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The Protective Effects of Felodipine on Methotrexate-Induced Hepatic Toxicity in Rabbits

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Abstract

Background Methotrexate is folic acid antagonist, used in the therapy of various types of diseases. Oxidative stress and inflammation have the major role in methotrexate toxicity.

Objective To study the protective effects of felodipine against methotrexate-induced hepatotoxicity in rabbits.

Methods Twenty four rabbits divided randomly into three groups. Group I was left without treatment, group II received a dose of 20 mg/kg methotrexate (MTX) intraperitoneally from 3rd day for three successive days and group III received a dose of 0.5 mg/kg felodipine orally for 7 days in addition to MTX therapy similar to group (2). On 8th day the following parameters (liver enzymes, liver tissue homogenate of glutathione, malondialdehyde and tumor necrosis factor-α) were monitored. As well as assessment of histological changes on liver tissue sections after scarification.

Results Administration of felodipine significantly decreased the elevated levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), malondialdehyde and tumor necrosis factor-alpha. It also elevated glutathione levels significantly, with improvement of histological features related to MTX exposure in group III compared with group II.

Conclusion Felodipine can protect hepatic tissue against MTX-induced hepatotoxicity.

Keywords Hepatotoxicity, Methotrexate, Felodipine, Glutathione, Malondialdehyde, Tumor Necrosis Factor-alpha.

List of abbreviation: ALP = Alkaline phosphatase, ALT = Alanine Aminotransferase, AST = Aspartate aminotransferase, DHFR = Dihydrofolate reductase, D.W = Distilled water, DNA=Deoxyribonucleic acid, E=Eosin, HAI = Histological Activity Index, H = Hematoxylin, GSH=Glutathione, iNOS = Cytokine-induced nitric oxide synthase, L-type = Long-Lasting calcium channels, MDA = Malondialdehyde, MTX = Methotrexate, NADP = Nicotinamide adenosine diphosphate, NADPH = Nicotinamide adenosine diphosphate hydrogen, NO = Nitric oxide, NOS = Nitric oxide synthase, ROS = reactive oxygen species, SD = Standard deviation, SPSS = Statistical Package for social Science, TNF-α=Tumor necrosis factor alpha

Introduction

The liver plays a major role in transforming and clearing chemicals which lead to increase its susceptibility to the toxicity from these agents; drugs are important causes of liver injury, more than 900 drugs, toxins, and herbs have been attributed to cause hepatic injury [(1)].

Methotrexate (MTX) is one of the folic acid antagonists, which is widely used in the therapy of various types of diseases [(2)]. It is used in high doses for different types of malignancies such as breast and lung carcinomas in addition to leukemia, while low doses used to treat inflammatory conditions and in the treatment of various autoimmune diseases including rheumatoid arthritis, juvenile idiopathic arthritis and psoriasis [(3,4)]. Moreover it is the first choice treatment for ectopic pregnancy [(2,5)]. MTX induced toxicity appears to be a consequence of the interaction of many factors that include the length of treatment, dosing schedule, type of disease, patient risk factors and the presence of genetic

and molecular apoptotic factors [2,6,7]. Methotrexate's therapeutic and toxic effects are a result of its capability to limit DNA and RNA synthesis by inhibiting dihydrofolate reductase (DHFR) and thymidylate synthetase that are essential for DNA synthesis [5,6]. This blocking in the synthesis of nucleic acids, certain amino acids and consequently proteins might lead to damage of organelles and plasma membranes of the hepatic parenchymal cells and interfering with their function and allowing leakage of enzymes [8]. Under normal conditions, NADPH is used by glutathione reductase to maintain the reduced state of cellular glutathione, which is a well-known as an important cytosolic antioxidant protecting against reactive oxygen species (ROS). It has been established that the cytosolic NAD (P)-dependent dehydrogenases [9] and NADP malic enzyme are repressed by MTX, indicating that the drug could reduce the availability of NADPH in cells via inhibiting pentose cycle enzymes [10]. MTX may also depress nucleic acid metabolism, due to the interference with the pentose phosphate shunt. Therefore, the significant reduction in glutathione levels promoted by methotrexate leads to a reduction of efficiency of the antioxidant enzyme defense system, which lead to sensitizing the cells to ROS [9]. Thus, the damaging effect of methotrexate is partly due to its direct toxic effect via increasing ROS production [6].

Taking into consideration the relationship between glutathione and the toxic effects of methotrexate, interest has focused on compounds that have the ability to work as antioxidants [11]

Felodipine is a calcium channel antagonist belongs to the second generation dihydropyridines; its targeting long lasting (L-type) calcium channels [12,13] and used in treatment of essential hypertension, or prophylaxis of angina [13,14] and symptomatic relief in raynaud's disease [15]. It is considered as a chain-breaking antioxidant [16] according to Janero et al who reported a direct effect of calcium antagonists on the lipid peroxidation of cardiac membranes [17].

This study was designed to evaluate whether the hepatotoxic effects caused by administration of methotrexate could be prevented or amileroated by concomitant felodipine treatment.

Methods

This prospective randomized controlled study was conducted from January 2014 through May 2014 at the Department of Pharmacology, College of Medicine, Al-Nahrain University. Experimental protocols were approved by the Institutional Review Board (IRB).

Twenty four healthy, domestic rabbits aged 3-4 months and weighing (600-1300) gm. of both sexes were studied. Before starting the experiment, rabbits were left for 72 hours to acclimatize to the animal room conditions and were maintained on an environment of controlled temperature with 12 hours light/dark cycle with free access to food and tap water. They were divided randomly into three groups, each group including eight animals. Group I as a (Negative control): rabbits were left without treatment. Group II as (Positive control): rabbits were given MTX injection (Ebwe, Australia) as an intraperitonial dose of (20 mg/kg) [18] from 3rd day of the experiment for three successive days. Group III (felodipine + MTX): rabbits were given felodipine tablet (Astrazeneca, Sweden) in a dose of 0.5 mg/kg orally once daily [19] for 7 days, and then MTX was given intraperitonially in a dose of (20 mg/kg) similar to group 2. At the end of experiment, the rabbits were subjected to blood collection under anesthesia by ether inhalation, the blood collected directly from the heart, centrifuged to get serum, which stored at -20°C for biochemical analysis.

After sacrification, the liver tissue were excised by thoracic section, two portions was isolated one of them were fixed in 10% formalin for 24 hours and embedded in paraffin blocks and underwent cutting by microtome into 5 mm thick sections, in addition to staining with

hematoxylin-eosin (H-E) stains. These sections were examined by Olympus CH-2 light microscope by histopathologist. The other portion was mobilized into the cooling box quickly to prevent autolysis and homogenization was done by rinsed the liver piece with chilled phosphate buffer saline (1X PBS) at 4 °C, blotted with filter paper and weighed. Half gram of liver tissue was homogenized in 5 ml of (1X PBS) utilizing tissue homogenizer [20] for 1 minute at 4 °C, then after two freeze thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g at 4°C. The supernatant was obtained and stored at -20°C for the assay of reduced glutathione, malondialdehyde and tumor necrosis factor alpha levels in the tissue.

Chemicals:

Reagent kits for assay of transaminases were purchased from BioMerieux-France, ALP assay kit was purchased from Biolabo Sa France, total bilirubin assay kit was obtained from Randox-United Kingdom, and kits for total protein and albumin were purchased from Linear Chemicals – Spain. Reagent ELISA kits for determination of tissue malondialdehyde (MDA), reduced glutathione (GSH) and tumor necrosis factor alpha (TNF-α) were purchased from Cusabio - China. The work was done in accordance with the method prescribed in each diagnostic kit.

Histological Analysis:

Score of liver damage severity was semi quantitatively assessed using the modified Histological Activity Index [21] 'Modified HAI' (table 1).

Statistical Analysis

Statistical analysis was performed by using computer program SPSS -19. Crude data was analyzed to obtain mean and standard deviation (SD). Student paired *t- test* was used to compare between two groups. P of ≤ 0.05 was considered significant and P-value of ≤ 0.001 considered as highly significant. The histological score comparison between each group were done by *Chi square* test [22].

Table 1: Modified Histological Activity Index (Modified HAI) grading: necro-inflammatory scores [21]

	SCORE
A. Periportal or periseptal interface hepatitis (piecemeal necrosis)	
Absent	0
Mild(focal, few portal areas)	1
Mild\moderate(focal, most portal areas)	2
Moderate(continuous around<50% of tracts or septa)	3
Severe(continuous around>50% of tracts or septa)	4
B.Confluent necrosis	
Absent	0
Focal confluent necrosis	1
Centrolobular necrosis in some areas	2
Centrolobular necrosis in most areas	3
Centrolobular necrosis +occasional portal-central (P-C)bridging	4
Centrolobularnecrosis+ multiple P-C bridging	5
Panlobular or multilobular necrosis	6
C.Focal (spotty) lytic necrosis, apoptosis and focal inflammation	
Absent	0
One focus or less per 10× objective	1
One to four foci per10× objective	2
Five to 10 foci per 10× objective	3
More than 10 foci per 10× objective	4
D.Protal inflammation	
None	0
Mild, some or all portal areas	1
Moderate, some or all portal areas	2
Moderate/marked, all portal areas	3
Marked, all portal areas	4
Maximum possible score for grading	18

Results

Analysis of t-test revealed a highly significant increase ($P \leq 0.001$) in the level of S. ALP, S. AST, tissue MDA and TNF-α in positive control group II (MTX treated) in comparison with negative control group I, in addition to significant decrease (P ≤ 0.05) in the level of S. total protein, S. albumin in group II compared with group I. It is also observed that group II showed statistically highly significant decrease ($P \leq 0.001$) in S. GSH than group I. The level of S. ALT show significant increase ($P \leq 0.05$) in group II also unlike the level of S. total bilirubin (table 2). Comparison between group II rabbits with group III (after concomitant use of felodipin) revealed statistically significant decrease (P ≤ 0.05) in the level of S. ALP, S. ALT and S. AST in the latter group with no significant difference in the level of S. total protein, S. albumin and S. total bilirubin in both

of them. There is a high significant increase ($P \leq 0.001$) in the level of T. GSH while high significant decrease ($P \leq 0.001$) in the level of tissue MDA and TNF-α in group III rather than group II (table 3).

Table 2: Comparison between –ve control group (Group I) and +ve control group (Group II) in relation to S. Total Protein, S. Albumin, S. Total Bilirubin, S. ALP, S. ALT, S. AST, T. GSH, T. MDA and T. TNF-α.

Parameter	Group I -ve control (Not treated) N=8 Mean±SD	Group II +ve control (MTX treated) N=8 Mean±SD	*P* value
S. Total Protein (g/dl)	5.63±0.91	4.28±0.47	0.0023*
S. Albumin (g/dl)	2.63±0.35	2.1±0.21	0.0029*
S. Total Bilirubin (mg/dl)	0.11±0.11	0.12±0.13	0.878
S. ALP (U/l)	59.25±6.02	128.13±22.52	< 0.0001**
S. ALT (U/l)	49.25±11.02	183.75±97.54	0.0017*
S. AST (U/l)	42.25±6.5	232.75±116.25	0.0004**
T. GSH (nmol/l)	35.76±3.6	12.33±0.63	< 0.0001**
T. MDA (ng/l)	122.28±0.69	135.2±4.2	< 0.0001**
T. TNF-α (pg/l)	85.53±3.73	170.89±14.8	< 0.0001**

* significant difference at $P \leq 0.05$ ** highly significant difference at $P \leq 0.001$

Table 3: Comparison between +ve control group (Group II) and MTX + Felodipine treated group (Group III) in relation to S. Total Protein, S. Albumin, S. Total Bilirubin, S.ALP, S.ALT, S. AST, T. GSH, T. MDA and T. TNF-α.

Parameter	Group II +ve control (MTX treated) N=8 Mean±SD	Group III (MTX+Felodipine) N=8 Mean±SD	*P* value
S. Total Protein (g/dl)	4.28±0.47	4.66±0.32	0.0762
S. Albumin (g/dl)	2.1±0.21	2.24±0.15	0.1592
S. Total Bilirubin (mg/dl)	0.12±0.13	0.14±0.12	0.798
S. ALP (U/l)	128.13±22.52	91.88±20.55	0.0046*
S. ALT (U/l)	183.75±97.54	94.0±17.22	0.0225*
S. AST (U/l)	232.75±116.25	115.5±23.85	0.0143*
T. GSH (nmol/l)	12.33±0.63	20.83±1.51	< 0.0001**
T. MDA (ng/l)	135.2±4.2	125.66±4.57	0.0007**
T. TNF-α (pg/l)	170.89±14.8	122.73±10.88	< 0.0001**

* significant difference at $P \leq 0.05$ ** highly significant difference at $P \leq 0.001$

There is also a statistically significant increase ($P \leq 0.05$) in the "modified HAI" scoring in group II when compared with group I (table 4). The histopathological examination of negative control group (group I) reveals normal hepatic tissue, no portal or periportal inflammation, necrosis and fibrosis as shown in figure 1, while there was a significant loss in hepatic architecture in positive control group (group II) demonstrated as portal inflammation with periportal interface hepatitis (piecemeal necrosis), centrilobular necrosis and bridging necrosis as in figure 2. However; this score showed a statistically significant decrease ($P \leq 0.05$) in group III when compared with group II (table 5). The histopathological examination of

MTX+felodipine treated group (group III) reveals significant restoration of hepatic architecture with mild portal inflammation of mononuclear cells infiltrate as shown in figure 3.

Table 4: Comparison of histopathological changes (by modified HAI scoring) between –ve control group (Group I) and +ve control group (Group II).

Score	Group I (-ve control) (Not treated) N=8		Group II (+ve control) (MTX treated) N=8		*P* value
	No.	%	No.	%	
0	8	100	0	0.0	0.003*
4	0	0.0	4	50.0	
5	0	0.0	2	25.0	
6	0	0.0	1	12.5	
8	0	0.0	1	12.5	

* Denote significant difference at $P \leq 0.05$

Table 5: Comparison of histopathological scores between +ve control group (Group II) and MTX+felodipine treated group (Group III)

Score	Group II (+control) (MTX treated) N=8		Group III (MTX+felodipine treated) N=8		*P* value
	No.	**%**	**No.**	**%**	
1	0	0.0	4	50.0	0.015*
2	0	0.0	3	37.5	
3	0	0.0	1	12.5	
4	4	50.0	0	0.0	
5	2	25.0	0	0.0	
6	1	12.5	0	0.0	
8	1	12.5	0	0.0	

* Denote significant difference at $P \leq 0.05$

Discussion

The results of the present study indicate that MTX lead to oxidative tissue damage by increasing lipid peroxidation and consequently inflammation in the liver tissue and decreasing the level of antioxidant enzymes. Also, increased AST, ALT and ALP with decreased levels of total protein and albumin, which considered as biochemical indicators of liver damage; the histopathological findings support this. Subsequent results were shown that felodipine provided significant protection from the effects of MTX on the liver. The damage of liver tissue after MTX exposure is a well known phenomenon, and the clear sign of hepatic injury is the leakage of hepatic enzymes into plasma.

Definitely, both the biochemical parameters and histological manifestations supported a diagnosis of liver damage. The elevated levels of serum enzymes of ALT, AST and ALP in MTX-treated rabbits indicate the increased permeability, damage or necrosis of hepatocytes [23], these findings have been agreed with other studies [27,29].

The decreased levels of serum total proteins were due to the dissociation of polyribosomes from endoplasmic reticulum and also due to defects in protein biosynthesis [24]. Consequently, albumin level reduced as it represents the larger portion of serum proteins and due to increased renal loss of albumin secondary to MTX induced nephrotoxicity, in agreement with Jwied in 2009 and Rizvi in 2012 who reported that liver disorders are related to a decrease in the serum levels of total proteins [25,26].

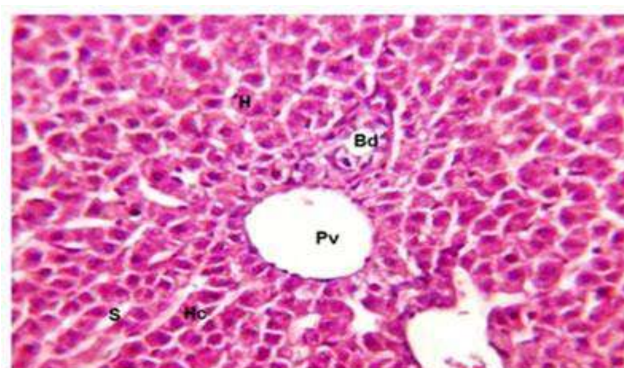

Figure 1: Section of liver tissue of group I (control group) on day 8 of the experiment shows normal hepatic tissue, no portal or periportal inflammation, necrosis and fibrosis. H & E stain, (40X). H: hepatocyte, Hc: hepatic cord, S: sinusoid, Pv: portal vein, Bd: bile duct.

It is well known that oxidative stress plays an important role in the tissue damage due to MTX [7,27,28]. The extent of severity caused by MTX-associated liver injury was linked to both the dose and the treatment interval [27]. The significant lowering in glutathione (GSH) levels induced by MTX as expressed above could produce a reduction of effectiveness in the antioxidant enzyme defense system and increased sensitivity of the cells to ROS [9]. MDA was a stable metabolite of the free radical caused lipid peroxidation cascade [29]. It is used usually as a marker of oxidative stress and destroying of lipid layers [30]. As described above, methotrexate leads to lipid peroxidation via significant elevations in MDA levels.

The lipid peroxidation mediated by oxygen-free radicals was thought to be an important cause of destruction and damage to the cell membranes and was suggested to be a contributing factor of the development of MTX- mediated tissue damage [29].

The free-radicals were seem to trigger the accumulation of leukocytes in the tissues involved, and thus exacerbate tissue injury indirectly through the activation of neutrophils. It has been exposed that activated neutrophils secrete enzymes and liberate oxygen radicals [31] also free radicals have a direct damaging effects on these tissues [27]. Moreover, it has been determined that methotrexate leads to histological damage including portal inflammation with centrilobular necrosis. The histological alterations may occur though methotrexate oxidative properties. These results are confirmed with other previous studies [23,27] with difference in the severity due to the difference in the duration of the toxicity induction.

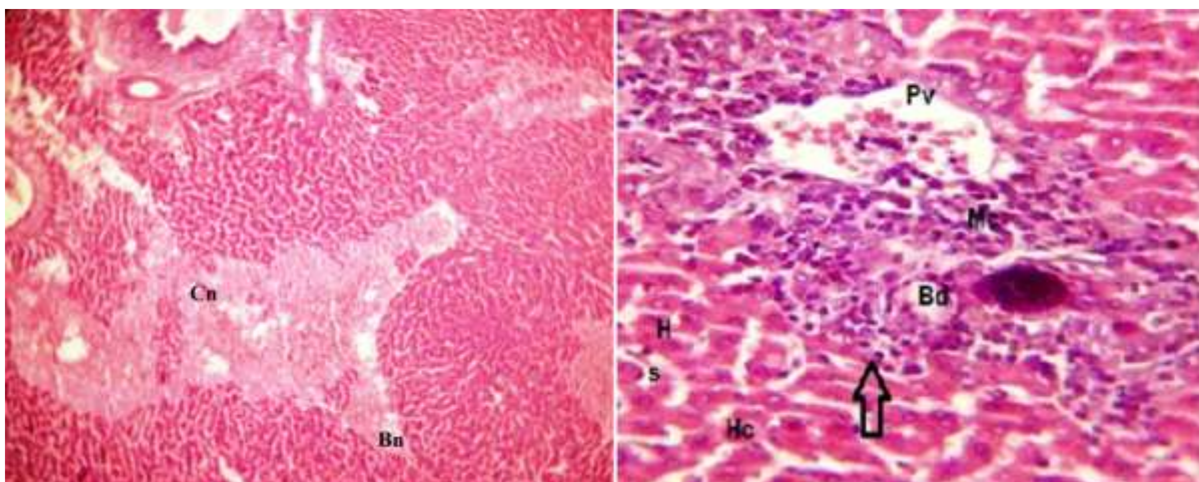

Figure 2: Sections of liver tissue of group II (MTX 20 mg/kg) on day 8 of the experiment shows portal inflammation with periportal interface hepatitis (piecemiel necrosis) (arrow on the right) and centrilobular necrosis (Cn) and bridging necrosis (Bn) (on the left). H & E stain, (40X) on right & (20X) on left. H: hepatocyte, Hc: hepatic cord, S: sinusoid, Pv: portal vein, Bd: bile duct, Mc: mononuclear cells infiltrate.

The current study reported that the administration of felodipine with MTX lowered the levels of MDA significantly and exhibited a marked elevation in the level of GSH in the hepatic tissue as compared to MTX group. This observation increase thoughts that felodipine showed antioxidant properties [32] by acting as lipophilic chain-breaking antioxidant.

Calcium antagonists including felodipine have also been shown to prevent glutathione loss [33], and this might be a possible mechanism too. Felodipine inhibited cytokine-induced nitric oxide production (iNOS) and nitric oxide synthase (NOS) mRNA induction. In addition, felodipine inhibited cytokine-induced superoxide production both in the presence and absence of an (NOS) inhibitor, suggesting

that it acted as a superoxide scavenger. The attenuation of intracellular calcium by felodipine can also modulate free radicals and inflammatory mediators production [34].

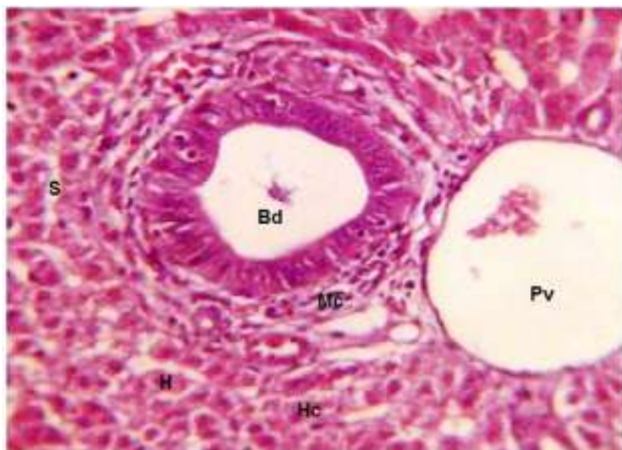

Figure 3: Section of liver tissue of group III (MTX 20 mg/kg +felodipine 0.5mg/kg) on day 8 of the experiment, shows mild portal inflammation. H & E stain, (40X). H: hepatocyte, Hc: hepatic cord, S: sinusoid, Pv: portal vein, Mc: mononuclear cells infiltrate.

Monocyte-derived macrophages were rich in nicotinamide-adenine-dinucleotide phosphate (NADPH) oxidaselead to amplifying the generation of (ROS) reactive oxygen species [35]. Again, ROS production was associated with intracellular calcium (iCa) elevation, protein kinase C activation and NADPH oxidase activation, leading to a vicious cycle of inflammation and progressive necrosis and fibrosis [36]. These results suggest that the antioxidant and consequently anti-inflammatory properties of felodipine may have beneficial effects in protecting against cellular damage caused by lipid peroxidation. The histological picture of the group treated with felodipine showed mild portal inflammation, which is a reversible damage as a result of the antioxidant and inflammatory modulation effects that reported above, which prevent further damage. Matsubara et al in 2010 reported that the hisopathological picture of the cardiac and kidney tissue were improved after felodipine had been administered, indicating the protective effects of felodipine on the myocardium and kidney by decreased perivascular inflammation and myocardial necrosis and fibrosis in aldosterone-high salt intake hypertensive unnephrictomized rats [37].

In conclusion, felodipine seems to have hepatoprotective activity through its favorable effects on liver function tests, oxidative stress, TNF-α and histopathological scores.

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Author contributions

The first author involved in the collection of samples, arrangement and writing of the study under supervision of the second and third authors, histopathological grading done by the third author.

Conflict of interest

There are no any financial and personal relationships that could bias this work.

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