

Protective and Therapeutic Effects of Quercetin on Methotrexate Induced Hepatotoxicity in Adult Albino Rats: Biochemical, Histological and Immunohistochemical Study

Eman El Bana and Kamal M. Kamal

Anatomy and Embryology Department, Faculty of Medicine, Benha University, Egypt

ABSTRACT

Background: Methotrexate (Mtx) is an antineoplastic and immunosuppressive drug. That may cause hepatotoxicity, whereas quercetin has anti-inflammatory and antiproliferative properties.

Aim of the Work: This study aimed to investigate the possible protective and therapeutic effects of quercetin against methotrexate (Mtx)-induced hepatotoxicity with biochemical and histopathological studies in rats.

Materials and Methods: Twenty four adult male wister albino rats were equally divided into four experimental groups: control (group I), Mtx group (group II) rats received (single dose of Mtx 20 mg/kg i.p.). QCT protective group (group III) rats pre-treated with QCT (20 mg/kg orally daily for 7 days before Mtx administration. QCT therapeutic group (group IV) rats cotreated with Mtx (20 mg/kg for i.p, single dose) then QCT were given as before. Following treatment, the animals were sacrificed, and liver tissue samples were histopathologically evaluated using H&E, masson's, PCNA staining, and serum transaminases were measured and statistically compared across all groups.

Results: Group II (Mtx group) demonstrated hydrobic degeneration of hepatocytes, congestion of hepatic sinusoids, central veins and portal veins. An apparent increase in collagenous fibers distribution around the central vein and portal tract was detected. In groups III (protected QCT), and group IV (QCT therapeutic groups), showed less histological injury compared to Mtx group as regards liver sections, but pretreatment with QCT in group III was more effective and liver section appeared highly improved except for mild dilation of central vein and blood sinusoids.

Conclusion: Methotrexate has a deleterious effect on the liver. Quercetin may be a potential adjuvant drug to reduce the hepatic side effects observed during Mtx therapy for various clinical conditions.

Received: 21 June 2019, **Accepted:** 19 September 2019

Key Words: Drug Effects; liver; methotrexate; quercetin; rats.

Corresponding Author: Eman El Bana, MD, Anatomy and Embryology Department, Faculty of Medicine, Benha University, Benha, Egypt, **Tel.:** +20 1028003776, **E-mail:** emanelbana88@gmail.com

ISSN: 1110-0559, Vol. 43, No. 1

INTRODUCTION

Cancer is characterized by abnormal growth and proliferation of cells. Variable techniques are used for the treatment of cancer including chemotherapy, radiation and surgery. Chemotherapy is the most common method of treatment^[1].

Chemotherapy is an essential cancer treatment and many anti-cancer agents have been developed. Although, the systemic toxicity and side effects induced by these agents limits their application^[2,3].

It is given systemically to patients to control the cancerous cells proliferation. But chemotherapy unfortunately has no selectivity and cannot differentiate between cancer cells and healthy cells^[4].

Methotrexate (Mtx), a folic acid analogue, and has been widely applied chemotherapeutic agent for treatment of malignancies, so it is considered an effective cytotoxic agent. It is known that the application of chemotherapeutics causes acute toxic effects in multiorgan systems.

Methotrexate is also an effective immunosuppressive and anti-inflammatory agent and prescribed for treatment of many chronic inflammatory disease including psoriasis, rheumatoid arthritis, Crohn's disease^[5,6].

Mtx is considered the primary choice as the cost effective and well-experienced treatment option in most cases. However, hepatotoxicity is the most serious side effect of Mtx and high doses may cause steatosis, stellate cell hypertrophy, anisonucleosis, and hepatic fibrosis^[7].

Natural antioxidants have been investigated as potential nutraceuticals to increase the effectiveness of chemotherapeutic agents and restrict their adverse effects^[8]. Quercetin (3,3', 4', 5,7-pentahydroxyflavone; QR) is a large class of polyphenolic compounds present more in plants and food sources. It is primarily present in vegetables, fruits, red wine, tea and other aromatic plants^[9]. QCT was considered a therapeutic agent to eliminate various toxicities, including nephrotoxicity^[10] cardiotoxicity^[11], neurotoxicity^[12] and hepatotoxicity^[13]. In addition, QCT has been proved to have antioxidant^[14], antimicrobial^[15],

anti-inflammatory^[16] and anticancer activities^[17] The antioxidant ability of quercetin may be explained due to its high diffusion into cell membranes permit it to sweepoxyradicals^[18].

The present study aimed to investigate the useful effects of QCT on Mtx-induced hepatotoxicity

MATERIALS AND METHODS

Animals and experimental protocols

Chemicals

Mtx (25 mg/mL injectable) was purchased from Hospira (UK) and quercetin (3,3',4',5,7-pentahydroxyflavone, L21600) was from Enzo Life Sciences (Farmingdale, New York, USA).

Animals

Twenty-four adult male Sprague–Dawley rats weighing 200–250 g aged 8 weeks were used in this experiment. The animals were obtained from Animal House, Faculty of veterinary Medicine, Benha University, Mushtohar, Animal Ethics Committee approved the design of the experiment. Animals were fed ad libitum on a standard diet and water under controlled temperature and light (24°C; 12 h light/12 h dark cycles, respectively). The rats were randomly divided into four experimental groups consisting of six animal each, as follows:

1. Control group (I): received distilled water single dose equivalent to of Mtx by intraperitoneal injection.
2. Mtx-treated group (II): animals received Mtx (single dose 20 mg/kg i.p.) according to^[19].
3. QCT protected group (III): animals received quercetin (20 mg/kg) orally daily for 7 days^[20] before Mtx administration.
4. QCT therapeutic group (IV): animals received QCT (20 mg/kg b.w.) daily orally for 7 days after Mtx administration.

At the end of the experiment, the rats were killed under anesthesia (xylazine 10 mg/kg i.p. and Ketalar 50 mg/kg i.p.), blood samples were collected and liver tissues were dissected. The livers of rats were fixed in 10% neutral formalin for histopathological examinations.

Histopathological analysis

Fixed materials were dehydrated in ascending grades of ethanol and embedded in paraffin wax and of 5-micrometer thick sections were prepared and subjected to staining with hematoxylin-eosin (Hx&E), and masson's Trichrome (MT).

Immunohistochemical staining of PCNA

Paraffin-embedded rat liver sections were deparaffinized and hydrated. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 5 minutes.

Sections were incubated over night with PCNA monoclonal antibody and rinsed with phosphate buffer saline (PBS) for 5 minutes. The monoclonal antibody was then linked with biotinylated goat anti-mouse IgG antibody for 30 minutes. After being washed with PBS for 3-5 minutes, the sections were incubated with streptavidin-conjugated peroxidase for 35 minutes. A brown colored reaction was developed by exposing sections to 3, 3'-diaminobenzidine tetrahydrochloride solution (DAB) for 5 minutes and washed in distilled water. Sections were counterstained with hematoxylin^[21] PCNA positive cells were counted in 10 randomly selected, non overlapping fields and were expressed as the number of PCNA positive cells/mm².

Biochemical analysis

One ml of blood sample was collected from all the experimental animals in a disodium EDTA tubes through retro-orbital plexus.¹⁴ The samples were centrifuged at 3000 rpm for 20 min. The plasma was separated and immediately processed for biochemical estimations. The plasma sample was subjected to analysis of biochemical parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin (TB) estimation using commercial biochemical kits and REFLOTROTRON 'PLUS machine by Roche, USA.

Morphometric study

Masson stain

- a. The mean area percentage of collagen deposition was quantified in five images from five non-overlapping fields of each rat using Image-Pro Plus program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA).
- b. The mean area percentage of PCNA immun-expression was quantified in five images from five non-overlapping fields of each rat using Image-Pro Plus program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA).

Statistical analysis

All the data collected from the experiment was recorded and analyzed using IBM SPSS Statistics software for Windows, Version 19 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) with Post Hoc LSD test was used to compare differences among the groups. In each test, the data was expressed as the mean (M) value, standard deviation (SD) and differences were considered to be significant at $P \leq 0.05$ and non-significant at $P > 0.05$.

RESULTS

Biochemical results

(Table 1,2 and Histogram 1,2) The present data showed that rats exposed to MTX (20 mg/kg body weight) revealed hepatic toxicity such as increased serum activities of

enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and The total bilirubin(TB) serum levels. Administration of QRC led to improvement in these markers.

The parameters of liver function in group II (Mxt group) were significantly increased ($P<0.05$) when compared to group I (control) and to groups III, IV. But no significant change detected in groups III, IV when compared with control.

Histopathological changes

Heamatoxylin and eosin stain

The examination of control liver tissues (group I) showed normal hepatic architecture .Each hepatic lobule consisted of anastomosing radially distributing hepatic cells (hepatocytes). The hepatocytes were polygonal in shape with well-defined boundaries. Their cytoplasm was acidophilic. The majority of cells had a single rounded, vesicular, centrally placed nucleus. The hepatic sinusoids were seen as narrow spaces in between adjacent plates. The hepatic portal tracts were seen at the periphery of the lobule. Portal tracts had branches of the portal vein, hepatic artery and bile duct (Figure 1).

The liver sections of rats exposed to methotrexate (group II) showed that the hepatocytes still retained the lobular pattern of cords radiating from the central vein, but the cell plates were very thin and widely separated because of the dilatation of sinusoids between them. Affected and necrotic hepatic cells with vacuolations in the cytoplasm. The central veins were dilated and congested with cellular infiltration. The inflammatory cells infiltrations were detected around the region of portal triads and around central veins. The portal veins were dilated and congested. In addition to proliferation of the bile ductules were observed (Figure 2).

On examination of liver section of protected quarcien group (group III), there were marked improvement of liver section. The hepatic cords were normally arranged around sinusoids which appeared more or less normal except the persistence of mild dilatation of sinusoids in some areas. portal tracts were nearly normal with some congestion in hepatic artery (Figure 3).

The liver sections of therapeutic QCT (group IV) showed mild dilatation of sinusoids and dispersed inflammatory cells through the parenchyma of the liver.

Dilatation and congestion of portal vein were present (Figure 4).

Masson's trichrome stain

On examination of control group (I) liver section showed that normal distribution of collagen fibers around central veins. And fine threads of collagen fibers were detected in the portal area around the hepatic portal vein and bile duct (Figure 5). In Group II (Mtx treated) there were marked increase of the collagen fibers deposition around hepatic central vein and portal tracts (Figure 6). In group(III) showed less collagen fibers deposition in portal tract and around central vein (Figure 7).Group (IV) showed moderate deposition of collagen fibers in portal tract vein (Figure 8).

PCNA stain:Control group (group I) showed few number of cells with +vePCNA immunoreactivity (Figure 9) Group II showed massive +ve PCNA reaction (Figure 10) .Liver section of rats which protected QCT (group III) showed few hepatocytes with +ve PCNA reaction (Figure 11) .But in therapeutic QCT (group IV) showed more hepatic cells with +ve PCNA reaction (Figure 12).

Morphometric results

Masson stain

The mean area % of collagen deposition for all groups was represented in (Table 3) and (Histogram 3). There was insignificant increase in collagen deposition mean area% ($P>0.05$) in groups III and IV as compared with control group. But area % of collagen deposition was highly significantly increased in methotrexate treated group as compared to control, III and IV groups ($P<0.01$). Also, area % of collagen deposition was insignificantly increased in group IV as compared to group III ($P>0.05$).

PCNA immunostaining

The mean area % of PCNA immuno-expression for all groups was represented in (Table 4) and (Histogram 4). There was insignificant increase in PCNA immuno-expression ($P>0.05$) in groups III and IV as compared with control group. But area % of PCNA immuno- reactivity was highly significant increase in methotrexate treated group as compared to control, III and IV groups ($P<0.01$). Area % of PCNAimmuno- reactivity was insignificantly increased in group IV as compared to group III ($P>0.05$).

Table 1: Showing mean values of AST and ALT ± SD in the 4 groups

(IU/L) Mean ± SD	Group I	Group II	Group III	Group IV	F test	P value
ALT	17.5± 9.54	94± 20.85	18.75± 8.22	37.5 ± 14.9	25.28	0.000
AST	17.5± 9.54	79± 17.17	18.25± 12.34	27.25± 9.84	21.69	0.000
Significance ≤ 0.05	With groups II	With groups I,III & IV	With group II	With group II		

Table 2: Showing mean values of ALP and TB \pm SD in the 4 groups

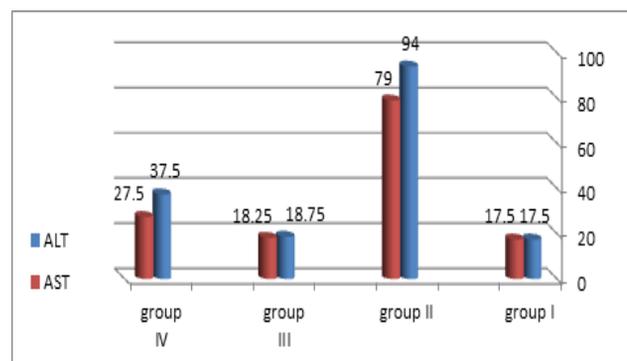
Mean \pm SD	Group I	Group II	Group III	Group IV	F test	P value
ALP (IU/L)	55.75 \pm 16.36	145.75 \pm 11.79	62.5 \pm 14.57	77 \pm 21.43	25.31	0.000
TB (mg/dl)	0.58 \pm 0.4	6 \pm 2.9	0.7 \pm 0.39	0.93 \pm 0.17	12.35	0.001
Significance \leq 0.05	With groups II	With groups I,III & IV	With group II	With group II		

Table 3: Showing the mean area %, SD of collagen fibers deposition in groups I, II, III and IV

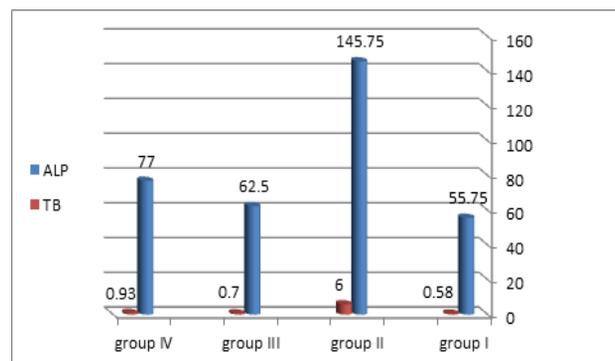
Mean% \pm SD	Group I	Group II	Group III	Group IV	F test	P value
MA	0.303 \pm 0.358	8.88 \pm 2.45	1.3 \pm 0.41	2.68 \pm 1.17	23.266	0.000
Significance \leq 0.05	With groups II	With groups I,III & IV	With group II	With group II		

Table 4: Showing mean values of area % PCNA immunoreactivity \pm SD in the 4 groups

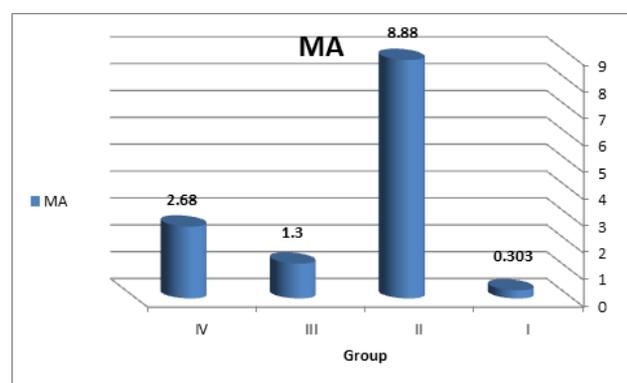
Mean% \pm SD	Group I	Group II	Group III	Group IV	F test	P value
MA	6.26 \pm 3.58	39.1 \pm 13.63	8.65 \pm 3.29	18.32 \pm .56	12.66	0.002
Significance \leq 0.05	With groups II	With groups I,III & IV	With group II	With group II		



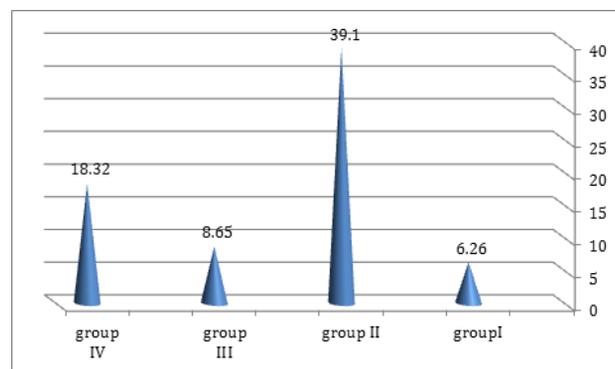
Histogram 1: showing mean values of AST and ALT in the 4 groups



Histogram 2: Showing mean values of ALP and TB in the 4 groups



Histogram 3: Showing the mean area % of collagen fibers deposition in groups I, II, III and IV.



Histogram 4: Showing mean values of area percent PCNA immunoreactivity in the 4 groups.

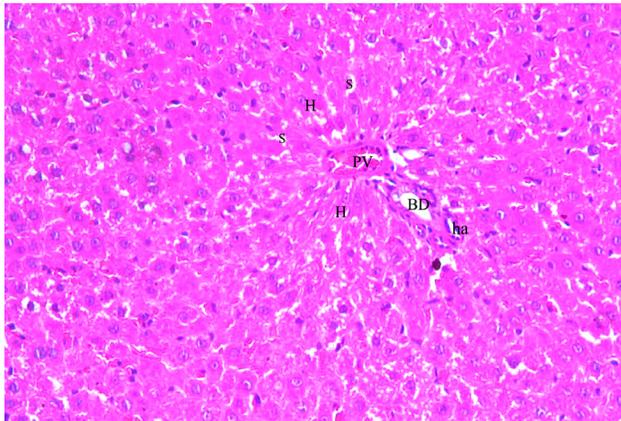


Fig. 1: Photomicrograph of a liver section of control rat showing normal hepatic architecture with cords of hepatocytes(H) separated by normal sinusoids(S),with normal portal tract formed of branches of hepatic artery(ha),portal vein(PV) and bile duct(BD). (HX&E 200)

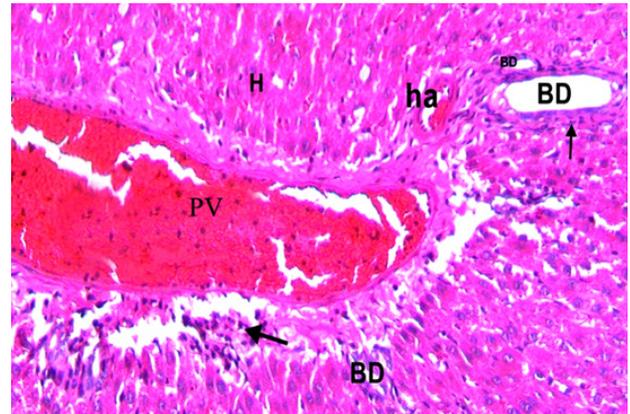


Fig. 4: Photomicrograph of a liver section of QCT therapeutic (group IV) rats showing more or less normal hepatocytes(H) . Congested and dilated portal vein(PV) .Proliferation of bile ducts(BD) and inflammatory cellular infiltrations (arrows) in the portal tract. (HX&E 200)

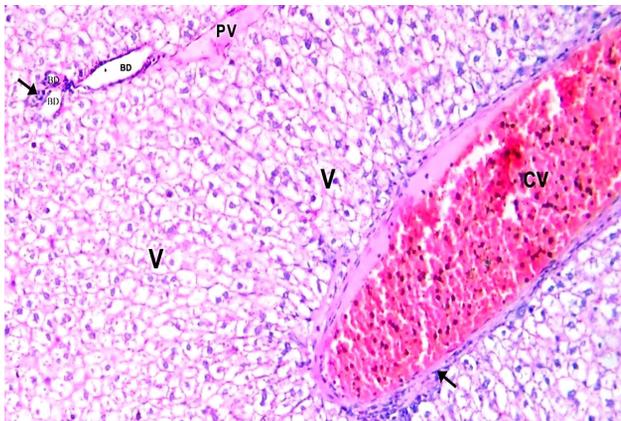


Fig. 2: Photomicrograph of a liver section of Mtx treated rat showing loss of hepatic architecture, marked hepatocytes vacuolations(V), dilated and congested central vein(CV) with inflammatory cellular infiltrations (Arrows),The portal tract shows congested portal vein (PV)& proliferated bile ducts(BD). (HX&E 200)

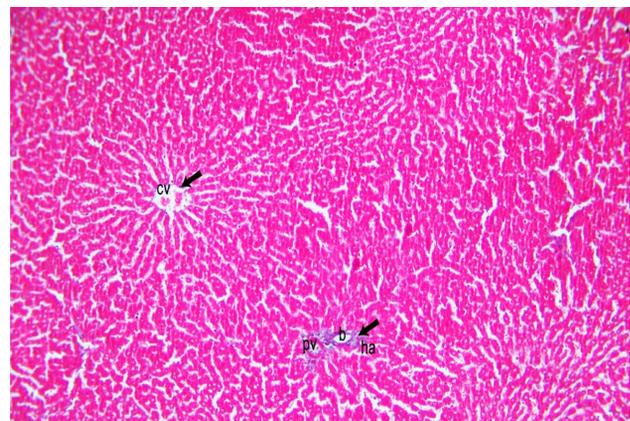


Fig. 5: Photomicrograph of a liver section of control rat (group I) showing normal distribution of collagen fibers in portal tract & around central vein. (Masson Trichrome X 100)

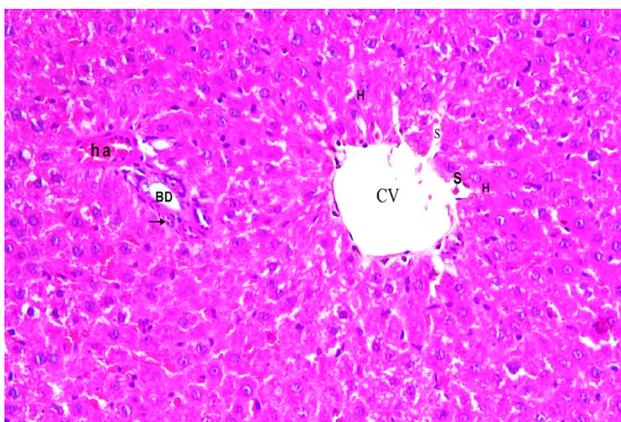


Fig. 3: Photomicrograph of a liver section of quercetin protected group (group III) rats showing restoration of normal arrangement of hepatocytes(H),although dilatation in some blood sinusoids(S)& central vein(CV). The portal tract shows mild congestion of hepatic artery(ha)minimal cellular inflammation(Arrow). (HX&E 200)

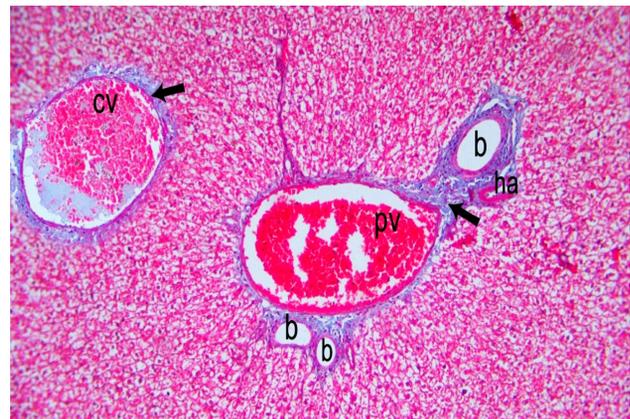


Fig. 6: Photomicrograph of a liver section of Mtx treated (group II) rats showing marked increase in deposition of collagen fibers in portal tract & around central vein. (Masson Trichrome X 100)

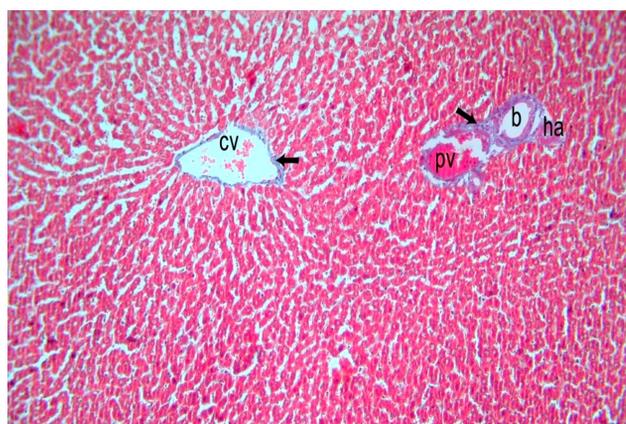


Fig. 7: Photomicrograph of a liver section of QCT protected Mtx treated (group III) rats showing less collagen fibers deposition in portal tract & around central vein. (Masson Trichrome X 100)

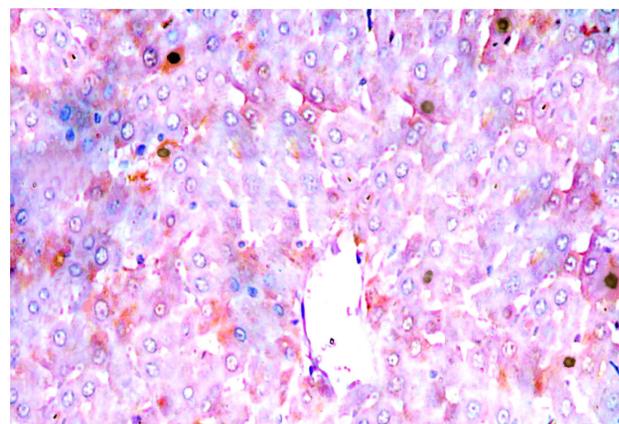


Fig. 10: Photomicrograph of a liver section of MTX treated rats (group II) showing abundant hepatocytes with positive PCNA. (PCNA x 200)

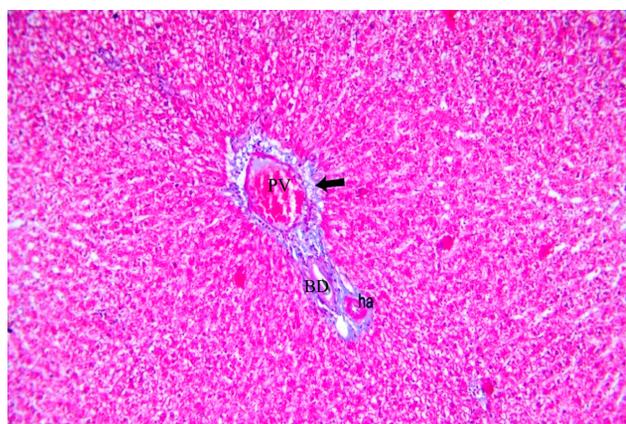


Fig. 8: Photomicrograph of a liver section of QCT cotreated with Mtx treated (group IV) rats showing moderate deposition of collagen fibers in portal tract. (Masson Trichrome X 100)

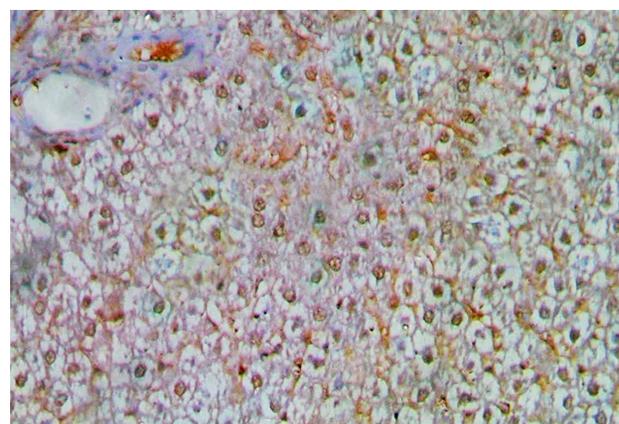


Fig. 11: Photomicrograph of a liver section of QCT protected MTX (group III) treated rats showing few hepatocytes with positive PCNA. (PCNA x 200)

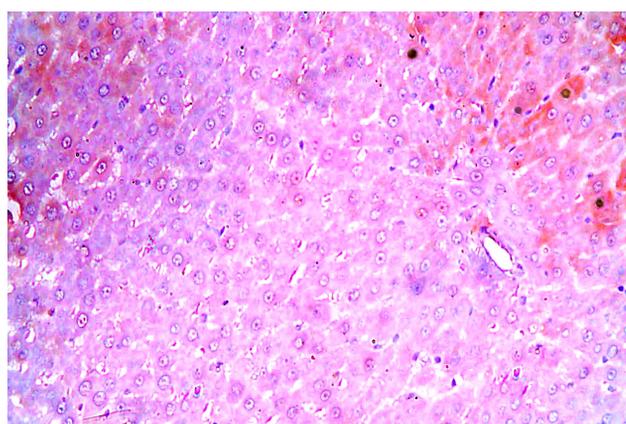


Fig. 9: Photomicrograph of a liver section of control rats (group I) showing hepatocytes with few number of PCNA positive nuclei. (PCNA x 200)

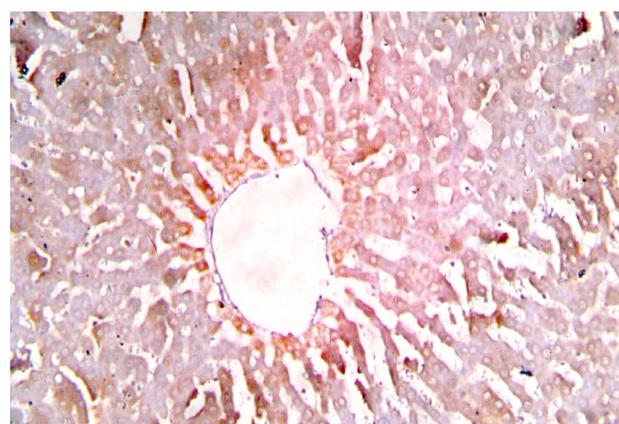


Fig. 12: Photomicrograph of a liver section of QCT cotreated with Mtx treated rats (group IV) showing most hepatocytes with positive PCNA. (PCNA x 200)

DISCUSSION

Hepatotoxicity is one of the major side effects of methotrexate (Mtx), which restricts its clinical use. Mtx increases the production of free radicals that are released by stimulated neutrophils, and thus increases cellular damage that can lead to, inflammation, necrosis. Patients treated with Mtx show nonspecific liver histological changes such as focal hepatic necrosis, fatty change, portal tract inflammation, and fibrosis^[22].

Enzyme levels such as ALT, AST, and LDH are often used to assess damage of liver. Liver injury causes damage or necrosis of membrane, which lead to intracellular enzymes to diffuse and be detected in serum. Elevation of the levels of these enzymes in the serum indicate that the hepatic membrane's functional integrity has been lost. Serum, ALP, and total bilirubin levels were also associated with liver cell function. The rise in serum ALP is affected by the increased bile pressure^[23,24].

In the present study, results showed that Mtx administration increased the levels of ALT, AST, ALP, and total bilirubin in the serum of rats which was significantly increased as compared to the normal control group (all $p < 0.001$) and this data agreed with many studies^[25,26].

Quercetin administration significantly restored these parameters. In this study, pre- and cotreatment administration of quercetin with Mtx protected against the increase of plasma levels of ALT, AST, ALP, bilirubin, which is a marker of protection of liver by QCT. The observations of hepatoprotective activity of QCT in this study were confirmed in another previous studies^[27,20,28].

This reversal in enzyme levels after QCT treatment might be due to membrane-stabilizing activities that suppress the leakage of intracellular enzyme. This was compatible with the accepted view that liver cell regeneration, liver parenchyma recovery, and serum aminase levels return to normal^[24,29,30,25].

In the current study it was proved that Mtx administration caused marked deterioration of the histopathological architecture of the liver tissue when compared to normal control group. Such as the hepatocytes appeared large and vacuolated with small dense nuclei. The nuclear changes indicated decrease in cellular activity. This is confirmed by^[31] who stated that hepatocyte ballooning and spotty necrosis especially in zone 3 of the acinus, which indicated decrease in cellular activity.

The exact mechanisms of Mtx hepatotoxicity are unknown. Many studies stated that Mtx-induced hepatic injury may be due to oxidative stress^[5]. Recently, some studies reported that the intestinal flora might play a critical role in liver fibrosis and hepatocellular carcinoma^[32,33,34].

In the study, Mtx-treated group demonstrated various liver histological changes such as dilated and congested central veins, periportal inflammation, congested portal veins. These observations in accordance with the study

of^[35]. Dilatation of sinusoids due to the very thin cell plates which are widely separated. The thinning of cell plates is explained by the compression of the hepatocytes in the cords adjacent to the dilated and congested sinusoids where the initial toxic effects probably occurred^[36].

Proliferated bile ductules was noticed in liver sections and this in agreement with the study of^[37] in his study on the effect of Mtx administration on the liver of rats.

Mtx administration led to increase in collagenous fibers around the central vein, portal tract and between hepatocytes was clearly observed in this group as demonstrated by masson's trichrome stain. This observation confirmed the studies which had been reported concerning the hepatic fibrosis and this data approved in many studies^[38,39,40].

The causes of progressive liver damage may be explained due to cytostatic effects of Mtx on water content and dry weight of nucleus and cytoplasm and this leads to decrease hepatic repair and regeneration^[41].

Quercetin, an antioxidant flavonoid present enormously in plants, green leafy vegetables and fruits has been reported beneficial effects in many diseases^[42].

In the current study detected that quercetin limit the methotrexate induced hepatotoxicity in rats. The histological and histochemical changes in the liver of QCT protected group were slightly less than therapeutic QCT group and both groups more better than group II. This means that using quercetin as prophylactic agent is more beneficial than as therapeutic one.

The hepatoprotective effect of quercetin against methotrexate-induced hepatotoxicity occurs by induction of phase I enzymes by methotrexate without changing the outcome of methotrexate metabolism^[43].

pretreatment with QCT restored the hepatic architecture except for only mild sinusoidal dilatation while in group IV liver tissue sections improved but still congested and dilated central vein and sinusoids were noticed and this in accordance with^[44] who stated that quercetin has preliminarily protective effect on liver injury in rats in his study on the effect of QCT on ethanol-induced cirrhosis and recovers necrosis and inflammation as the study of^[45] on his study of QCT on ccl-induced cirrhosis.

Quercetin demonstrates antioxidant property by scavenging free radicals and inhibiting the oxidation of various molecules^[46,47] anti-inflammatory response and induction antifibrotic potential^[48].

CONCLUSION

QCT supplementation may suppress Mtx induced hepatic toxicity during chemotherapy, Pre- and cotreatment with QCT restored the hepatic structure and antioxidant status

CONFLITS OF INTEREST

There are no conflicts of interests.

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المخلص العربي

التأثير المحتمل للحمايه بالكيرسيتين ضد السمية الكبدية الناجمة عن الميثوتريكسات في الجرذان البيضاءالبالغه: دراسة كيميائية ونسجية وهستولوجية مناعية

ايمان علي البنا – كمال مصطفى كمال

كلييه طب بنها – قسم التشرييح والاجنه

الخلفية: الميثوتريكسيت هو دواء مضاد للورم ومثبط للمناعة يستخدم في علاج الاورام الخبيثة. قد يتسبب في تسمم الكبد، في حين أن كيرسيتين خصائص مضادة للالتهابات ومضادة للحساسية ومضادة للتكاثر.

الهدف من العمل: تهدف هذه الدراسة إلى استكشاف الآثار الوقائية والعلاجية المحتملة للكيرسيتين ضد السمية الكبدية الناجمة عن الميثوتريكسات مع الدراسات الكيميائية والنسجية والكيميائية الحيوية في الجرذان.

المواد والطرق: تم تقسيم الجرذان البالغه البيضاء الاربع والعشرون إلى أربع مجموعات وهم تجريبية: الضابطه (المجموعة الأولى)، الميثوتريكسات (المجموعة الثانية) تم اعطاء الجرذان جرعة واحدة من الميثوتريكسيت (٢٠ ملج / كج) / كج) بالحقن في الغشاء البريتوني. المجموعة الثالثة (الكيرسيتين الحمايه) تم اعطائها كيرسيتين ٢٠ ملج / كج عن طريق الفم يوميا لمدة ٧ أيام ثم الميثوتريكسات جرعه واحده. المجموعة الرابعه (كيرسيتين العلاجية) تم اعطائها الميثوتريكسات جرعة واحدة ثم الكيرسيتين يوميا لمدة ٧ ايام، في نهايه التجربه تم التضحية بالحيوانات، وتم تقييم عينات أنسجة الكبد. باستخدام صبغات هيماتوكسلين وماسون وصبغه الاميون بيكنا. وتم قياس ترانسامينازات المصل ومقارنتها إحصائياً في جميع المجموعات.

النتائج: أظهرت المجموعة الثانية (مجموعة الميثوتريكسات) أضراراً لبعض خلايا الكبد، واحتقان الجيوب الكبديه والأوردة المركزية. وزيادة في توزيع الألياف الكولاجينية حول الوريد المركزي والقنوات البوابية. في المجموعتين الثالثة (الكيرسيتين الحمايه)، والرابعة (الكيرسيتين العلاجية)، أظهرت إصابة نسيجية بالكبد أقل بالمقارنة بمجموعة الميثوتريكسات، ولكن المجموعة الثالثة (الكيرسيتين الحمايه) أظهرت تحسناً أكثر عن المجموعة الرابعة (الكيرسيتين العلاجية) باستثناء تمدد خفيف في الجيوب الكبديه.

الخلاصة: الميثوتريكسات له تأثير ضار على الكبد. قد يكون كيرسيتين دواءً مساعداً للحد من الآثار الجانبية الكبدية التي لوحظت خلال علاج الميثوتريكسات لمختلف الحالات السري.