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Epigallocatechin Gallate Alleviated Methotrexate-Induced Nephrotoxicity in Rats

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Abstract

LONG-TERM USAGE of methotrexate drugs caused adverse reactions in a variety of organs, including stomach, intestine, kidney, liver, lung, bone marrow, brain, and testis. The current research aimed to ascertain the beneficial effects of epigallocatechin gallate, a biologically antioxidant polyphenol flavonoid, on methotrexate-induced nephrotoxicity in rats. There were four male rats groups (n=10): Control group, EGCG group 100 mg/kg b.wt. three times a week, MTX group 20 mg/kg b.wt. (i/p) once a week, and MTX + EGCG group. Serum creatinine, Blood urea nitrogen, Cystatin-C, Neutrophil gelatinase-associated lipocalin, and oxidative stress markers were estimated. Additionally, histopathological changes were examined by hematoxylin and eosin staining (H and E), and gene expression of the interleukin-1 beta and tumor necrosis factor alpha-1 were examined using quantitative real-time PCR. Data analysis recorded a notable amelioration of oxidative stress in groups that supplemented with EGCG comparing MTX-treated group as confirmed by lowering malondialdehyde and nitric oxide contents and elevation of superoxide dismutase and catalase activities in kidney tissue homogenate and returned to the basal levels of the control group. EGCG enhanced renal function parameters compared to the MTX-treated group ($p<0.05$). Also, histopathological examination confirmed the protective effect of EGCG against MTX. Epigallocatechin gallate administration as a natural antioxidant markedly decreased methotrexate-induced nephrotoxicity due to its anti-cancer, anti-inflammatory, and antioxidant impacts on impairment of oxidative stress and improving kidney function.

Keywords: epigallocatechin gallate (EGCG), methotrexate (MTX), nephrotoxicity, oxidative stress.

Introduction

In general, nutritional antioxidants are safe substances present in medicinal plants that have desirable effects in alternative medicine, such as helping to reduce oxidative stress. Epigallocatechin gallate (EGCG) is especially rich in polyphenols and catechins, constituting two important groups of nutritional antioxidants [1]. These nutrients have anti-carcinogenic, antihypertensive, anti-fungal, anti-inflammatory, anti-atherothrombogenic, anti-oxidative, and anti-diabetic properties in addition to their antioxidant properties [2]. Consequently, consumption of epigallocatechin gallate has been related to a lower mortality rate, especially from heart diseases. It has been believed that the antioxidant properties of epigallocatechin gallate's polyphenols and catechins may have a role in protecting against chronic diseases, albeit the exact process by which this happens is unknown [3].

EGCG is described by its high content of flavonoids, chiefly catechins which represent 20 to 30% dry weight. Catechins that are colorless, water-soluble flavan-3-ols can donate the astringency and bitter taste of green tea implantation [4]. The chemical composition of EGCG is widely recognized. EGCG, (−)-epigallocatechin (EGC), (−)-epicatechin (EC) and (−)-epicatechin-3-gallate (ECG) are its main catechins or polyphenolic components. Catechin, epigallocatechin digallates, gallocatechin, 3-O-methyl gallocatechin gallate, and epicatechin digallate are additionally found yet in lower amounts [5].

EGCG, a flavonoid present in high amounts in cocoa powder, chocolate, black tea and green tea, is responsible for the antioxidant characteristics of these foods to scavenge certain radical species. Flavonoids can also recycle antioxidants like α-tocopherol that disrupt chemical chains, by giving a hydrogen atom to the tocopheryl radical [6].

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Methotrexate (MTX) is an antagonist to folic acid that is frequently considered a chemotherapeutic drug treating cancers like leukemia, osteosarcoma, breast cancer, lung cancer, and used in the management of other inflammatory illnesses [7]. Furthermore, it has an effective role in rheumatoid arthritis and multiple sclerosis treatment. Side effects of MTX include hematological failure, hypersensitivity pneumonia, toxicity of the central and peripheral nervous system, and dysfunctions of the gastrointestinal system and the liver [8].

Long-time administration of MTX caused adverse reactions in numerous organs, including the kidney, the liver, and others. Nephrotoxicity is the main common side effect because the drug is excreted out of the kidneys through glomerular filtration and via active transport [9].

Our research centered on the protective effect of orally administrated EGCG against oxidative stress of MTX-mediated nephrotoxicity.

Materials and methods

Drugs

Methotrexate: It was bought from Shanxi PUDE pharmaceutical Co., Ltd, China.

Epigallocatechin gallate (E4143-100MG). It was purchased from Sigma Aldrich (St.lousiana, USA).

Experimental animals

The study protocol was carried out on 40 male Sprague Dawley rats weighing 225±20 g. Rats were obtained from Medical Experimental Research Center (MERK), Faculty of Medicine, Mansoura University. Animals were sustained under constant temperatures around 25°c and 12 hours of light-dark cycles in individual metal cages. They were fed a standard diet according to MacArthur and Sun [10] and water *adlibitum* through the experimental period for four weeks. The current research work was accepted by Mansoura University, Animal care and Use Committee (MC-ACUC) with ethical approval number Ph/D 45.

Experimental design

Four identical groups of ten animals each were randomly distributed. Group (1) was kept as a control. In Group (2), rats were orally administrated with freshly prepared epigallocatechin gallate 100 mg/kg b.wt per os using stomach tube [11] three times a week, while in Group (3), rats were injected with 20 mg/kg b.wt of methotrexate via intra-peritoneal route once weekly for 4 weeks according to the research performed by Ismael et al., [12]. Finally, rats in Group (4) were received epigallocatechin gallate (100 mg/kg.b.wt) and injected intraperitoneally with methotrexate at dose of 20 mg/kg.b.wt once weekly during the experimental period.

Collection of blood samples and measurement of renal function tests

At the end of the experimental protocol, blood samples were withdrawn (3 mL from each rat) through intra-cardiac puncture under light halothane anesthesia. The blood samples were placed on a vacuum tube, then centrifuged at 3000 xg for 15 minutes to separate serum that was used to determine colorimetrically the levels of creatinine [13], blood urea nitrogen (BUN) [14] using commercial kits (Diamond Diagnostic Co. Cairo, Egypt) and cystatin-C. Measurement of biochemical parameters; Neutrophil gelatinase associated lipocaline (N-GAL) and cystatin-C based on ELISA kits (Clinilab Co., Maadi, Cairo, Egypt).

Collection of kidney specimens:

After blood collection procedures, the rats were anesthetized by sodium thiopental with a dose of 50 mg/kg b.wt. [15], which was injected intraperitoneally, then the rats were dissected and the right kidney was rapidly extracted and then divided into three equal parts using a sharp scalpel, one part was rapidly immersed in a container with 10% neutral buffered formalin for histopathological examination and the 2nd and 3rd parts were immediately saved in liquid nitrogen in cryo-tubes for molecular analysis and oxidative stress markers.

Homogenization of kidney tissues for oxidative stress and antioxidant

One hundred milligrams of kidney tissues were homogenized in 900 µL ice-cold buffer which consists of (50 mM potassium phosphate, pH 7.5,1 mM EDTA) using mortar and pestle then centrifuged at 4000 xg for 15 minutes at 4°C. The clear supernatant was soon stored at - 20° C for oxidant and antioxidant markers analysis [16]. These markers were estimated in the kidney homogenate's supernatant using the colorimetric technique by the manufacturer's instructions (Bio-Diagnostics, Dokki, Giza, Egypt).

Measuring of MDA, NO, SOD and CAT in kidney tissues

Renal oxidative stress after MTX treatment was determined with estimations of renal cortical malondialdehyde (MDA) (as index for lipid peroxidation) according to Lahouel *et al*. [17] and nitric oxide (NO) Singh *et al*. [18]. Renal superoxide dismutase activity (SOD) was colorimetrically determined in line with the method described by Weydert and Cullen, [19] using NADH oxidation. And catalase activity (CAT) was determined colorimetrically using the procedure provided by Hadwan and Abed [20].

Histopathological inspection of kidney tissues

Renal tissues used for histopathological technique were kept at 10% neutral buffer formalin that was processed using hematoxylin and eosin technique according to the technique of Suvarna *et al*. [21]. The tissue section was cut with a 3µm in thickness and was

examined using several magnification powers (4x, 10x and 40x).

Gene expression analysis of kidney tissues

The kidney tissues were immediately minced into small pieces about 0.1 gm using a sharp and clean scalpel blade, inserted in an eppindorff tube, and stored as soon as possible at -80ºC. RNA from kidney tissues was extracted using a TRIzol Reagent (ThermoFisher Scientific- USA) for the determination of tumor necrosis factor-alpha- 1 (TNFα-1) and interleukin-1 beta (IL-1β) gene expression using SYBR Green (according to Solis BioDyne Kit) (Table 1) real-time PCR (Applied bio system kits). Fold expression was calculated using the equation $2^{-\delta\delta ct}$ method against beta actin. qRT-PCR protocol was adjusted as initial denaturation step of 95°C for 9 minutes that was followed by 35 cycles of denaturation, annealing and elongation for 95°C (30 sec), 55°C (40 sec) and 72°C (30 sec), respectively. The reaction was ended by final elongation step at 72°C for 5 minutes.

Statistical Analysis

All data were formatted as mean±SE. Statistical analysis was achieved by SPSS Version 16 using one away ANOVA and Tuckey's test as a post hoc test to determine statistical differences. All data with p-Value less than 0.05 were regarded as significant.

Results

Effect of MTX and EGCG on renal functions (serum creatinine, BUN, Cyst-C and N-GAL)

The results explained that rats injected with MTX recorded a considerable increase in the serum concentrations of renal functions (creatinine, BUN, cyst-C and N-GAL) contrasted with the control group ($p< 0.05$). In contrast, rats supplemented with EGCG recorded significant reductions in elevated serum renal injury markers ($p< 0.05$) compared to the control group (Fig. 1). On the contrary, rats co-administrated EGCG with MTX showed a notable decrease in serum creatinine, BUN, Cyst-C and N-GAL levels ($p< 0.05$) contrasted with MTX-injected group.

Effect of MTX and EGCG on oxidative stress and antioxidant markers (MDA, NO, SOD and CAT) in renal tissues

The results presented in Fig. 2 indicated that the treatment of MTX achieved a notable elevation in the concentrations of renal tissue malondialdehyde (MDA) and nitric oxide (NO) ($p< 0.05$) along with a great decline in the activity of antioxidant enzymes (SOD and CAT) comparing the control. Meanwhile, EGCG supplementation to MTX-treated rats demonstrated a significant decrease ($p< 0.05$) in peroxidative levels (MDA and NO) and elevation in the activity of renal tissue antioxidants (SOD and CAT) ($p< 0.05$).

Histopathological examination

Histopathological examination revealed that EGCG conserved the histological architecture of renal tubular and glomerular epithelium (Fig. 3B). The group of rats that was intoxicated with MTX showed diffuse tubulorrhexis that was accompanied with vacuolation and sloughing of renal tubular epithelium and glomerular damages (Fig. 3C). Similarly**,** MTX group showed edema and hemorrhage admixed with few inflammatory cells widely separated with dilated tubules beside multifocal peritubular fibrosis (Fig. 3D). Moreover, the intoxication with MTX induced diffuse tubular necrosis represented by hyperesinophilia of tubular epithelial cytoplasm with pyknotic nucleus or complete loss of tubular architecture with few leukocytic infiltrations admixed with RBCs and fibroblast (Fig. 3E). The supplementation of EGCG to MTX intoxicated group indicated the presence of few dilated tubules with attenuated epithelium and shrunken, hypocellular glomerular tufts with dilated uriniferous tubules (Fig. 3G).

Effect of MTX and EGCG on IL-1β and TNFα-1 gene expression

The kidney mRNA expression data was showed several markers of proliferation, fibrosis, inflammation and angiogenesis in MTX-treated group comparing control group. Furthermore, there was a notable over-expression of IL-1β and TNFα-1 ($p< 0.05$). On the other hand, both EGCG and co-administrated EGCG with MTX groups indicated a significant down-regulation in the expression of IL-1β and TNFα-1 compared to controls ($p< 0.05$) concerning markers of inflammation and angiogenesis (Fig. 4).

Discussion

MTX is a dihydrofolate reductase inhibitor on tetrahydrofolate synthesis of folic acid as MTX penetrates the cell through the reduced folate carrier and passes into polyglutamation mediated by folylpolyglutamate synthetase. By consuming cells of reduced tetrahydrofolate cofactors, MTX and its polyglutamates caused apoptosis in many cell types and impaired purine and pyrimidine synthesis as well as protein synthesis, which are necessary for DNA and RNA synthesis [22]. Treatment with MTX can cause kidney toxicity at low or high doses. There are two possible mechanisms by which MTX–induced nephrotoxicity occur; a) direct toxic effect on renal tubules, resulting in tubular injury from MTX precipitation in kidney tubules and a decline in glomerular filtration rate, which is typically mitigated by hydration and alkaline urine; b) enhancement of reactive oxygen species, causing elevation of lipid peroxidation and oxidative stress, which may be the cause of damage to cells or organs [23].

It was discovered that MTX increased the levels of serum creatinine, BUN, Cyst-C and N-GAL levels in MTX animal models, which led to renal damage. The

increase in renal function levels was caused by cystic dilation in the tubular lumen of kidney tissue, atrophy and hypertrophy in some glomeruli and severe leucocyte inflammatory cell infiltration [24, 25].

According to several researches, oxidative stress contributes to kidney damage caused by MTX. By activating polymorphonuclear neutrophils, MTX not only inhibits the antioxidant defense system but also, enhances reactive oxygen radical levels [26]. Organ damage develops as a result of excessive free radicals production and an impaired endogenous antioxidant defense system, which also results in cell membrane disintegration [27]. In order to diminish the amount of NADPH in cells, MTX inhibits cytosolic nicotinamide adenosine diphosphate (NADP)-dependent dehydrogenases and NADP malic enzymes. As a result, MTX increases MDA levels (a sign of lipid peroxidation) and NO levels, while decreasing the activity of antioxidant enzyme such as SOD, CAT [28].

Lipid peroxidation with oxidative stress is caused by an increase in ROS and/ or decrease in antioxidant defense system activity, which results in an excess of malondialdehyde. MDA is one of the byproducts of membrane polyunsaturated fatty acids peroxidation, which affects membrane fluidity and permeability and results in degradation of membrane integrity. It has been shown that nitric oxide (NO) influences the pathophysiology of multiple diseases including nephrotoxicity [29]. In this case, rats given MTX showed a significant increase in the lipid peroxidation indicators, MDA and NO. The ROS-mediated increased MDA level is a significant contributor to MTX-induced renal tissue damage. Peroxidase activity is induced by the rise in NO generation, a recognized catalase activity inhibitor.

As a result, hydrogen peroxide accumulates, which combines with the superoxide radical to produce hydroxyl radicals, an activator of lipid peroxidation. This process plays a role in MTX nephrotoxicity and oxidative stress development. The slowdown in SOD and CAT activity and a rise in MDA levels indicated that MTX-induced toxicity in the renal tissues [30]. Additionally, the slowdown in SOD activity may lead to a rise in the amounts of oxygen radicals that will interact with NO to produce the peroxynitrite anion, another harmful oxidant radical species [31].

EGCG greatly improved the antioxidant defense mechanism due to its antioxidant capabilities. According to the current data, there was a crucial rise in SOD and catalase activity in renal tissues in comparison to the MTX-treated group, along with a decline in MDA and NO levels [32].

Due to the capability of MTX to instigate oxidative and nitrative stresses, with stimulation of nuclear factor-κB (NF-κB) like inflammatory cell infiltrations, tubular cell necrosis, hemorrhages, glomerulosclerosis and proteinous materials in tubules in MTX group, oxidative stress-induced renal damage primarily occurred at the histopathological findings in this study [33]. MTX caused significant degenerative changes, such as tubular degeneration, dilatation, cell swelling, and damage, in a previous study using the same dose [34].

Polyphenol compounds in EGCG are strong antioxidants that assist in protecting against oxidative stress and inhibit cell deterioration. Furthermore, the connective tissue showed huge reduction in the number of dilated and congested blood vessels in relation with the MTX treated group, accordingly the blood stream to the renal tubules were decreased, consequently, the kidney's availability of chemotherapeutic drugs was reduced. This could be due to the anti-angiogenic property of EGCG [35].

The inactivation of the signaling pathway of transcription factor nuclear factor-kappa B (NF-κB) mediated I kappa B kinase complex pathways is the most prominent inhibitory effect of EGCG polyphenol, and the release of TNFα-1, which is involved in regulating various inflammation genes, and anti-inflammatory activity of EGCG [36].

Additionally, EGCG catechins through its antioxidant and anti-inflammatory characteristics may harm the expression of inflammatory cytokines TNFα-1 and IL-1β that can cause damage through increase the reactive oxygen species (ROS), such as nitric oxide and inflammatory mediators like prostaglandin E2 [37].

Systemic inflammatory response and proinflammatory cytokines activation were linked to MTX-induced kidney toxicity. In this study, the MTX-administrated group has significantly elevated expression of the acute inflammation markers IL-1β and TNFα-1. Also, EGCG treatment as a strong antioxidant, provided significant reduction in the expressions of these elevated parameters: IL-1β and TNFα-1, due to the anti- inflammatory effects of EGCG in protecting the kidney from MTX-induced nephrotoxicity [38].

Our results came following the results of Singh *et al*. [39]; Hus *et al*. [40]; Kesavalu *et al*. [41]; El-Mowafy *et al*. [42] & Subramani and Natesh [43] as they reported that MTX's progressive kidney damage was prevented by the anti-inflammatory effects of EGCG, which also reduced prostaglandin synthesis, decreased the production of pro-inflammatory cytokines, and decreased the expression of the TNFα-1and IL-1β genes in the kidneys.

Conclusion

The protective effect of EGCG was attributed to the fact that it is rich in polyphenolic compounds principally, catechins which increase the antioxidant activity by reducing the oxidative stress produced by MTX by increasing SOD and Catalase activities in

kidney tissues, it also, improve kidney function. MTX by increasing SOD and Catalase activities in kidney tissues, it also, improve kidney function.

Ethics approval and consent to participate

The current study complies with national and international guidelines. The current study was approved by the research ethics committee at Mansoura University. The current study was conducted based on the guidelines of ARRIVE guidelines 2.0.

Competing interests

There are no conflicts to declare.

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TABLE 1. Oligonucleotide primers and probes used in SYBR Green real time PCR.

Gene	Primer sequence (5'-3')
Rat ß-actin	CACTGTCGAGTCGCGTCC
	CGCAGCGATATCGTCATCCA
IL-1β	TGCCACCTTTTGACAGTGATG
	AAGCTGGATGCTCTCATCAGG
TNFα-1	ACTGAACTTCGGGGTGATCG
	CCACTTGGTGGTTTGTGAGTA

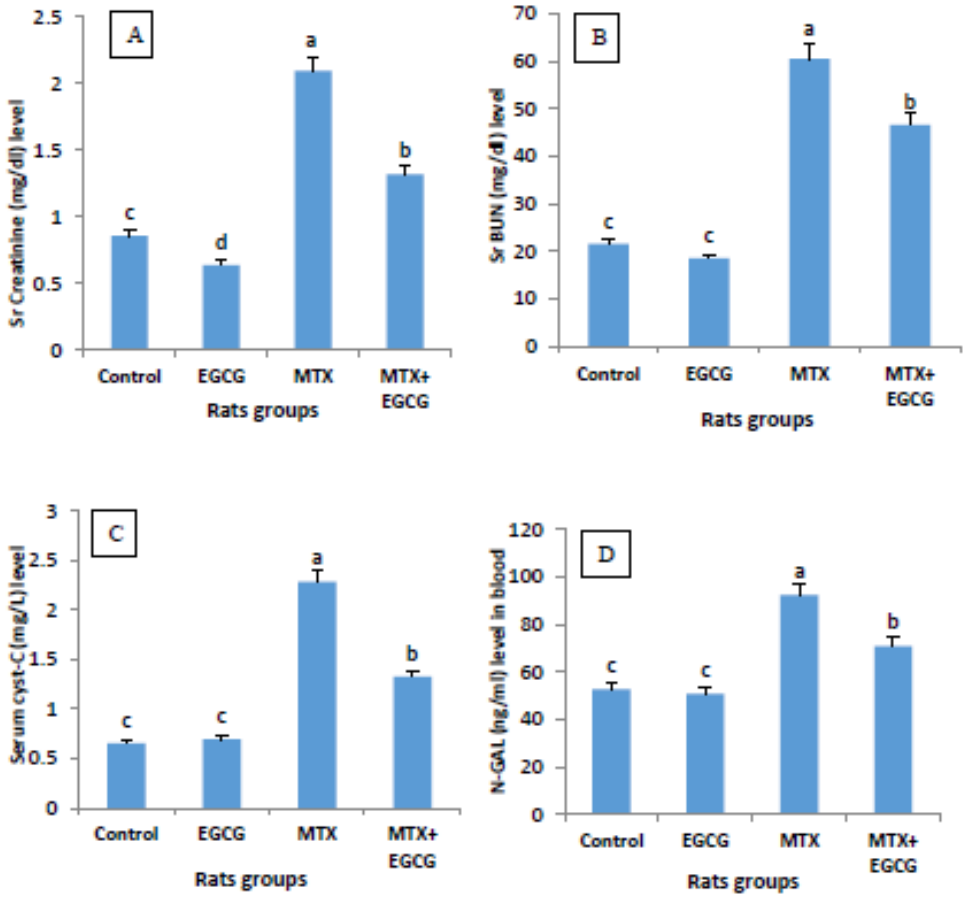

Fig. 1. Effect of EGCG and MTX-intoxicated rats on renal function parameters. (A) serum creatinine concentration; (B) serum blood urea nitrogen levels (C) serum cystatin-C levels & (D) N-GAL concentration in blood.

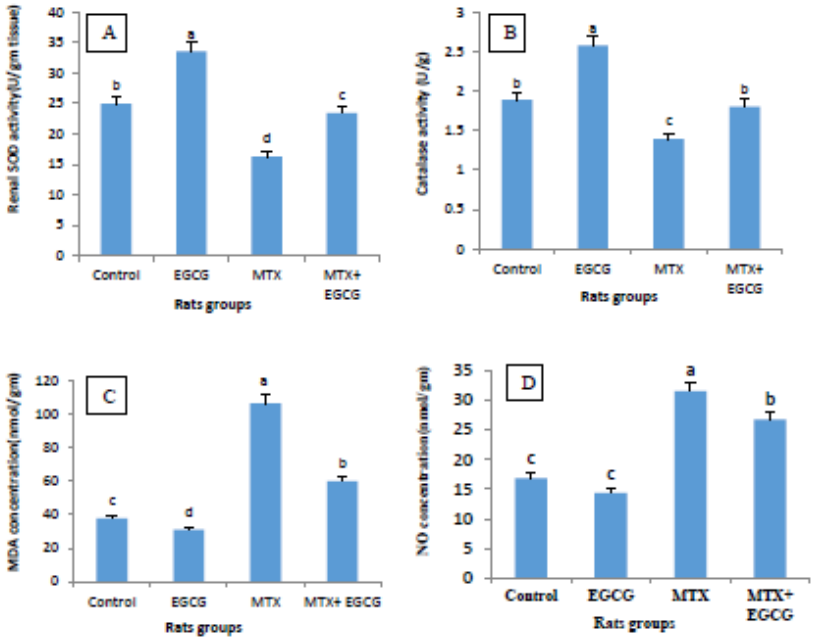

Fig. 2. Renal oxidative stress and antioxidants markers of rats intoxicated with MTX and treated with GT. (A) renal SOD activity; (B) renal catalase activity (C) renal MDA concentration & (D) renal NO concentration.

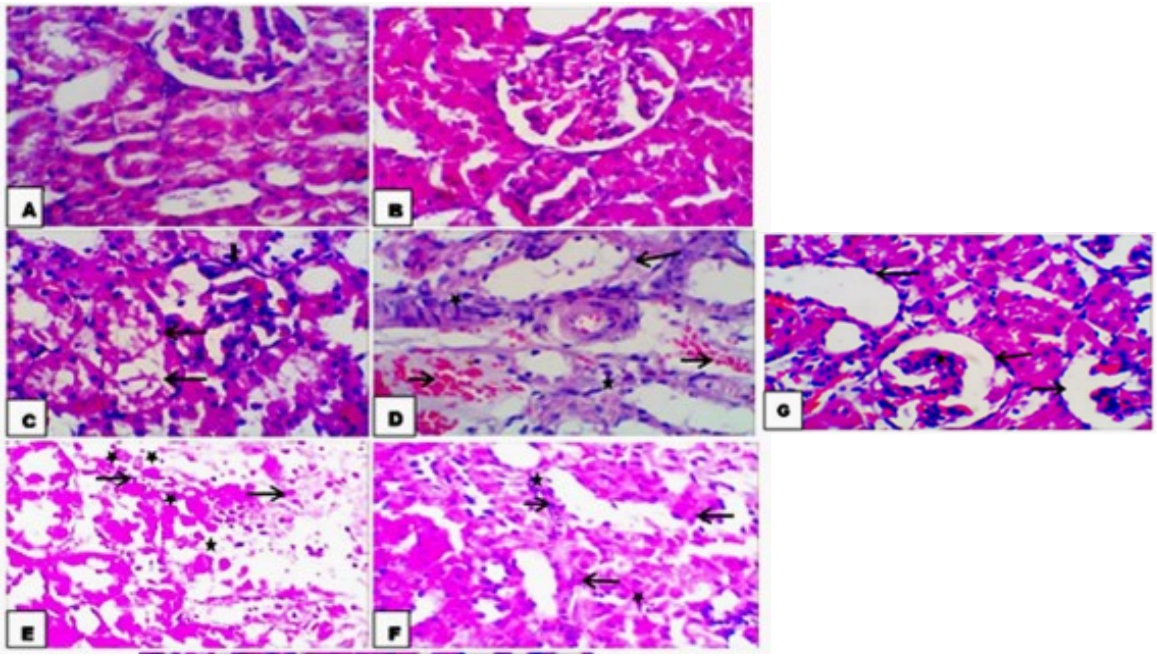

Fig. 3. Histopathological examination of kidney tissues of rats exposed to MTX-induced renal toxicity and treated with EGCG (H and E). A) Kidney tissue samples from the control group (1) using H&E stain, 400x **B)** kidney samples from Group 2 **C, D, E and F)** Tissue samples from Group (3) [Tubulorrhexis with tubular epithelial vacuolation and sloughing (thin arrows) beside glomerular damage (thick arrow), edema and hemorrhage (thick arrow) admixed with few inflammatory cells widely separated with dilated tubules (thin arrow) beside multifocal peritubular fibrosis (star), Diffuse tubular necrosis represented by hyperesinophilia of tubular epithelial cytoplasm with pyknotic nucleus or complete loss of tubular architecture (thick arrows) with few leukocytic infiltrations admixed with RBCs and fibroblast (stars), Ectatic tubules lined with attenuated epithelium (thin arrows) beside atrophied, slit like tubules (thick arrow) with peritubular, interstitial fibrosis (stars)], **G)** Kidney samples from Group 4 **[**few dilated tubules with attenuated epithelium (thin arrow) and shrunken, hypocellular glomerular tufts (star) with dilated uriniferous tubules (thick arrow)].

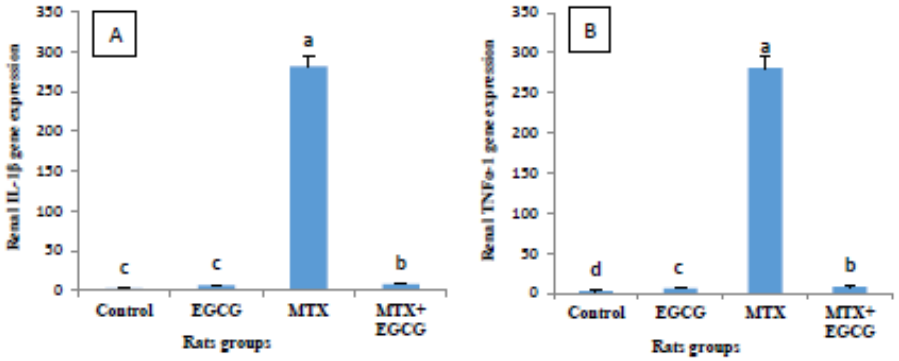

Fig. 4. Gene expression analysis of proinflammatory markers of rats exposed to MTX-induced renal toxicity and treated with EGCG. (A) renal interleukin-1 (IL-1β) gene expression & (B) renal tumor necrosis factor alpha (TNF*α*-1)gene expression.

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التأثير الوقائي للإيبيجالوكاتكين جالات على السمية الكلوية المحدثة بالميثوتريكسيت في الجرذان

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الملخص

تسبب الاستخدام طويل الأمد لعقار الميثوتريكسيت في حدوث انتكاسات واضحة فى مختلف اعضاء الجرذان، بما في ذلك الكلى والكبد والرئة ونخاع العظام والدماغ والخصية. يهدف البحث الحالي إلى التأكد من التأثيرات المفيدة لـ epigallocatechin Gallate، وهو فلافونويد بوليفينول مضاد للأكسدة بيولوجيًا، على التسمم الكلوي الناجم عن الميثوتريكسيت في الجرذان. كانت هناك أربع مجموعات من الفئران (العدد = 10): مجموعة التحكم، مجموعة EGCG 100 ملجم/كجم من وزن الجسم. ثلاث مرات في الأسبوع، مجموعة MTX 20 ملغم/كغم من وزن الجسم. (i/p) مرة واحدة في الأسبوع، ومجموعة MTX + EGCG. تم تقدير الكرياتينين في الدم، نيتروجين اليوريا في الدم، السيستاتين-C، الليبوكالين المرتبط بالجيلاتيناز المتعادل، وعلامات الإجهاد التأكسدي. بالإضافة إلى ذلك، تم فحص التغيرات النسيجية المرضية عن طريق تلطيخ H&E، وتم فحص التعبير الجيني لبيتا إنترلوكين-1 وعامل نخر الورم ألفا-1 باستخدام تفاعل البوليميراز المتسلسل الكمي في الوقت الحقيقي. سجل تحليل البيانات تحسنا ملحوظا في الإجهاد التأكسدي في المجموعات التي تستكمل بـ EGCG مقارنة بالمجموعة المعالجة بـ MTX كما تم تأكيد ذلك عن طريق خفض محتويات المالونديالدهيد وأكسيد النيتريك وارتفاع أنشطة ديسموتاز الفائق الأكسيد والكاتلاز في تجانس أنسجة الكلى والعودة إلى المستويات القاعدية للتحكم. مجموعة. عزز EGCG معلمات وظائف الكلى بالمقارنة مع المجموعة المعالجة بـ MTX ($P < 0.05$). كما أكد الفحص النسيجي المرضي التأثير الوقائي لـ EGCG ضد MTX. أدى تناول Epigallocatechin Gallate كمضاد للأكسدة طبيعي إلى تقليل السمية الكلوية الناجمة عن الميثوتريكسيت بشكل ملحوظ بسبب تأثيراته المضادة للسرطان والمضادة للالتهابات ومضادات الأكسدة على ضعف الإجهاد التأكسدي وتحسين وظائف الكلى.

الكلمات الدالة: ميثوتريكسات ، ابيجالوكانكين ، اجهادات الأكسدة ، التسمم الكلوى.