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Research Article

THE EFFECT OF PRETREATMENT WITH TOLL-LIKE RECEPTOR 4 ANTAGONIST RESATORVID ON METHOTREXATE-INDUCED LIVER INJURY IN RATS: HISTOPATHOLOGICAL STUDY

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ABSTRACT

Objective: This research aims to evaluate the histopathological changes after pretreatment with resatorvid against methotrexate induced-liver injury.

Methods: 28 male albino-wistar rats divided into random 4 groups (7 rats in each). Control group: Rats left untreated. Vehicle pre-treated group: Rats were administered dimethyl sulfoxide (DMSO) followed by methotrexate (MTX). Methotrexate treated group: Rats left untreated then administered MTX. Resatorvid pre-treated group: Rats were administered resatorvid followed by MTX. 24 h after the end of treatment, the animals were sacrificed. Liver tissue samples dissected out immediately and fixed in 10% formalin. The traditional procedures (paraffin-embedded method) was used to prepare liver tissue for microscopic evaluation by none alcoholic fatty liver disease (NAFLD) Activity Score Components.

Results: Liver tissue sections of MTX-treated group show moderate-to-severe steatosis of hepatic cells and micro- and macro- hepatocellular fatty degeneration and giant fatty cysts with chronic inflammatory cells infiltration. While liver tissue sections of the resatorvid pre-treated group show moderate hepatic cellular fatty degeneration, with a decreased number of fatty cysts chains and the inflammation disappeared.

Conclusion: Resatorvid hepatoprotective effect against MTX-induced injury was promising throughout resolving the accompanying inflammation and partial restoring histopathological fatty alterations.

Keywords: Liver steatosis, Methotrexate sodium, Resatorvid, TLR4 receptor.

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INTRODUCTION

Resatorvid is a selective inhibitor of toll-like receptor 4 (TLR4) signal transduction pathway. It interferes with the intracellular toll/interleukin-receptor (TIR) domain adaptor molecule interaction [1,2]. Its structure (Fig. 1) with α-β unsaturated carbonyl group would allow it to act as Michael acceptor, via its cyclohexene ring which covalently bonds the nucleophilic cysteine 747 residue (Cys747) located at TIR domain that is necessary for TLR4 homodimeraization phase [3,4,5].

TLR4 is a member of the pattern recognition receptor (PRR), the family that are widely expressed by hepatic, parenchymal and/non-parenchymal cells as well as activate hepatic tissue inflammatory response [6,7]. Hepatocytes are the main site for PRR expression despite their weak response to TLR4 ligand. Hepatic kupffer cells (KCs), stellate cells (HSCs), and dendritic cells directly respond to TLR after their activation producing pro-inflammatory cytokines (CKs) that are the end product of TLRs pathways while playing an indefinite role in sinusoidal endothelial cells (SECs). TLRs are also expressed by lymphocytes [6-8]. Therefore, TLR4 is perhaps an attractive target for MTX-induced liver injury since MTX hepatotoxicity involves generation of free radicals (FR), oxidative stress and imbalance of cellular oxidant/antioxidant enzymes, these mechanisms lead to immune stimulation and production of proinflammatory CKs that may involve TLR4 induced inflammatory pathways [9-12].

Aim of the study

This research aims to evaluate the histopathological changes after pre-treatment with resatorvid against methotrexate induced-liver injury in an albino-wistar rat model.

METHODS

Experimental design

A total of 28 male albino Wistar rats (aged 4–6 mo and weighed 125–225 g) taken from Kut Technical Institute, University of Wasit, were maintained under non-specific pathogen-free conditions in wire-meshed cages (7 rats in each cage) with *ad libitum* access to water and regular diet. The animals were kept under a constant temperature 24 ± 3°C with 12:12 h light dark cycle [13-15]. Animal handling and housing have proceeded in accordance with the international guidelines for the care and use of laboratory animals of the National Research Council [13,14]. The ethical committee of the Pharmacology Department, College of Medicine, Mustansiriyah University, approved the experiment.

The animals were divided into random four groups (7 rats in each group) [14,16]: Control group: Rats kept on a regular diet and distilled water (D/W) throughout the 14 experimental d. Vehicle pre-treated group: Rats were administered intraperitoneal (i.p.) DMSO once for 7 d [16,17], followed by 7 d of oral MTX 0.2 mg/kg/d once via rat oral gavage [14]. Methotrexate treated group: Rats were left untreated for 7 d followed by 7 d of oral MTX 0.2 mg/kg/d once dependent on the adult dose for rheumatoid arthritis to stimulate model of induced liver injury [14]. Resatorvid pre-treated group: Animals were administered i.p. resatorvid 5 mg/kg/d once for 7 d [16], followed by 7 d of oral MTX 0.2 mg/kg/d via rat oral gavage [14].

Chemical and drug preparation

Resatorvid ($C_{15}H_{17}ClFNO_4S$ - a white crystalline powder with DMSO solubility of ≥360 mg/mL according to the manufacturer) was purchased from MedChemExpress, New Jersey, USA. It was dissolved in

DMSO and diluted with D/W to a final concentration of 17 mg/ml 1 h. Before it was administered i.p. according to rat's weight [16,17]. MTX ($C_{20}H_{22}N_8O_5$ - 50 mg/ml injectable solution) was purchased from KOÇAK Farma, ÍSTANBUL, Turkey, diluted with D/W to a final concentration of 0.333 mg/ml and administered via rat oral gavage according to rat weight [14]. DMSO: It was purchased as 99.5% solution from Central Drug House (P) Ltd., New Delhi, India. It was diluted with D/W to 8% w/v solution (the concentration used to dissolve resatorvid) and administered i.p. according to resatorvid protocol and rats weight [16,17].

Tissue sample collection and histopathological study

After 24 h of the end of the treatment, the rats were anaesthetized with Ketamine 91 mg/kg-Xylazine 9 mg/kg intramuscularly (I.M.), both were achieved from Alfasan woerden, Woerden, and Kepro, Deventer, Holland respectively [18,19]. A cut was done to rats' abdomen using sharp scissor after animal scarification, and the liver was dissected out immediately. Liver tissue samples were fixed in containers with 30 ml of 10% formalin and stored in −28°C until their processing [16,20,21].

The traditional processing procedures of the paraffin-embedded method were used to prepare liver tissues for microscopic evaluation [22-24]. The procedures include liver tissues' fixation in formalin 10%, cross-sectional slicing, and tissue step-by-step dehydration in graduated ethanol series clearing the dehydrant using xylene [25,26]. Infiltration followed, tissue blocks embedding in paraffin and being placed in metal mold, labeled, discarding any paraffin excess, and allowing the paraffin to congeal at room temperature then placed in refrigerator. Steps continued with infiltration, tissue blocks embedding in paraffin and then placing them in a metal mould, labelling was done, followed by discarding paraffin excess, and allowing the paraffin to congeal at room temperature then transfer them to refrigerator [26]. Manual tissue sectioning utilizing microtome was done, followed by marking the cleansed slides with a diamond pen and adjustment of obtained tissue sections on slide surfaces by utilizing water bath at 45°C. Pulling the slides out to allow tissue section adherence to the slide surfaces. Drying the obtained slides in a storage box for 24 h. Tissue sections clearing and rehydration by xylene solution, alcohol and water performed for several times. Moving the slide box's into a hot oven with 65°C for 15 min followed by staining with hematoxylin and eosin (H & E) stains [21,26]. Finally, cover slides mounting using few drops of clear resin (a mixture of distyrene, a plasticizer, and xylene) to the bottom before setting the cover on the tissue section slowly. Finally allowing the obtained sections to dry on a slide warmer for 24 h [23].

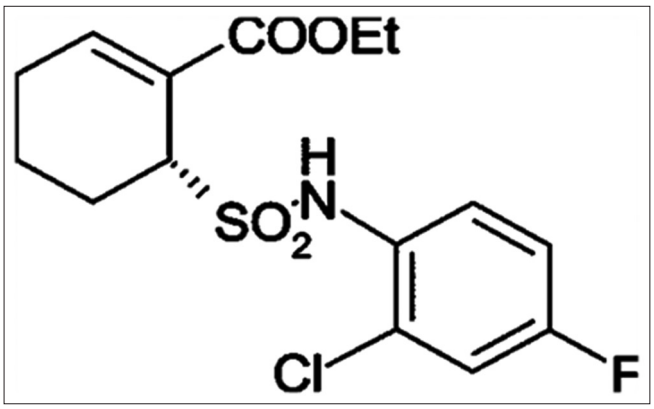

Fig. 1: Chemical structure of resatorvid [3]

Assessment of liver histopathology

Liver structure evaluation after methotrexate-induced injury done by professional pathologist utilizing a light microscope and assess the grade of the induced histopathological changes using histological scoring system for NAFLD [27]. This scoring system comprehends 3 main changes in the liver: Steatosis (S) range from 0-3, Lobular inflammation (L) range from 0-3, and Ballooning of hepatocytes (B) ranges from 0-2. The total scores represent the sum of all the hepatic changes according to the following equation and ranges from 0-8 (table 1) [27]. The digital photoshop software used to simplify the range of histopathological changes by X100 magnification.

Total NAFLD Activity Score (NAS) = S + L + B [27]

RESULTS

In this experiment, the resulted histopathological findings of the treated groups were graded as mild, moderate and severe findings. These findings were examined in 4 treatment groups each contain 7 rats according to NAFLD component scoring system seen in Table 2.

Liver histopathological findings of the control group

control group liver tissue sections show normal hepatic cellular tissue, normal lobular rearrangement with total NAS (NAFLD activity scores) of 0 as seen in (Table 2) as well as (Figs. 2, 3,4 and 5).

Liver histopathological findings of the methotrexate-treated group

Liver tissue sections of this group showed moderate to severe steatosis of hepatic cells, degeneration of hepatic cell, micro- and macro-fatty vacuoles seen to join with each other forming giant fatty cysts with chronic inflammatory cells infiltration. Total NAS scores of 8 as seen in (Table 2) and in (Figs. 6, 7, 8 and 9).

Liver histopathological findings of the resatorvid pretreated group

liver tissues sections' of this group show moderate hepatic cellular fatty degeneration, with a decreased number of fatty cysts chains and no inflammation observed as seen in Fig. 10, 11, 13 and 13. Total NAS scores obtained for this group of animals was 3 (Table 2).

DISCUSSION

Steatosis histopathologically characterized by the large fat vacuoles resulted from triglycerides (TG) deposition in the liver [28-30]. Their typical

Table 1: NAFLD activity score (NAS) components [27]

Item	Score	Extent (%)	Definition and comment
Steatosis	0	<5	Refers to the amount of surface area involved by steatosis as evaluated on low-to-medium power examination (40X–100X); minimal steatosis (<5%) receives a score of 0 to avoid giving excess weight to biopsies with very little fatty change
	1	5–33	
	2	>33–66	
	3	>66	
Lobular inflammation	0	No foci	Acidophil bodies are not included in this assessment nor is portal inflammation
	1	<2 foci/200x	
	2	2–4 foci/200x	
	3	>4 foci/200x	
Hepatocyte ballooning	0	None	The term "few" means rare but definite ballooned hepatocytes
	1	Few balloon cells	
	2	Many cells/prominent ballooning	

NAFLD: Non-alcoholic fatty liver disease, NAS: NAFLD activity scores

Table 2: NAFLD activity score (NAS) components of the treatment groups

Score components	Groups*		
	Control group**	**MTX group****	**Resatorvid pre-treated group****
Steatosis			
Score	0	3	2
Extent	<5%	>66%	33–66%
Lobular inflammation			
Score	0	2	0
Extent	None	2–4 foci/200x	No inflammation
Ballooning degeneration			
Score	0	2	1
Extent	None	Many	Few cells
Total scores	0	8	3

NAS: NAFLD activity scores, * n=28 rats for the treatment groups, **n=7 rats for each group, ***Total score graded as severe (>= 5), moderate (3-4) and mild (< 3).

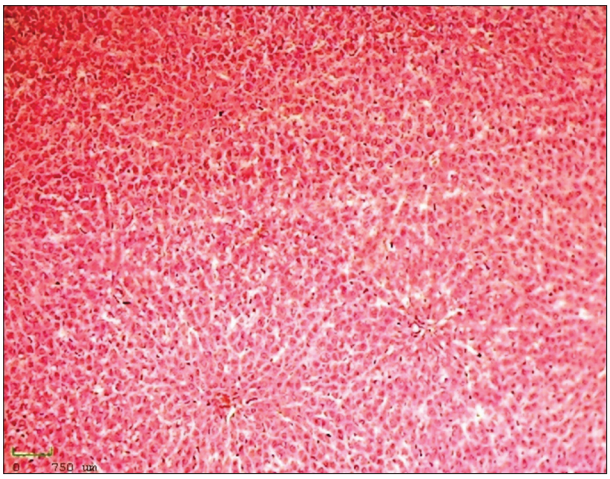

Fig. 2: Liver section of normal control rats (no abnormality), H and E ×100

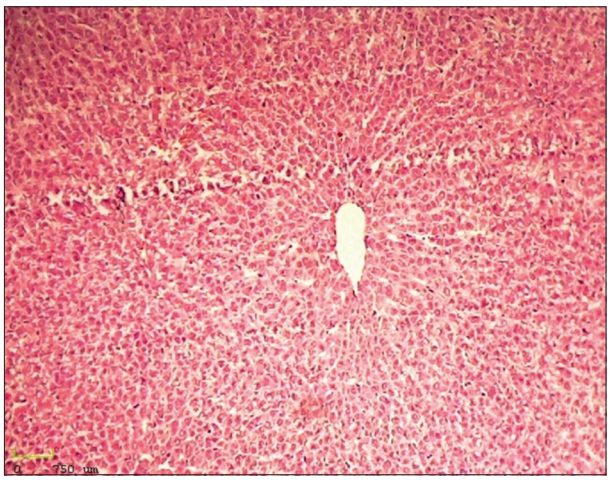

Fig. 3: Liver section of normal control rats (no abnormality), H and E ×100

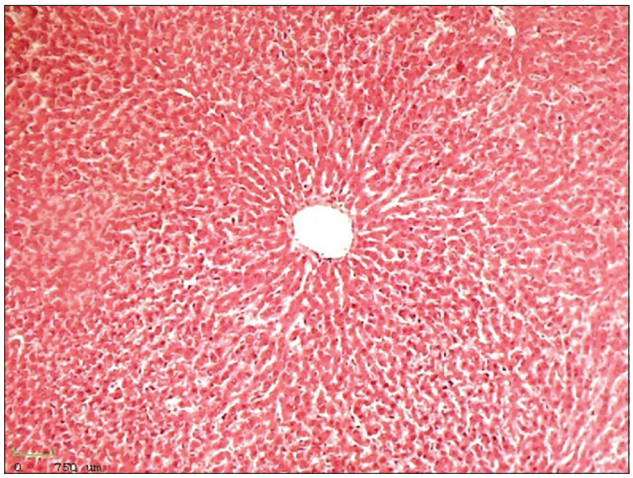

Fig. 4: Liver section of normal control rats (no abnormality), H and E ×100

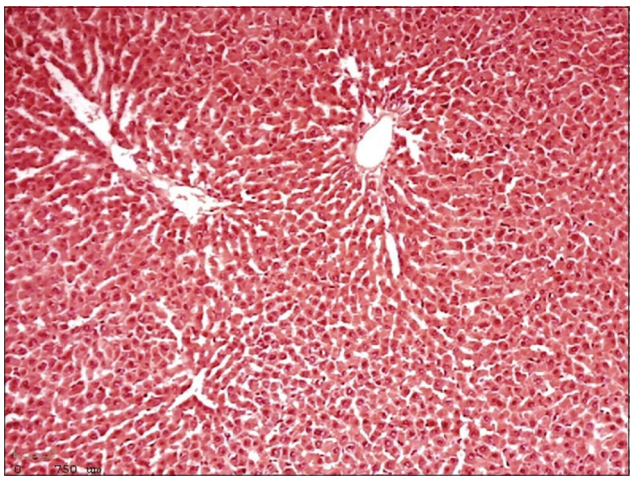

Fig. 5: Liver section of normal control rats (no abnormality), H and E ×100

presentation involves microvesicular changes: the presence of small fat vacuoles (liposomes) surrounding the nucleus at the centre of hepatocytes due to mitochondrial injury [31,32]. The macrovesicular changes: these are the sequel follows microvesicular vacuoles that represent "small and large fat droplets" which occupies the whole hepatocyte and pushes the nucleus to the edge of the cell. These droplets can re-join together resulting in the development of irreversible fatty cysts which later appears empty vacuoles due to lipolysis [28,32,33], and Finally, Steatohepatitis refers to steatosis coexisting with hepatocellular ballooning, fibrosis, and lobular inflammation. It is also attributed to oxidative stress and mitochondrial loss of function [10,31,32].

Chemotherapeutic drugs are the usual cause of multiple organ damage [34], especially those with self-renewing tissues. Since the liver is the main site for drug metabolism, so it is highly vulnerable to drug-induced liver injuries [11,28,31]. MTX is a classical chemotherapeutic and immunosuppressive

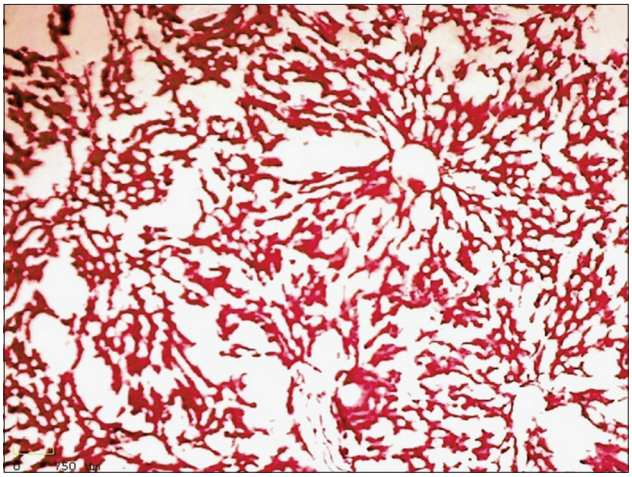

Fig. 6: Liver section of methotrexate-treated rats (moderate-to-severe steatosis) showing fatty degeneration of hepatic cells, microvesicular and macrovesicular fat vacuoles are shown joining each other and forming fatty cystic chains, H and E ×100

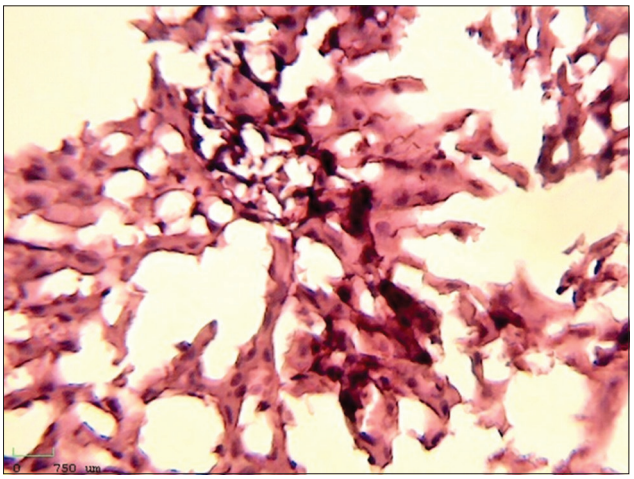

Fig. 7: Liver section of methotrexate-treated rats (moderate to severe steatosis) showing fatty degeneration of hepatic cells, micro-vesicular and macro-vesicular fat vacuoles are shown joining each other and forming fatty cystic chains, H and E ×100

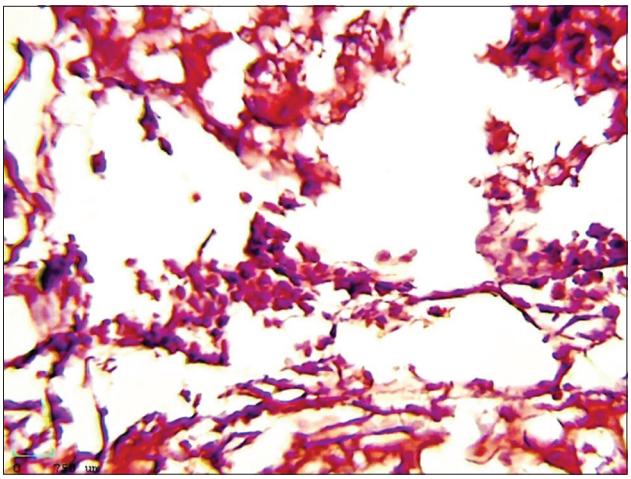

Fig. 8: Liver section of methotrexate-treated rats (moderate to severe steatosis) showing fatty cyst chains with chronic inflammatory cells infiltration, H and E ×100

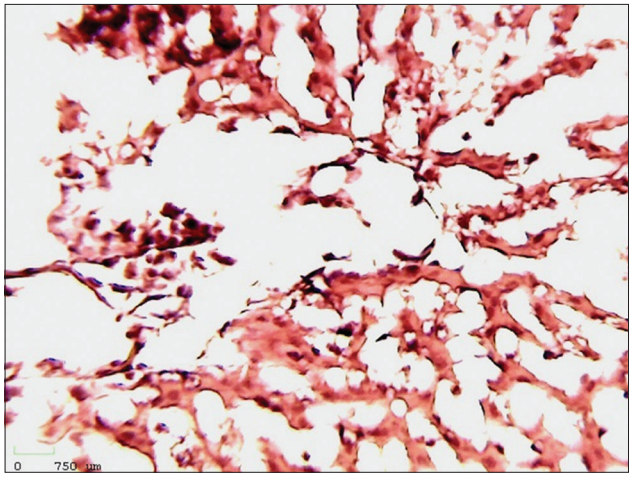

Fig. 9: Liver section of methotrexate-treated rats (moderate to severe steatosis) showing fatty cyst chains with chronic inflammatory cells infiltration, H and E ×100

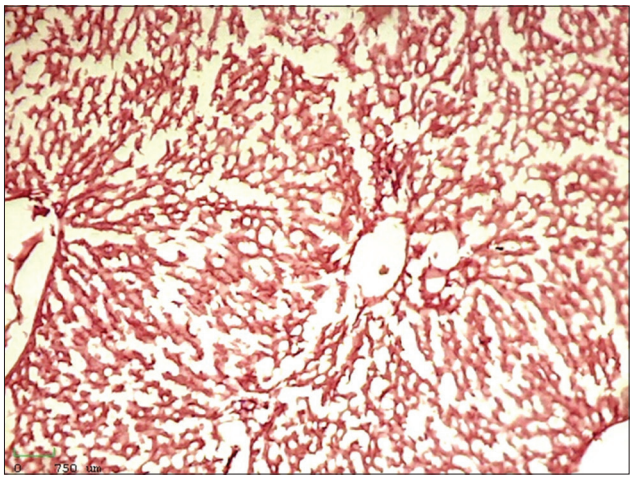

Fig. 10: Liver section of resatorvid pre-treated rats showing moderate steatosis with macrovesicular and microvesicular hepatic cell fatty degeneration and fewer numbers of fatty cysts chains, H and E ×100

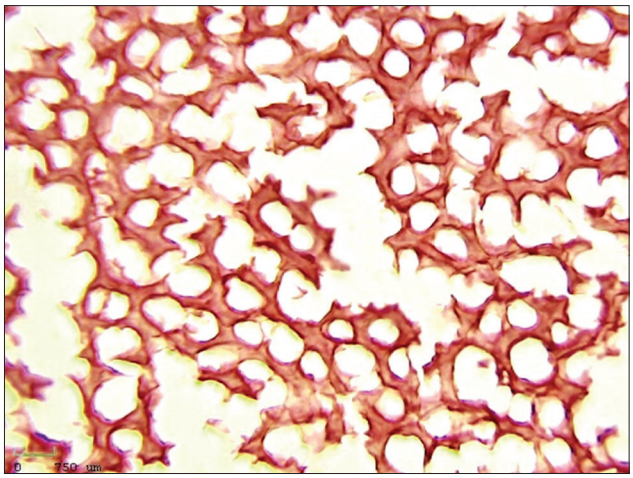

Fig. 11: Liver section of resatorvid pre-treated rats showing moderate steatosis with hepatic cell fatty degeneration and fewer number of fatty cysts chains. No inflammation observed H and E ×100

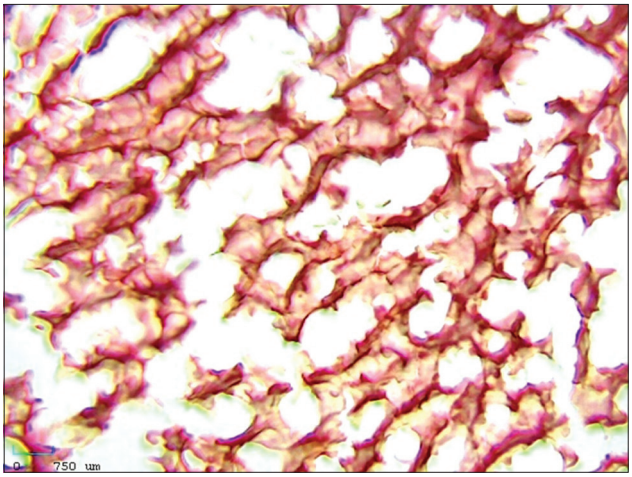

Fig. 12: Liver section of resatorvid pretreated rats showing moderate steatosis with macrovesicular hepatic cells fatty degeneration and fewer number of fatty cysts chains. No inflammation observed H and E ×100

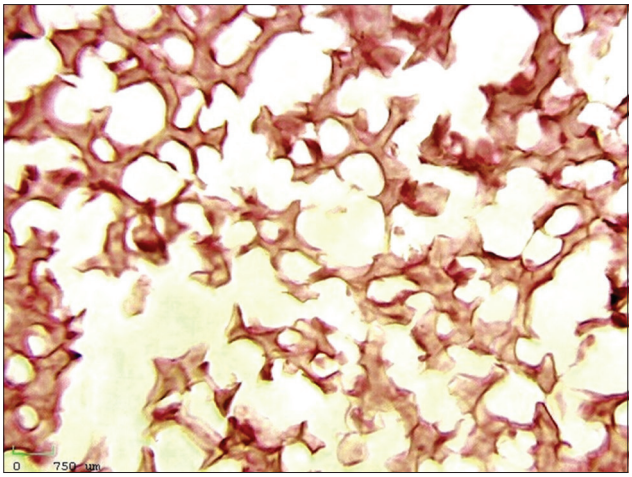

Fig. 13: Liver section of resatorvid pretreated rats showing moderate steatosis with hepatic cell fatty degeneration and fatty cysts chains. No inflammation observed H and E ×100

drug [35]. It resulted in haematological and gastrointestinal toxicities even when administered in low-dose regimen [36,37], and it is a usual cause of hepatocellular injury and death [38,39]. A methotrexate-induced liver injury may present as steatosis, cholestasis, fibrosis, cirrhosis, and in rare case acute liver failure [11,31,34,40]. In this study, we found that MTX-treated animals showed moderate to severe steatosis in comparison with animals in the control group. Both microvesicular and macrovesicular fatty vacuoles that are rejoining and form a fatty cyst (ballooning degeneration), pyknosis of chromatin in the nucleus was seen with a peripheralized nuclei that result in a shape of a signet ring. Moderate inflammation observed in the histological section of treated animals with infiltration of chronic inflammatory cells. The histopathological changes we achieved in this study are in consistent with the previous studies, which described severe focal necrosis accompanied by hepatic granular damage, abnormal hepatocytes rearrangement around central vein with shrunken and inflected nuclei, infiltration of mononuclear cells, vascular congestion, increased KCs activation and proliferated connective tissue in hepatic sections of those rats treated with MTX [15,20,21,34,41-43]. Despite, our finding represent the moderate-severe patterns of MTX-induced liver injury and thus we described milder steatohepatitic changes [11,31,34,39,40].

These changes attributed to MTX increment in both oxidative and nitrosative stress, that resulted from liver exposure to high levels of MTX-oxidizing metabolites [11,31]. Methotrexate inhibition of cytoplasmic de novo synthetic pathways of purines, pyrimidines as well as polyamines that leads to diminished hepatic folate reservoir, restrain folate entrance to the mitochondria, affect nucleic acid synthesis in S-phase and thus render hepatic cell death and further generation of FR [9,23,44]. Imbalance in cellular oxidant/antioxidant enzymes, which causes decreased availability of cytosolic nicotinamide adenosine diphosphate hydrogen (NADPH) that in turn affect the reduced state of cellular glutathione, also the decreased the level of superoxide dismutase and catalase [20,21,39,42]. These mechanisms will obstruct hepatic cholesterol and TG metabolism resulting in fatty infiltration, also would increase cellular sensitization to FR leading to stimulation of inflammatory response by activating KCs, HSCs, and macrophages (MQ). Such scenarios lead to fibrosis, leukocyte accumulation, neutrophils secretion of pro-inflammatory enzymes and CKs like inducible nitric oxide synthase (iNOS), Nuclear factor-κB (NF-κB) and tumour necrosis factor-α (TNF-α). This, in turn, causes more production of FR causes sinusoidal congestion, dilation, hepatic fatty vacuolation, focal necrosis and portal inflammation which is the typical pattern of drug-induced steatohepatitis (DISH) produced by FR [20,31,34,40].

Liver sections of resatorvid pre-treated rats showed moderate steatosis with NAFLD scores of 3, some restoration of macrovesicular and microvesicular hepatocytes fatty degeneration, and decrease in number of fatty cysts chains, with total reservation of infiltrated inflammatory cells but with incomplete ameliorating of the hepatic histological injuries induced by MTX. This protective effect is promising but not satisfying. It may indicate the requirement of longer treatment courses or coadministration courses. Still, it could be explained by resatorvid antagonism of TLR4 activation and upregulation caused by intracellular and extracellular damage-associated molecular patterns released during oxidative stress [45]. As a selective antagonist of TLR4, resatorvid inhibits both TLR4-Myeloid differential88 (MyD88)-dependent and MyD88-independent signaling pathways which induce inflammatory immune response and release of CK as TNF-α and interleukin-6 [46-48]. This inhibition is rather attributed to the inhibition of FR generation resultant from the activation of TLR4-MyD88-dependent signaling cascade that stimulates mitogen-activated protein kinase and c-Jun phosphorylation which are involved in FR generation [47,49].

CONCLUSION

Resatorvid hepatoprotective effect against MTX-induced injury was promising throughout resolving the accompanied inflammation and partial restoring histopathological fatty alterations.

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AUTHORS' CONTRIBUTION

Bassim S. Ahmed- contributed to the definition of intellectual content, experimental study, data analysis, manuscript revision as well as being guarantor, also contributed in the financial support. Alaa F. Hassan - contributed to the design, experimental study, data analysis and acquisition, manuscript preparation and review, and guarantor and also contributed in the financial support. Samer F. Hassan - contributed in the financial support, manuscript revision, and editing.

CONFLICTS OF INTEREST

the authors have no conflicts of interest.

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