Hepatoprotective Effect of the Aqueous Extract of *Camellia sinensis* Against Methotrexate-induced Liver Damage in Rats

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Abstract

Methotrexate (MTX) is a folate antagonist widely used in the treatment of neoplastic diseases; its biotransformation in the liver produced active metabolites that promote hepatotoxicity. The present study was designed to evaluate the hepatoprotective effect of aqueous extract of *Camellia sinensis* (Green tea) against MTX-induced liver damage in rats. A model of liver injury in rats was induced by intraperitoneal injection of 20mg/kg MTX as a single dose followed by saline and 1.25% and 2.5% aqueous extract of green tea (GTE) were orally administered 7 days prior and 5 days after MTX-intoxication as a sole source of drinking water. After killing the animals, blood samples were obtained for evaluation of serum levels of alanine and aspartate aminotransferases (ALT and AST) and alkaline phosphatase (ALP) activities, while liver tissue homogenate was prepared to evaluate tissue levels of glutathione (GSH) and malondialdehyde (MDA). Additionally, liver tissue sections were prepared and stained with hematoxylin and eiosin for histological evaluation. The results showed that administration of green tea extract (GTE) significantly decreased the elevated levels of ALT, AST and ALP activities in the serum compared to MTX-treated group. Treatment of animals with GTE 7 days before and 5 days after MTX also elevates GSH levels and decreases MDA levels significantly compared to MTX-treated group, this was associated with improving histological features that already impaired due to exposure to MTX. In conclusion, treatment of rats with GTE protects hepatic tissue against MTX-induced liver damage in dose dependent manner.

Key words: Green tea, Hepatotoxicity

الخلاصة

مثوتركزيت هو نظير للفوليت ذو استعمال واسع في علاج الامراض السرطانية, يتحول الميثوتركزيت بعد ايضه داخل الكبد الى ناتج سام يؤدي الى تسمم الكبد.صممت هذه الدراسة لتقييم فعالية جرع مختلفة من المستخلص المائي للشاي الاخضر(*Camellia sinensis*) في حمايةكبد الجرذان المختبرية من المثوتركزيت.تم استحداث التسمم الكبدي الجرذ بواسطة حقنه داخل الغشاء البريتوني ب (٢٠ ملغم /كلغم)من مادة الميثوتركزيت لمدة خمسة ايام متتالية وذلك يتم بالتتابع مع معالجة الجرذ ب(١,٢٥% - ٢,٥%) من مستخلص المائي الشاي الاخضر عن طريق الفم كبديل عن ماء الشرب لمدة خمسة ايام ماقبل و سبعة ايام ما بعد الحقن بالميثوتركزيت.بعد قتل الحيوان اخذت عينة من مصل الدم لقياس مستوى فعالية الالانين(alanine) و الاسبارتيت امينوترانسفيريز (aspartate aminotransferases) , من جهه اخرى ناخذ مستخلص انسجة الكبد و نقيم الكمية النسيجية من الجلوتاثيون (glutathione) و المالونداياالدهايد (malondialdehyde) والالكلاين فوسفتيز (alkaline phosphatase) بالاضافة الى اخذ جزء من نسيج الكبد للفحص النسيجي بواسطة صبغه بمواد الايوسين و الهيماتوكسلين.اظهر تحليل النتائج ان اعطاء مستخلص الشاي الاخضر يقلل و بشكل ملحوظ مستويات فعالية كل من الالانين , الاسبارتيت امينوترانسفيريز و الالكالاين فوسفتيز(alkaline phosphatase) وذلك بالمقارنة مع الجرذان غير المعالجة بالشاي الاخضر و التي تم حقنها بالميثوتركزيت وادى ايضا الى زيادة مستوى الجلوتاثايون وخفض مستوى المالونديالدهايد بصورة ملحوظة بالمقارنة مع الحيوانات التي اعطيت الميثوتركسيت و كان هذا واضحا من تحسن الشكل النسيجي للكبد و الذي كان متضررا من مادة الميثوتركزيت .كاستنتاج ,المعالجةالمبكرة بالجرع المعتمدة من الشاي الاخضر تحمي انسجة الكبد من الضرر الناتج عن الميثوتركزيت.

Introduction

Methotrexate (MTX) , a folate antagonist is widely used in the treatment of neoplastic diseases . It has also been used successfully as anti-inflammatory and immunosupressive agent in non - neoplastic diseases such as psoriasis, arthritis biliary cirrhosis and Reiter's syndrome [(1, 2)]. Methotrexate is actively accumulated in the liver where it is metabolized and stored in polyglutamated form. The major side-effect of chronic methotrexate administration is hepatotoxicity, which is characterized by fatty infiltration, inflammation, cellular necrosis and apoptosis, steatosis, fibrosis and cirrhosis [(3, 4)]. The mechanisms of methotrexate induced hepatotoxicity are not fully understood. From the results of *in vitro* experiments it has been suggested that increased oxidative stress contributes to methotrexate hepatotoxicity [(5, 6)], both through increased reactive oxygen species activity and impaired anti-oxidative defense via depleted intrahepatic glutathione depots [(7)].

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Typical histopathological findings in MTX induced liver disease include nuclear atypia, vacuolization, and mild fatty metamorphosis.

Flavonoids have been found to play important roles in the non-enzymatic protection against oxidative stress [8, 9], especially in case of cancer. Flavonoids are group of polyphenolic compounds that occur widely in fruit, vegetables, tea, cocoas and red wine [10, 11, 12].Fresh tea leaves are rich in flavanol monomers known as catechins such as epicatechins [13], which are 13.6 g/100 g in green tea and 4.2 g/100 gm dry weight in black tea [14].In animal studies, it has been revealed that green tea may protect liver and brain cells against sequelae of oxidative stress induced by ethanol intoxication [15, 16, 17]. Supplementation of green tea extract (GTE) attenuates cyclosporine A-induced oxidative stress in rats [18]. Catechins derived from tea leaves are natural, safe for consumption, and have been proved to be very effective antioxidants. The present study was designed to evaluate the protective effect of GTE against MTX-induced hepatotoxicity in rats.

Material and Methods

Preparation of Aqueous Green Tea Extract

The aqueous extract of green tea was made according to the method of Maity *et al* (1998) [19] by soaking for 10 minutes 1.25 gm and 2.5 gm of green tea leaves respectively in 100 ml of distilled water at 90°C. Solutions of GTE were freshly prepared on daily bases, and then filtered to obtain final concentrations of 1.25% and 2.5% respectively. These solutions were used as substituent for water as the sole source of drinking fluid.

Experimental protocol

Twenty eight Sprague-Dawley rats (150-250 g) of both sexes were housed in the animal house of the College of Pharmacy, University of Baghdad under controlled conditions of temperature and humidity and fed standard chow died and drinking water *ad libitum*. The animals were allocated into 4 groups and treated as follow:

Group I: seven animals received normal saline by i.p. injection for 12 days, sacrificed by cervical dislocation on day 13 and served as controls.

Group II: Seven animals were injected with single 20 mg/kg i.p. MTX followed by saline for 5 days[20, 21]. The animals were sacrificed by cervical dislocation on day 6 and served as positive controls.

Groups' III and IV: seven rats in each group treated with either 1.25% or 2.5% GTE for 7 days before induction of hepatoxicity and 5 days after, and then the animals were sacrificed by cervical dislocation on day 13.

After sacrification of animals by cervical dislocation, blood samples were obtained by thoracic section and serum was prepared for the evaluation of the activities of alanine aminotransferases (ALT), aspartate aminotransferases (AST) and alkaline phosphatase (ALP). Moreover, liver were quickly excised, placed in chilled phosphate buffer solution (pH 7.4) at 4 ^{0}C, blotted with filter paper and weighed. One gram of liver was then taken to prepare 10% tissue homogenate using the same buffer solution utilizing tissue homogenizer [22] for 1 minute at 4 ^{0}C. All preparations were freshly prepared and kept frozen at -18 ^{0}C unless worked immediately. Tissue homogenate levels of GSH and MDA were evaluated using standard procedures [23, 24]. Liver tissues were prepared for histological examination using paraffin sections technique [25]. Blocks were cut by microtome into 5 mm thick sections, stained with hematoxyline and eosin and then examined under light microscope. All data were expressed as mean ± S.E. Unpaired t-test was carried out to compare populations using Graph Pad Prism software (San Diego, USA). A 0.05 level of probability was used as the criterion for significance.

Results

Significant increase in ALT, AST and ALP was observed in the serum of rats treated with MTX compared to control group. After treatment of rats with green tea extract 7 days prior & 5 days after MTX, a significant improvement in the levels of ALT, AST, and ALP was reported, their levels were significantly decreased (P<0.001) compared to MTX-intoxicated rats (Figures 1, 2 and 3).

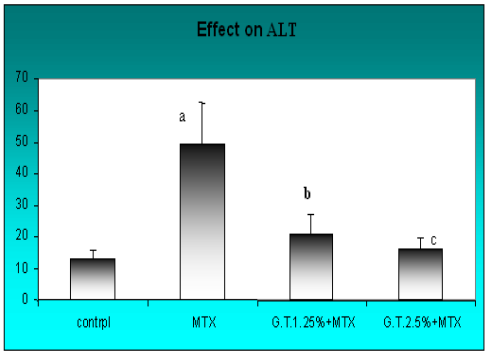

Figure 1: Effect of different treatment approaches on the serum activity of alanine aminotransferase (ALT) in rats.
- Each value represents mean ± S.D.
-*Significantly different with respect to control.
-Values with non-identical superscripts (a,b,c) with each parameter are significantly different (P<0.05).

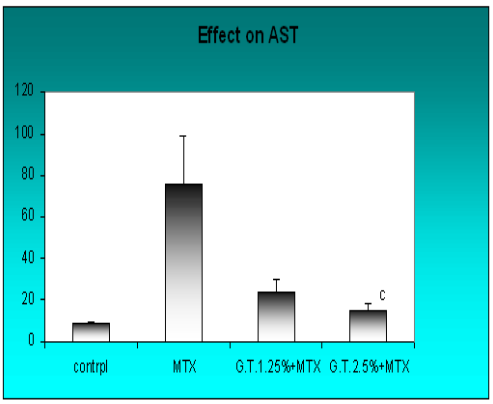

Figure 2. Effect of different treatment approaches on the serum activity of aspartate aminotransferase (AST) in rats.
- Each value represents mean ± S.D.
- * Significantly different with respect to control.
-Values with non-identical superscripts (a,b,c) with each parameter are significantly different (*P*<0.05)

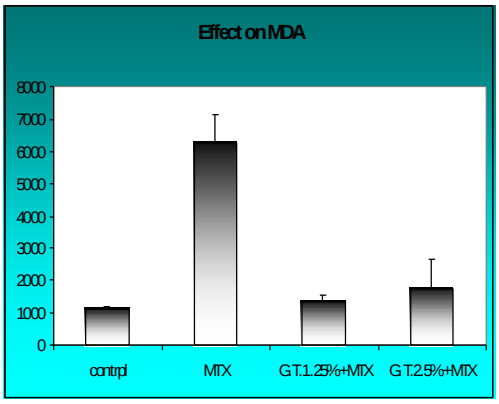

Figure 4. Effect of different treatment approaches on the malondialdehyde (MDA) contents in rats liver homogenate.
- Each value represents mean ± S.D.
-* Significantly different with respect to control.
-Values with non-identical superscripts (a,b,c) with each parameter are significantly different (*P*<0.05).

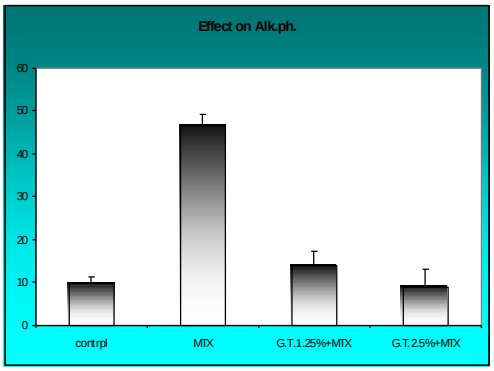

Figure 3: Effect of different treatment approaches on the serum level of alkaline phosphatase in rats.
- Each value represents mean ± S.D.
-* Significantly different with respect to control.
-Values with non-identical superscripts (a,b,c) with each parameter are significantly different (*P*<0.05).

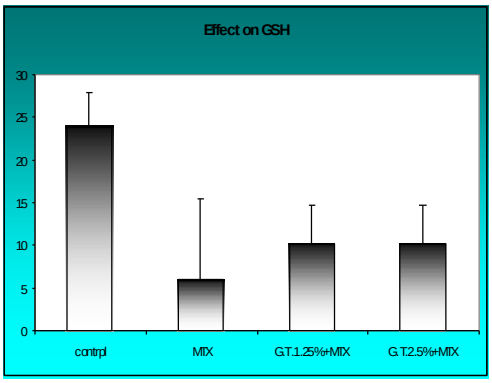

Figure 5. Effect of different treatment approaches on the glutathione (GSH) level in rats liver homogenate.
- Each value represents mean ± S.D.
-* Significantly different with respect to control.
-Values with non-identical superscripts (a,b,c) with each parameter are significantly different (*P*<0.05).

Rats treated with MTX resulted in a significant increase ($P<0.05$) in hepatic lipid peroxidation measured by the amount of MDA formed, associated with significant decrease ($P<0.05$) in the liver tissue GSH levels. However, treatment of rats with GTE for 7 days prior & 5 days after MTX, led to a significant decrease in MDA levels ($P<0.001$) and elevation in GSH level ($P<0.001$) compared to MTX-treated group (Figures 4 and 5). Concerning the histological finding (figure 6), uses of MTX produces several pathological changes in liver tissues, including fatty infiltration, macrovascular degeneration, pleomorphism, ballooning degeneration and hypertrophied hepatocytes (figure 7), while the liver section from rats treated with 1.25% of GTE for 7 days prior & 5 days after MTX showed moderate fatty change, mild apoptosis and moderate collapse of the structure (figure 8) and livers of animals treated with 2.5%GTE for 7 days prior & 5 days after MTX showed mild fatty changes of the mid zone, absence of fatty changes and preserved periventricular structures (figure 9).

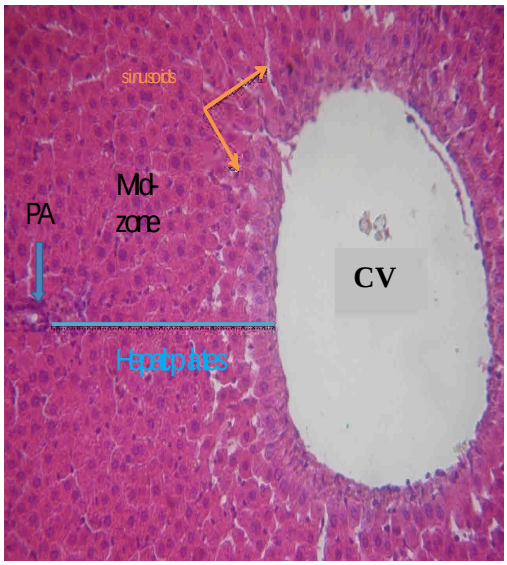

Figure 6. Control Section showed normal rat's hepatic tissue with normal portal (PA); cental vein (CV) and mid zone (X 400).

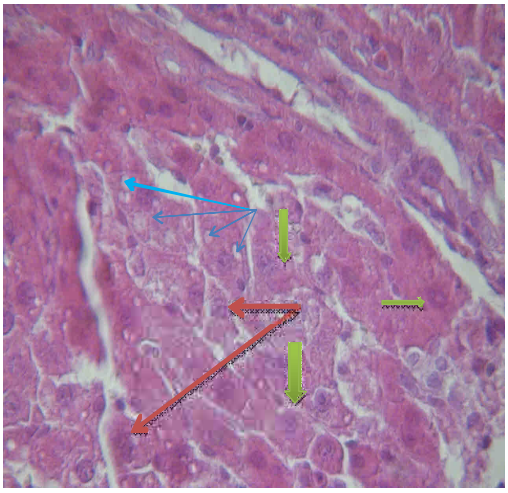

Figure 7. Liver section from rats treated with MTX showed magnification of periportal area, moderate fatty changes, macrovascular degenaration (blue arrows), pleomorphism, different cellular shapes (brown arrows), ballooning degeneration, hypertrophied hepatocytes (large size green-yellow arrows) (X 800).

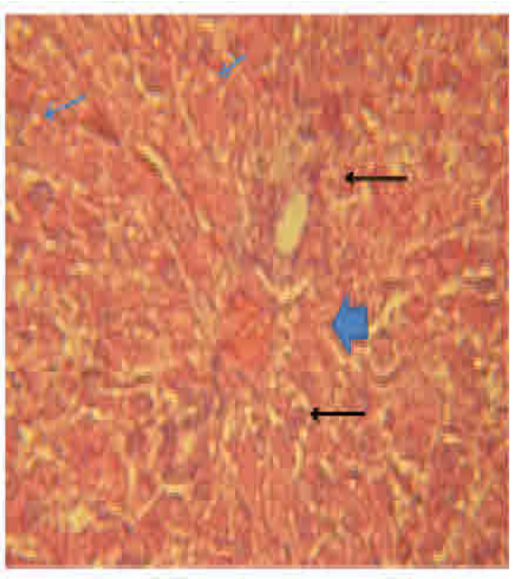

Figure 8. Liver section from rats pretreated with 1.25%GTE and challenged with MTX showed closer view of periportal area, revealing moderate fatty change (blue thin arrows), mild apoptosis (black arrows), moderate collapse of structure (wide blue arrow)(X 400).

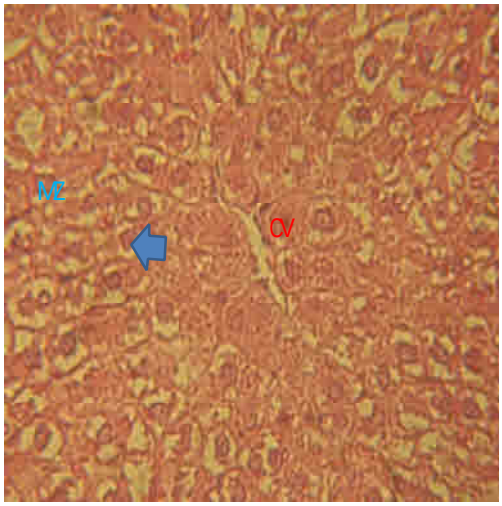

Figure 9. Liver section from rats pretreated with 2.5%GTE and challenged with MTX showed preserved periventricular (cv) structure, absence of fatty changes, while noticing mild fatty changes of the mid zone (MZ) (arrow) (X 400).

Discussion

Epicatechins (antioxidant present in green tea) scavenge a wide range of free radicals including the most active hydroxyl radical, which may initiate lipid peroxidation. It prevents the loss of lipophilic antioxidant α-tocopherol by repairing tocopheryl radicals and protection of the hydrophilic antioxidant ascorbate [26]. Therefore, it may decrease the concentration of lipid free radicals and terminate initiation and propagation of lipid peroxidation [27]. The data presented in this study demonstrated the implication of oxidative stress in hepatic tissue induced by MTX treatment (Fig. 3), manifested by increase in MDA contents in liver tissue. Epicatechins are effective scavengers of physiologically active reactive oxygen and nitrogen species including superoxide [28], peroxyl radical [27], peroxynitrite [29] and hypochlorous acid[30]. It was reported that, epicatechines can react with superoxide radical via one electron transfer mechanism or by a hydrogen abstraction mechanism to form the corresponding semiquinone [31]. Epicatechins may chelate metal ions, especially iron and copper, which, in turn inhibit generation of hydroxyl radicals and degradation of lipid hydroperoxides which causes reactive aldehyde formation [32]. The liver damage was determined by measuring serum levels of ALT and AST while level of TBARS in liver was used as an indicator of lipid peroxidation. The levels of the antioxidant thiol in liver homogenates (GSH) was significantly improved upon treatment of MTX-intoxicated rats with 2.5%GTE (Fig. 4) which inhibited MTX-induced hepatic injury and thereby the level of oxidative stress, as it can decrease lipid peroxidation and enhance antioxidant enzyme activities, whereas the level of MDA was significantly decreased comparable to MTX-intoxicated group. In agreement with the results obtained in this study, administration of green tea to ethanol-intoxicated rats resulted in the normalization of lipid peroxidation as well as glutathione concentration and ALT activity in liver [17]. Damaging liver tisse after MTX exposure is a well-known phenomenon, and the obvious sign of hepatic injury is the leakage of hepatic enzymes into plasma. There is no doubt that both the histological appearance and biochemical parameters supported a diagnosis of liver damage. The increased levels of serum enzymes such as ALT, AST and ALP have been observed in MTX-treated animals, which indicate the increased permeability, damage or necrosis of hepatocytes. Green tea extract gave a high hepatoprotective effect by reversing these changes produced by MTX (Fig.1, 2 and 5). The observed decrease in the serum activities of these enzymes showed that GTE, to some extent, preserved the structural integrity of the liver from the toxic effect. It is well known that GTE is effective scavengers of reactive oxygen species and may also function indirectly as antioxidants through their effects on transcription factors and enzyme activities [33, 34]. Green tea extract, water-soluble antioxidants, has been demonstrated to inhibit iron-induced oxidation of synaptosomes by scavenging hydroxyl radicals generated in the lecithin/lipoxidase system [35]. On the one hand, GTE can penetrate the lipid bilayer, decreasing free radicals concentration or influencing antioxidant capability in biomembranes [36, 17]. On the other hand, they could reduce the mobility of free radicals into the lipid bilayer as well. Moreover, GTE can interact with phospholipid head groups, particularly with those containing hydroxyl groups, so they could decrease the fluidity in the polar surface of phospholipid bilayer [37]. In addition, GTE can prevent the loss of the lipophilic antioxidant _-tocopherol, by repairing tocopheryl radicals, and protection of the hydrophilic antioxidant ascorbate [38, 39]. Liver is the major site for synthesis of GSH and detoxification of different drugs and xenobiotics in the liver may involves use of this tripeptide[40]. Glutathione plays a common role in cellular resistance to oxidative damage as a free radical scavenger and by generation of ascorbate or tocopherol in liver [41]. The decreased hepatic GSH in MTX-intoxicated rats could be as a result of hexose monophosphate (HMP) shunt impairment due to MTX, thereby NADPH availability is reduced and the ability to recycle GSSG to GSH is decreased [42]. By blocking oxidative damage through lipid peroxidation and protein oxidation, green tea extract prevent the loss of membrane permeability and dysfunction of cellular proteins and decreases the endogenous level of hydroxyl radical and GSH [40]. In conclusion, green tea has hepatoprotective activity against methotrexate-induced toxicity in rats.

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