Research Article

"Possible Protective Effect of Montelukast on Methotrexate-Induced Hepatotoxicity in Albino Rat: Histological and Immunohistochemical Studies"

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Abstract

Background: Methotrexate (Mtx) is a very common anti-cancer drug, it had a lot of negative influences on the cells of the liver. Montelukast (Mnt) is used as anti-asthmatic drug, an elected reversible cysteinyl leukotriene D4 receptor contender. The goal of the current work was to study the injurious influences of Mtx on the adult male albino rat's liver and to assess the protecting outcome of Mnt. **Materials and Methods:** 80 albino male rats were separated to 4 independent groups in the following way: group-I: controls; group-II: treated by Mnt; group-III: treated by Mtx; group-IV: treated by both Mtx and Mnt. At the end of the experiment, the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were calculated from the blood samples of the rats. Furthermore, the tissues of the liver have been histologically tested. Immunocytochemical stain was done by means of polyclonal rat antibodies (anticleaved caspase 3 and iNOS). **Results:** Mtx produced nitrosative stress demonstrated by amplified nitric-oxide, by up-regulating the induced nitric-oxide synthase. Mtx as well lead to apoptotic results, as it upregulated caspase-3 in the liver tissues. Mnt inverted the oxidative-stress, in addition to inflammatory and apoptotic signs caused by Mtx. **Conclusion:** The current study demonstrates that Montelukast is useful in treatment of Mtx-induced hepatotoxicity, through its antioxidant, anti-inflammatory and antiapoptotic mechanisms. Efficacy of montelukast draws the consideration to be a probable effective adjuvant in Mtx.

Keywords: Montelukast, Methotrexate-Induced liver injury.

Introduction

Methotrexate (Mtx) is a common anti-cancer medication which is used for the treatment of leukaemias and many neoplasms including hepatic cell carcinoma, bronchogenic carcinoma, breast cancer and horiocarcinoma (Şener G. et al., 2006). Hepatotoxicity has been recognized as a potential major adverse effect that can occur with prolonged use of methotrexate especially in patients with preexisted liver disease. Methotrexate can cause liver injury by many mechanisms as methotrexate can activate hepatic stellate cells, which lead to increased collagen deposition. Also it leads to accumulation of metabolites (polyglutamates) resulting in prolonged folate inhibition with subsequent hepatic injury (Bridges et al., 1989 and Cronstein, 1996).

Montelukast (Mnt) is an elected, pharmacological antagonist of type-1 cysteinyl-leukotriene receptors (CysLT1Rs). It effectively antagonizes the proasthmatic and proinflammatory effects of cysteinyl-leukotrienes (CysLTs) and make a section of many universal guidelines of the asthma therapy. Recent evidence suggests that montelukast possesses a range of secondary antiinflammatory activities, apparently unrelated tothe antagonism of CysLT1 Rs, and also shows antioxidant activity (Tintinger et al., 2010).

Materials and Methods

Animals:

A number of 80 adult male albino rats weighing 200-220 grams were used. They were housed in clean plastic cages (as 5 rats /cage) in the laboratory room of the Anatomy Dep.in the

medicine faculty, Minia University under standard laboratory conditions. All animals were free to eat and drink.
Experimental protocols were approved by the Institutional Animal Ethics Committee of ElMinia University.

Drugs used & experimental design:
Methotrexate (Mtx, 50mg vial), montelukast (singulair tablet, 4 mg), anti-cleaved caspase 3 and iNOS were purchased from Sigma. The animals were grouped into 4-independent groups, 20 animals in every group.
Group I (control): rats were kept receiving only food and distilled water.
Group II (Mnt): the rats were received montelukast, 10 mg/kg daily for 10 days orally (Evren K. et al., 2012).
Group III (Mtx): the rats were received Methotrexate, single dose 20 mg/kg intraperitoneal (Evren K. et al., 2012).
Group IV (Mtx+Mnt): the rats were received Mtx , solo dosage of 20 mg / kg intraperitoneal followed by Mnt 10 mg/ kg orally every day for period of ten days, after 3 days methotrexate injection (Evren K. et al., (2012).
Twenty-four hours after the last injection, all rats involved in the study have been killed by decapitation after application of a little halothane anesthesia and the liver tissues of rats were removed for further analysis. The liver tissue specimens were placed in 10% formal-dehyde solution for routine histological examination by light microscopy (Kose et al., 2012).

Methods:
1. Biochemical Study:
Blood specimens have been taken from the tail vein of the rat and put to tubes for evaluation of biochemical tests. A centrifuge was applied to the blood at a speed of 3,000 round per minute for a period of 10-minutes, after standing at room-temp. For 15-min. The bio-chemical factors, counting AST and ALT, were checked in serum using commercial kits (Reactive GPL, Barcelona, Espana) (Cure et al., 2015)
2. Histological study:
The liver samples were putted in formalin of concentration (10%) and implanted in paraffin. A thickness of 5- μm paraffin sectors were made and then regularly marked with hematoxylin and eosin (H&E) colors. Stained slides were microscopically analyzed using light microscopy.
3-Immunohistochemical study:
Immunocytochemical stain was made by antibodies from polyclonal-rat (**anti-cleaved caspase-3 & iNOS**). Sections were de-paraffinized, hydrated then cleaned in 0.1M phosphate-buffer-saline (PBS). Endogenous peroxidases were handled by H_2O_2 in methyl alcohol (Peroxidase blocking solution) after that cleaning in tris buffer saline (TBS). Non-definite combining of IgG was stopped via ordinary goat serum, dilute 1:50 in 0.1% concentration serum albumin with TBS for 30-min.

The sections were incubated with the diluted initial anti-bodies (1:500) for cleaved caspase-3 (for 30-min.) at room-temp. Sections then were cleaned 3-times every time for 5-min using a buffer and incubation for additional 30-min then after that washing. Subsequent additional 30-min incubation with Vectastain ABC kits (Avidin, Biotinylated horse radish peroxidase Complex) and cleaning for 10-min, the substrate, diaminobenzidine tetra hydrochloride (DAB) in H2O was added for 5-10 min. The enzyme reactivity was progressing as explained before. The sections were slightly counterstained by hematoxylin to acquire a better morphological cells identification, and de-hydrated by passing through rising alcohol concentrations after that cleaned using xylene.
Statistical Analysis:
The consequences were reported in the form of means ± SD (standard deviation). The biochemical parameters were analyzed were performed using the one-Way ANOVA test (f-test) for numerical data among the 4-groups then Tukey post Hoc analysis among every 2-groups. The consequences are reported in the form of mean ± SD. P-values of below 0.05 were statistically considered significant.

Results
1-Biochemical Results:
The obtained data of the influences of Mtx and treatment of Mnt on serum ALT&AST levels

were introduced in Table-1. The results showed that injecting with methotrexate rise significantly the serum ALT&AST activities in comparison to controls. Concomitant treatment of methotrexate and montelukast significantly decrease ALT and AST values.

Table (1): Serum AST&ALT Levels in the various groups of the study (IU/L).

	Group-I (Control) (n=20)	Group-II Montelukast) (n=20)	Group-III Methotrexate (n=20)	Group-IV (Methotrexate + Montelukast) (n=20)	P value					
AST					<0.001*					
Range	(25-35)	(28-36)	(65-76)	(45-55)	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
Mean ±SD	30.3±3.1	31.7±2.7	69.9±3.4	49.9±3.2	0.520	<0.001*	0.001*	<0.001*	<0.001*	<0.001*
ALT					<0.001*					
Range	(25-40)	(30-40)	(58-67)	(43-50)	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
Mean ±SD	31.9±5.1	35.4±3.6	62.1±2.9	46.7±2.6	0.017*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*

- T-test for quantitative data among the 4-groups after that Tukey post Hoc analysis was performed among every 2-groups.
- *: Significant change at P-value less than 0.05.

2- **Histological Results:**

The controls and Mnt-group exhibited an ordinary look of the cells of liver presented in (Figs.1&2). In the Mtx group, Liver section showed deformed hepatic architecture and marked enlarged sinusoids especially in the centric areas of the liver acini with marked crowding of the centric vein (Fig.3). Degeneration signs of hepatocytes in the form of swollen cells up to ballooning with vacuolated cytoplasm.

Apoptotic cells were also scattered around the central vein. Apoptotic hepatocytes are characterized by having hypereosinophilic cytoplasm and condensed darkly stained nuclei, pyknotic nuclei (Fig.4). In the treated group, there is marked reduction of the previous changes. Inflammatory infiltration and hemorrhage appeared to be reduced, there was slight disturbance in lobular architecture and mildly dilated blood sinusoids (Fig.5). Also there are few apoptotic hepatocytes rounding the centric vein (Fig.6).

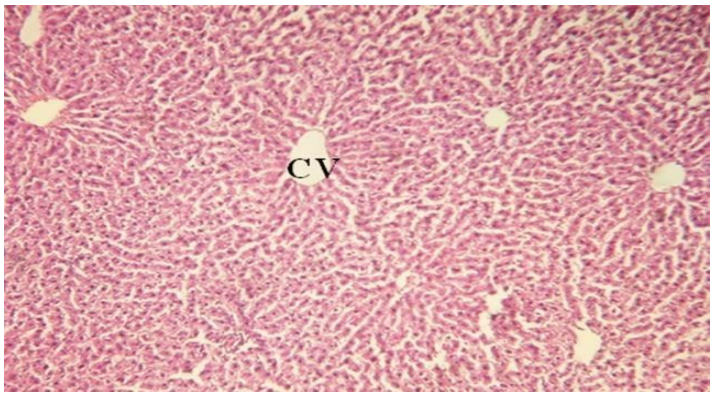

Fig. (1): A micrographic photo of liver tissue of rat for controls displaying ordinary lobular construction. Notice the central vein (CV). ((Hx&EX100).

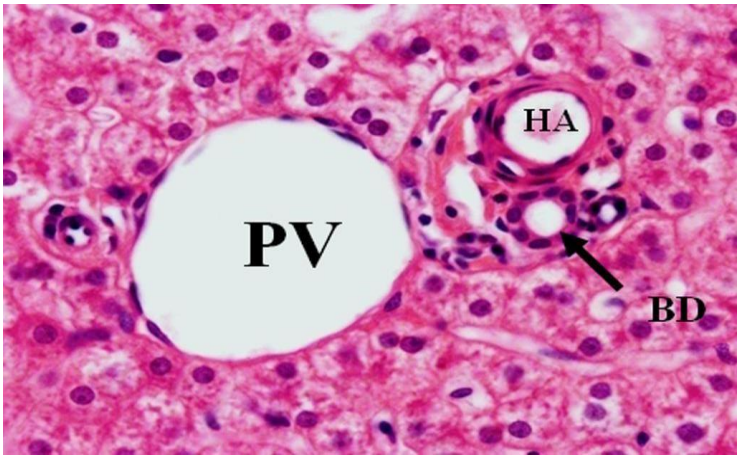

Fig. (2): A micrographic photo of liver tissue of rat for controls displaying gateway tract comprising branches of hepatic artery (HA), portal vein (PV) and bile duct (BD). (Hx&E X 400).

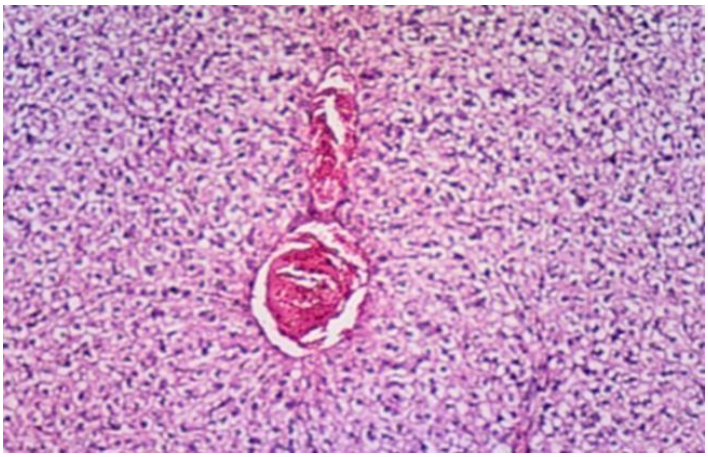

Fig. (3): A micrographic photo of liver tissue for rats of group-III displaying congested central vein with disturbed architecture. Hepatocytes are swollen with vacuolated cytoplasm. (Hx&EX100).

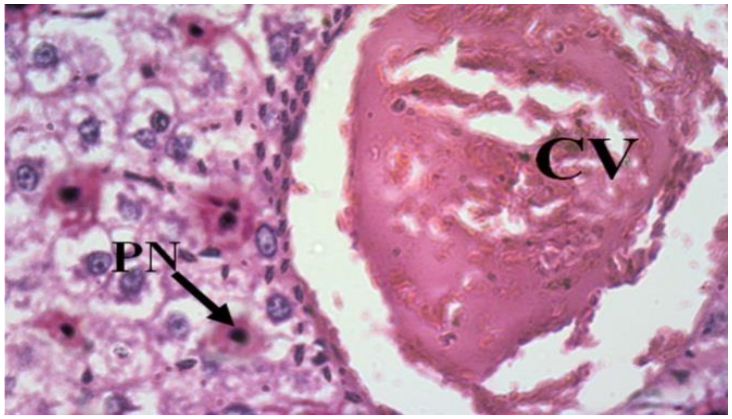

Fig. (4): A micrographic photo liver tissue for rats of group-III displaying congestion of central vein. Apoptotic cells appear with hyperoesinophilic cytoplasm and darkly stained, condensed and pyknotic nucleus PN (black arrow). ((Hx&EX400).

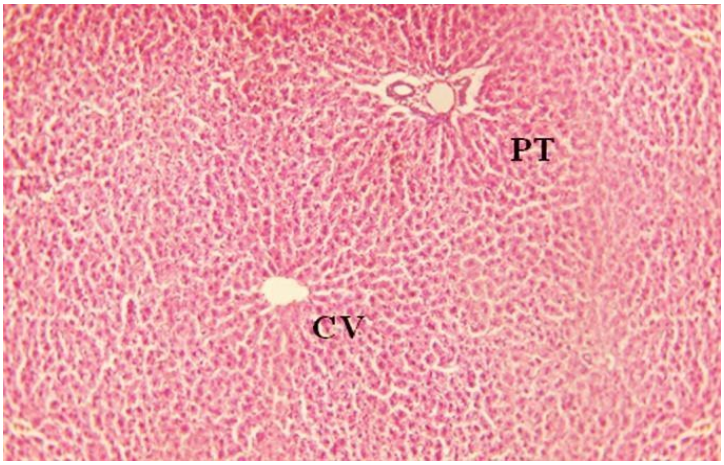

Fig. (5): A micrographic photo liver tissue for rats of group-II displaying conserved lobular architecture with central vein (CV) and portal tract (PT). (Hx&EX100).

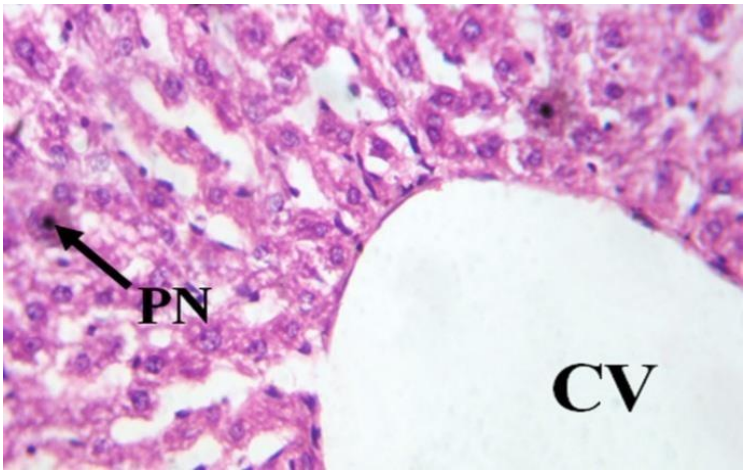

Fig. (6): A micrographic photo liver tissue for rats of group-IV at pericentral region displaying few apoptotic hepatocytes with hypereosinophilic cytoplasm and densely stained nucleus, pyknotic PN (black arrow). ((Hx&EX400).

3- (A) Immunohistochemical study for activated Caspase 3:

The positive immunoreactivity for activated caspase 3 appeared in the form of brown staining at the cytoplasm and/or nucleus of the immunoreactive cells.

The liver sectors of the controls & montelukast groups exhibit an ordinary lobular construction with negative immunolabelling for activated caspase 3 (Figs. 7 & 8).

In the Mtx group, there was obvious high immune-reactivity for active caspase-3 (casp3) in liver sections (fig. 9). In the treated group, there was a remarkable fall in the activated casp3 immunolabelling in hepatocytes as compared to Hepatocytes as compared to the high expression the high expression observed in the previous group (Fig.10).

(b) Immunohistochemical study for iNOS: The immunohistochemical reactivity for iNOS in sections of rat liver tissues appeared in the form of brown staining of the cytoplasm of the immunereactive cells.

Liver sections of control & montelukast groups showed normal lobular architecture with negative immunolabelling for iNOS (Figs.11 & 12). In the Mtx group, there is an apparent high immunoreactivity for iNOS. Most of the hepatocytes had shown cytoplasmic expression only (Fig.13). In the groups that was treated, there were aremarkable fall in iNOS immunolabelling in observed in the previous group (Fig.14).

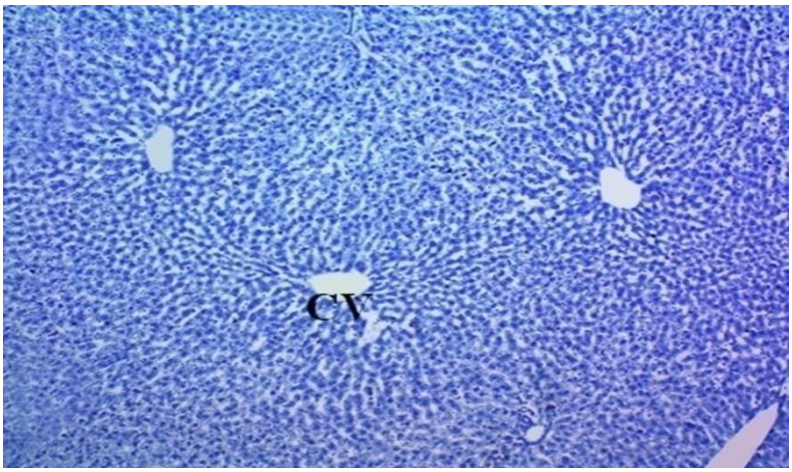

Fig. (7): A micrographic photo of liver tissue for **controls rats labeled for activated caspase 3, displaying no detectable immune-reactivity. Immunohistochemistry, counterstained with Hx.X100).**

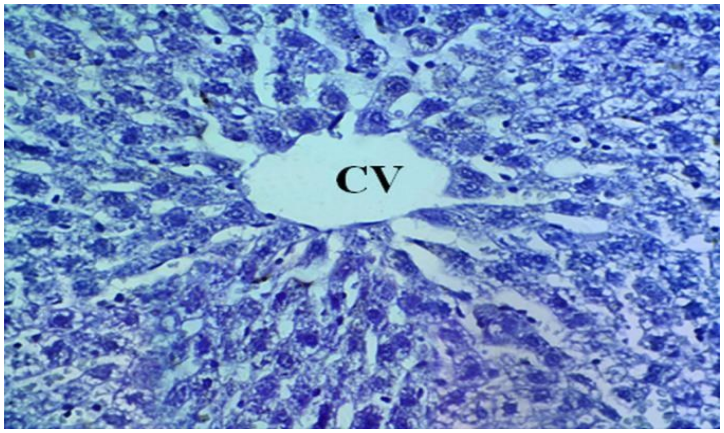

Fig. (8): A micrographic photo of liver tissue of group-II rats considered for activated casp- 3, displaying absence immunolabeled cells rounding the centric vein (CV). Immuno-histochemistry, counter-stained with Hx.X 400).

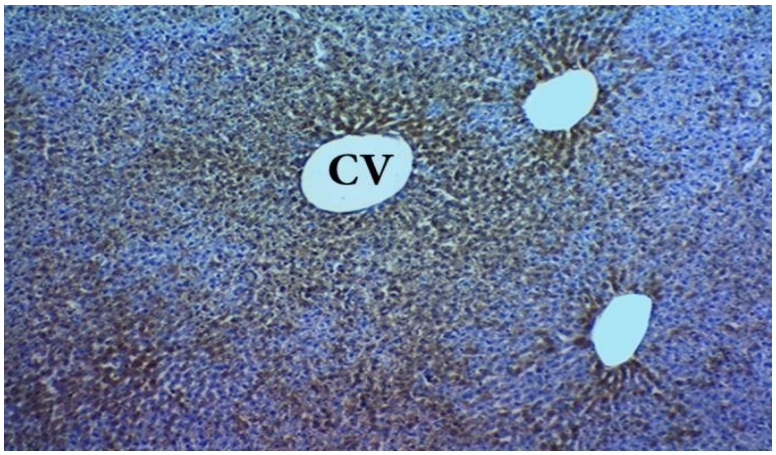

Fig. (9): A micrographic photo of liver tissue of group-III rats considered for activated casp-3, displaying a general immunolabeling of hepatocytes. Immunohistochemistry, counterstained with Hx.X100).

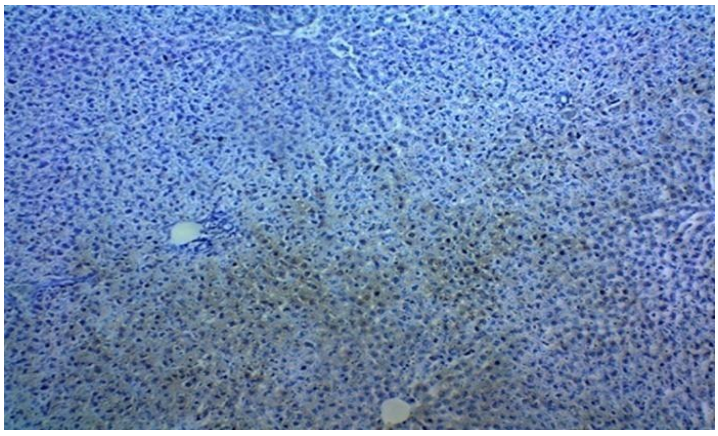

Fig. (10): A micrographic photo of liver tissue of group-IV rats considered for activated casp-3, showing decrease in activated caspase 3 expression. Immunohistochemistry, counterstained with Hx.X100).

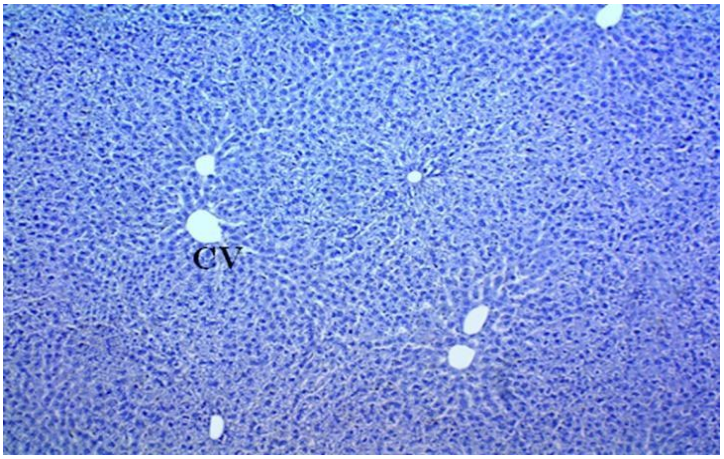

Fig. (11): A micrographic photo of liver tissue of controls rats considered for iNOS, displaying no detectable immune-reactivity immunohistochemistry, counterstained with Hx.X100).

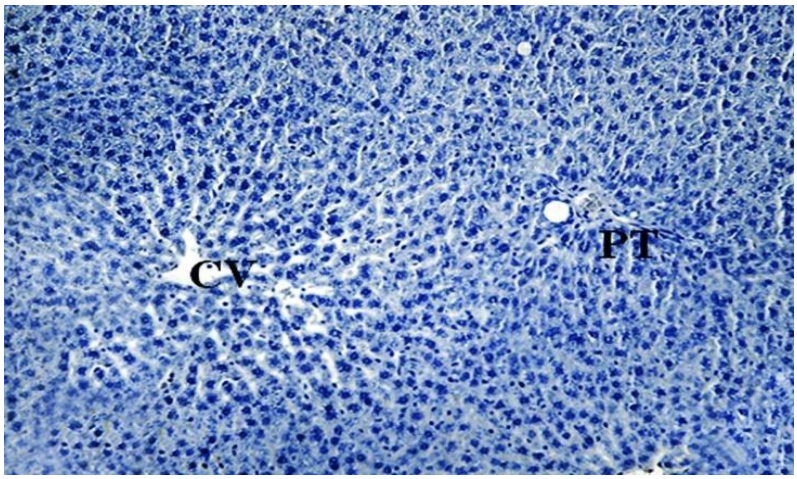

Fig. (12): A micrographic photo of liver tissue of group-II rats considered for iNOS, displaying a negative immunolabeling of hepatocytes Immunohistochemistry, counterstained with Hx.X100).

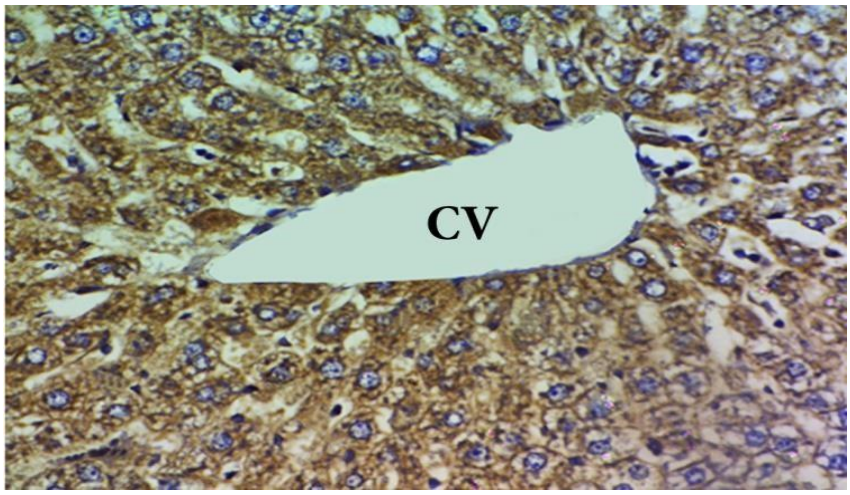

Fig. (13): A micrographic photo of liver tissue of group-III rats labeled of iNOS, displaying extensive immunolabeling in hepatocytes and endothelial cells. Immunohistochemistry, counterstained with Hx.X400).

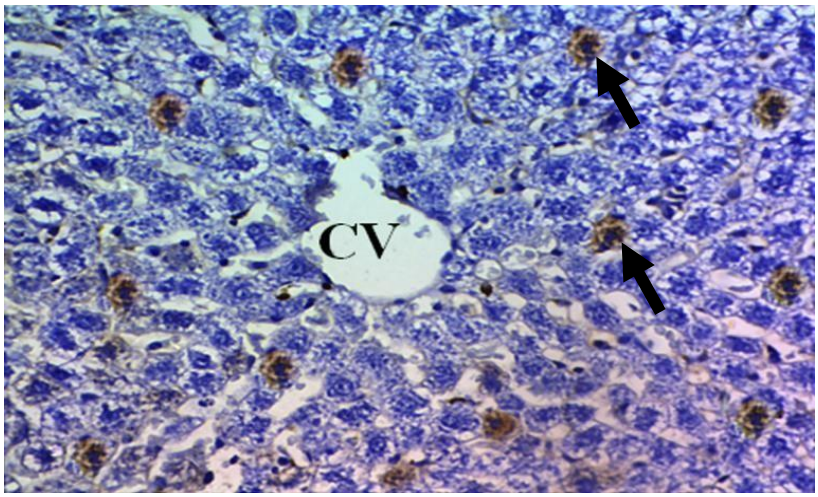

Fig. (14): A micrographic photo of liver tissue of group-IV rats labeled for iNOS, displaying little expression (black arrow) rounding the CV. Immuno-histochemistry, counter-stained with Hx.X400).

Discussion

Methotrexate may lead to liver hepatotoxicity, in the form of steatosis, cholestasis, fibrosis and cirrhosis (Vonen and Morland, 1984). The mechanisms of Mtxhepatotoxicity can be related to its accumulation in the cells with a polyglutamated arrangement. This arrangement leads to reducing the levels of folat and hepato-toxicity (Jahovic et al., 2003).

Montelukast, one of the selective reversible cysteinyl-leukotriene receptor antagonists, is used for the maintenance treatment of asthma and to relieve symptoms of seasonal allergies (Canbay et al., (2010). Mohamadin et al., (2011), Coskun et al., (2011) and Cuciureanu et al., (2009) concluded that montelukast has an anti-inflam-matory & anti-oxidant capacity.

The present study revealed that administration of methotrexate caused increase in serum ALT & AST levels. This consequence was in agreed with the earlier studies (Morsy et al., 2013 and Ali et al., 2014). ALT level of Mtx-group was significantly high in comparison with the controls and montelukast groups ($p< 0.05$). Treating of rats with montelukast resulted in a significantly improved hepatic markers com-pared to sole Mtx treatment ($p<0.001$). This is in agreement with Ozkan et al., (2010) who studied the protective effect of Mnt vs. hepatic ischemia/reperfusion wound of rats.

In the present study, in comparison between control (normal) group and group 3 which were treated by Mtx revealed marked distortion of hepatic architecture, congestion of the central vein and the blood sinusoids with inflammatory cell infiltration Moreover, there were signs of degeneration of hepatocytes in the form of swelling cells up to ballooning, vacuolated cytoplasm. Apoptotic hepatocytes are charac-terized by having hypereosinophilic cytoplasm and condensed darkly stained nuclei (pyknotic nuclei); these results are in agreement with the studies done by El-Sheikh et al., (2015); Morsy et al., (2013) and Ali et al., (2014) who detect a damaging effect by methotrexate in liver in the form of congestion, inflammation and apoptosis. Montelukast caused marked reduction of patho-logical changes as agreement with Mohamadin et al., (2011).

The concentration of the previous changes at pericentric area (third zone) may be clarified through the fact of that the cells in the third zone

Were specifically full of enzymes included in the medication absorption (Fawcett and Jensh, 2002), This is the most vulnerable zone to intoxication due to the little blood supply.

Maruf, (2014) has claimed that the necrotic cells were rarely found in Mtx-induced liver injury and said that Mtx produced more apoptotic cells rather than necrotic cells but Iqbal et al., (2001) claimed that they did not see apopotic lesions. Shigeyuki et al., (1998) found that there is variation in the individual response of the livers of the albino rats to Mtx toxicity and they explained the variation by the difference of cytochrome P450 activity in the rats.

Because caspase 3 is an important indicator of apoptosis, immunohistochemical localization of activated caspase 3 was performed in rat liver tissues to confirm the apoptosis as one of mechanisms of acute liver injury induced by methotrexate (Helal, 2010. Attoub et al., 2013 and Rajput et al., 2013).

For ordinary cells, casp-3 present as a procaspase wherein the probable splitting location is complete. Once splitted by activating the apoptotic sequence, this active caspase was considered a novel indicator not exist in ordinary cells. Thus, the discovery of this indicator must be a unparalleled and critical marker of apoptosis (Allen et al., 2002). Within the execution of apoptosis, splitted casp-3 is responsible, partially or completely, for the proteolytic splitting of a great amount of proteins and for apoptosis associated chromatin margination, DNA shatter and nuclear breakdown within apoptosis (Cohen, 1997).

In this study, it was found that methotrexate administration in group (3) caused marked increase in the expression of active caspase 3 in rat liver tissues in comparison to the control group. This was in agree with El-Sheikh et al., (2015) and Mukherjee et al., (2013). Our results show that administration of montelukast in group 4 reduce the number of positive immunoreactive cells for caspase 3 expression, this was in agree with Ibrahim et al., (2014).

Our results showed that methotrexate caused significant increase in iNOS expression in liver sections of Mtx group as a marker of oxidative stress agreed the results of (El-Sheikh et al., 2015) and (Mukherjee et al., 2013).

In the current study, immunohistochemical staining of liver sections with iNOS was performed to confirm oxidative stress. As inducible nitric oxide synthase (iNOS) is one of 3 main enzymes producing nitric-oxide (NO) via the amino-acid l-arginine which are eNOS (endothelial NOS) and nNOS (neuronal NOS), (Knowles & Moncada, 1994).

iNOS makes great number of NO depending on stimulation and proinflammatory cytokines (e.g. Interleukin-1, alpha Tumor necrosis agent and gamma Interferon). Introduction of the high-yield iNOS frequently happens in the oxidative background, and so high levels of NO have the chance to get reacted with super-oxide directing to peroxynitrite formation and cell toxicity (Green et al., 1994).

References

1. Şener G., Ekşioğlu-Demiralp E., Çetiner M., Ercan F., and B. Ç. Yeğen, (2006) "β-glucan ameliorates methotrexate-induced oxidative organ injury via its antioxidant and immune-modulatory effects," European Journal of Pharmacology, vol. 542, no.1–3,pp.170–178.
2. Bridges, S.L., Alaricon, GS. andkoopman, W.J. (1989): methotrexate – induced- liver abnormalities in rheumatoid arthritis. J Rheumatol 16:1180.
3. Cronstein, BN. (1996): Molecular therapeutics. Methotrexate and its mechanism of action. Arthritis Rheum. 39:1951-1960.
4. Tintinger, G. R., Feldman, C., Theron, A. J., & Anderson, R. (2010): Montelukast: more than a cysteinyl leukotriene receptor antagonist? The Scientific World Journal, 10, 2403-2413.
5. Kose, E., Beytur, A., Dogan, Z., Ekincioglu, Z., Vardi, N., Cinar, K., & Ekinci, N. (2012): The effects of montelukast against amikacininduced acute renal damage. Eur Rev Med PharmacolSci, 16(4), 503-511.

6. Cure, E., Kirbas, A., Tumkaya, L., Cure, M. C., Kalkan, Y., Yilmaz, A., & Yuce, S. (2015): Protective effect of infliximab on methotrexate-induced liver injury in rats: Unexpected drug interaction. Journal of cancer research and therapeutics, 11(1), 164.

7. Vonen, B., &Mørland, J. (1984): Isolated rat hepatocytes in suspension: potential hepatotoxic effects of six different drugs. Archives of toxicology, 56(1), 33-37.

8. Jahovic, N., Çevik, H., Şehirli, A. Ö., Yeğen, B. Ç., & Şener, G. (2003): Melatonin prevents methotrexate-induced hepatorenal oxidative injury in rats. Journal of pineal research,(4)43, 282-.782

9. Canbay, E., Agachan, B., Ozturk, T., Giris, M., Asoglu, O., Balik, E., & Bugra, D. (2010): Dual inhibition of wound healing and oxidative process by montelukast in experimental colon anastomoses. Surgical innovation, 17(3), 248-255.

10. Mohamadin, A. M., Elberry, A. A., Elkablawy, M. A., Gawad, H. S. A., & AlAbbasi, F. A. (2011): Montelukast, a leukotriene receptor antagonist abrogates lipopolysaccharide-induced toxicity and oxidative stress in rat liver. Pathophysiology, 18(3)235-.242،

11. Coskun, A. K., Yigiter, M., Oral, A., Odabasoglu, F., Halici, Z., Mentes, O., & Suleyman, H. (2011): The effects of Montelukast on antioxidant enzymes and proinflammatory cytokines on the heart, liver, lungs, and kidneys in a rat model of cecal ligation and puncture–induced sepsis. The Scientific World Journal, 11, 1341-1356.

12. Cuciureanu, M., Căruntu, I. D., Păduraru, O., Stoica, B., Jerca, L., Crauciuc, E., &Nechifor, M. (2009): The protective effect of montelukast sodium on carbon tetrachloride induced hepatopathy in rat. Prostaglandins & other lipid mediators, 88(3), 82-88.

13. Morsy, M. A., Ibrahim, S. A., Amin, E. F., Kamel, M. Y., Rifaai, R. A., & Hassan, M. K. (2013): Curcumin ameliorates methotrexateinduced nephrotoxicity in rats. Advances in pharmacological sciences, 2013.

14. Ali, N., Rashid, S., Nafees, S., Hasan, S. K., & Sultana, S. (2014): Beneficial effects of Chrysin against Methotrexate-induced hepatotoxicity via attenuation of oxidative stress and apoptosis. Molecular and cellular biochemistry, 385(1-2), 215-223.

15. Fawcett, D.; and Jensh, R. (2002): Bloom and Fawcett's concise histology, 2th ed., Arnold, London: 207-212.

16. El-Sheikh, A. A., Morsy, M. A., Abdalla, A. M., Hamouda, A. H., & Alhaider, I. A. (2015): Mechanisms of thymoquinone heaptorenal protection in methotrexate-induced toxicity in rats. Mediators of inflammation, 15-20

17. Iqbal, M.P., Sultana, F. and Mehboobali, DG. (2001): Folinic acid protects against suppression of growth by methotrexate in mice. Biopharm. Drug Dispos. 22:169-178.

18. Shigeyuki, K.; Keiko, N. and Kazumi, W.G. (1998): Strain differences of the ability to hydroxylate methotrexate in rats. Institute of Pharmaceutical Science, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minamiku, Hiroshima: 734-8551.

19. G. K. Helal, (2010): "Thymoquinone supplementation ameliorates acute endotoxemiainduced liver dysfunction in rats," Pakistan Journal of Pharmaceutical Sciences, vol. 23, no. 2, pp. 131–137.

20. S. Attoub, O. Sperandio, H. Raza et al., (2013): "Thymoquinone as an anticancer agent: evidence from inhibition of cancer cells viability and invasion in vitro and tumor growth in vivo, "Fundamental & Clinical Pharmacology, vol.27, no.5, pp. 557–569,

21. S. Rajput, B. N. P. Kumar, S. Sarkar et al., (2013): "Targeted apoptotic effects of thymoquinone andtamoxifen on XIAP mediated Aktregulation in breast cancer," PLoS ONE, vol. 8, no. 4, Article ID e61342, 13 pages.

22. Allen, M.; Gown; and Mark, C. (2002): Improved Detection of Apoptotic cells in Archival Paraffin Sections: Immunohistochemistry Using Antibodies to Cleaved

Caspase 3. The Journal of Histochemistry & Cytochemistry, 50(4): 449-454.

23. Cohen, G. (1997): Caspases: the executioners of apoptosis. Biochem. J., 326: 1-6.

24. Mukherjee, S., Ghosh, S., Choudhury, S., Adhikary, A., Manna, K., Dey, S., & Chattopadhyay, S. (2013): Pomegranate reverses methotrexate-induced oxidative stress and apoptosis in hepatocytes by modulating Nrf2-NF-κB pathways. The Journal of nutritional biochemistry, 24(12), 2040-2050.

25. Ibrahim, M. A., Amin, E. F., Ibrahim, S. A., Abdelzaher, W. Y., & Abdelrahman, A. M. (2014): Montelukast and irbesartan ameliorate metabolic and hepatic disorders in fructoseinduced metabolic syndrome in rats. European journal of pharmacology, 724, 204-210.

26. Knowles, R. G., & Moncada, S. (1994): Nitric oxide synthases in mammals. Biochemical Journal, 298(Pt 2), 249.

27. Green, S. J., Scheller, L. F., Marletta, M. A., Seguin, M. C., Klotz, F. W., Slayter, M., & Nacy, C. A. (1994): Nitric oxide: cytokineregulation of nitric oxide in host resistance to intracellular pathogens. Immunology letters, 43(1-2), 87-94.

28. Ozkan, E., Yardimci, S., Dulundu, E., Topaloğlu, Ü. Şehirli, Ö. Ercan, F., & Şener, G. (2010): Protective potential of montelukast against hepatic ischemia/ reperfusion injury in rats. Journal of Surgical Research, 159(1), 588-594.

29. Maruf, A. A. (2014): Evaluation of DrugInduced Liver Injury in an In Vitro Oxidative Stress Inflammation System (Doctoral dissertation).

30. Evren Kose, Hilal Irmak Sapmaz, Ediz Sarihan, Nigar Vardi, Yusuf Turkoz, and Nihat Ekinci1 (2012): Beneficial Effects of Montelukast against Methotrexate-Induced Liver Toxicity: A Biochemical and Histological Study. The Scientific World Journal Volume, Article ID 987508, 6 pages.