and apoptosis in ovarian tissue.[6,14,36,44] Conversely, the administrations of GAL following MTX has been observed to suppress the inflammation and apoptosis levels dose-dependently. In accordance with our findings, it has been demonstrated that GAL can exert protective and/or therapeutic effects by inhibiting inflammation and apoptosis in a range of experimental models.[20,45-47] The reduction in levels of inflammation and inflammation-induced apoptosis observed following the administration of MTX in conjunction with GAL treatment may be attributed to the *in vivo* anti-inflammatory activity of GAL. In support of this hypothesis, the anti-inflammatory activity of GAL is attributed to its capacity to inhibit the synthesis of pro-inflammatory mediators and activation of mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways.[24]

It should be noted that the present study is not without limitations. Firstly, no histopathological analysis was conducted on the ovarian tissues. In future studies, the therapeutic effect of GAL on MTX-induced ovarian damage should be confirmed through histological analysis. Secondly, the impact of three consecutive days of GAL administration was assessed in an experimental acute ovotoxicity model induced by a single dose of MTX. In future studies, the efficacy of GAL can be evaluated in a chronic toxicity model induced by repeated doses of MTX. Thirdly, the therapeutic efficacy of GAL was demonstrated using basic OS, inflammation, and apoptosis biomarkers. In future studies, the ovoprotective efficacy of GAL should also be evaluated in terms of cell signalling.

CONCLUSION

The findings of this study corroborate previous reports indicating that MTX can induce OS, inflammation, and apoptosis in ovarian tissue. GAL treatments (in particular at a dose of 5 mg/kg) administered after MTX were found to be highly effective in eliminating the damage caused by MTX, due to their antioxidant and anti-inflammatory properties. These findings suggest that GAL application may be a promising approach to alleviating chemotherapy-induced reproductive toxicity. Nevertheless, before clinical use, the ovoprotective activity of GAL should be supported by more comprehensive preclinical studies that elucidate the underlying molecular mechanisms.

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Authorship contribution statement

Consept and desing: AM and SD.

Acquisition of data: SD, NTA, EAD, AM and YA.

Analysis and interpretation of data: SD, NTA, EAD, AM and YA.

Drafting of the manuscript: SD.

Critical revision of the manuscript for important intellectual content: AM and 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: 2023/07) 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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