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Comparative Effect of Verapamil and Famotidine on Methotrexate Induced Hepatotoxicity in Rats

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Abstract

This research aims to evaluate possible protective effect of both Verapamil (VER) (150 mg∕kg) and Famotidine (FAM) (40 mg∕kg) on methotrexate (MTX) (5 mg∕kg) induced hepatotoxicity in rats. Forty adult male rats were divided into 5 groups; (n= 8). Group 1 received saline (control). Group 2 was subjected to single dose of MTX. Groups 3 and 4 were treated with VER and FAM respectively. Group 5 was treated with combined VER and FAM. At the end of treatment, the impacts of MTX on the hepatic damage and its prevention by VER and FAM were assessed by estimation of serum AST, ALT, total protein and albumin. Additionally, hepatic SOD activity, NO, MDA and TNF-α levels were measured in liver homogenates. MTX administration resulted in significant increases in ALT, AST, NO, MDA and TNF-α levels and reduces serum albumin, total protein and SOD levels; as well as severe inflammation and congestion in liver tissues compared to normal control group. On the other hand, treatment with VER and FAM ameliorated histopathological and biochemical deterioration induced by MTX. The oxidative stress plays an important role in MTX-induced hepatotoxicity in rats. Effects of VER on different parameters were better than FAM, while its combination was the best.

Keywords: Verapamil; famotidine; MTX; hepatotoxicity.

1. Introduction

Th Methotrexate (MTX) is a dihydrofolate reductase inhibitor; it is used in treatment of many cancer (as lymphomas and osteosarcoma) and chronic inflammatory diseases (as rheumatoid arthritis and psoriasis). Using of MTX in treatment of diseases is limited by its adverse effects, including its hepatotoxic effect. MTX is known to increase hepatic transaminase levels. However, its association with liver histological damage, especially fibrosis and cirrhosis, is of concern (**Bath et al., 2014**).

Verapamil (VER) is a calcium channel blocker; calcium regulates many cellular processes and is included in many pathological processes as major

cardiovascular complication, accumulation of calcium is an important factor of causing hepatocellular necrosis. Influx of calcium inside cells stimulates releasing inflammatory mediators under specific conditions. It can activate nitric oxide synthase and stimulate production of prostaglandins, leukotriene, and thromboxane (**Bernardi and Rasola, 2007**). Calcium also stimulates apoptosis through initiation of apoptotic cascade (**Cooper, 2002**). Calcium channel blockers have anti-inflammatory effects and antioxidant properties that allow them to decrease tissue damage (**Messiha and Abo-Youssef, 2015**). In addition to anti-inflammatory effects of VER, it also has protective effect function on liver and neurons independent of calcium influx blockade

(Ozogul et al., 2013).

Famotidine (FAM) is H2 receptor blockers; it has hepatoprotective and antioxidant effects which were investigated in different experimental animals, antioxidant activities were reported by previous study in vitro assay such as 1, 1-diphenyl-2-picryl hydrazyl, nitric oxide and hydrogen peroxide-scavenging activity assays (Ahmadi et al., 2011). Therefore, protective effects of these drugs on the liver may be due to their antioxidant activity in cleaning body from free oxygen species such as O_2^- and OH^-, which rise during MTX administration (Marnett, 2000).

Based on the facts mentioned above, our study was performed to evaluate possible protective effects of VER and FAM on MTX–induced hepatotoxicity in rats.

2. Materials and Methods

2.1. Drugs and chemicals

Methotrexate, VER and FAM were purchased from Sigma Aldrich Company, England. Kits for superoxide dismutase enzyme (SOD), nitric oxide (NO) and malondialdehyde (MDA) and tumor necrosis factor-α (TNF-α) assay were obtained from Bio-diagnostic Company in Egypt. Liver functions markers kits; Aspartate aminotransferase (AST) alanine aminotransferase (ALT), total protein and albumin were obtained from Egyptian Company for Biotechnology, Cairo, Egypt.

2.2. Animals

The study was conducted on forty adult male Wistar rats (average weight 200-250 g) that were obtained from animal house of Faculty of Medicine, Sohag University and housed in room temperature (23-25°C). They were given food and water freely. For acclimatization, rats were housed in same conditions before conducting the experiment. The protocol of this study was approved by the Institutional Animal Care and Use Committee of Sohag University, Sohag, Egypt (approval number: Sohag 5-5-11-2023-01).

2.3. Experimental design and procedure

Forty rats were randomly divided into five groups of eight rats each. All rats were treated daily by VER, FAM or both for 10 days except MTX was injected by intraperitoneal injection once on 4th day. Groups were divided as follows:

1. **Normal control group:** Administration of NaCl (0.9%) solution i.p daily.

2. **MTX group:** Single administration of MTX (5 mg/kg/ once on 4th day. i.p) (Ozogul et al., 2013).

3. **VER group:** Administration of VER (150 mg/kg/day. i.p) for 10 days and MTX (5 mg/kg once on 4th day. i.p) (Ahmadi et al., 2011).

4. **FAM group:** Administration of FAM (40 mg/kg/day. i.p) for 10 days and MTX (5 mg/kg/ once on 4th day. i.p (Ahmadi et al., 2011).

5. **Combined VER and FAM group:** Administration of VER (150 mg/kg/day. i.p), FAM (40 mg/kg/day. i.p) for 10 days and MTX (5 mg/kg/ once on 4th day. i.p).

All rats were fasted for a 24 hour before scarification by decapitation at end of the experiment. Blood samples were collected to be centrifuged, serum was stored quickly on -20˚c for biochemical analysis (AST, ALT, Total protein and albumin). Representative liver tissue sample was homogenized in phosphate buffered saline (pH 7.4) after washing in ice cold saline. The homogenate was centrifuged, and the supernatant was aliquoted and stored at -80°C for determination of hepatic SOD, NO, MDA and TNF-α levels. Another liver sample was fixed in formalin (10%) for further histopathological evaluation.

2.4. Assessment of liver function markers

AST, ALT, total protein and albumin were measured by spectrophotometer (Jenway 6051 colorimeter spectrophotometer). Serum ALT and AST values were described in U/L. Serum total protein and albumins were recoded as gm/dL.

2.5. Determination of hepatic SOD, NO and lipid peroxidation

Hepatic SOD, NO and MDA levels were assesed by using an enzyme linked immunosorbent assay (ELISA) according to manufacturer's instructions. SOD result was recorded as U/g tissue, NO result was described in µmol/g tissue and MDA value was expressed as nmol/g tissue.

2.6. Measurement of the inflammatory cytokine TNF-α

Hepatic TNF-α level were measured with ELISA kit. The assay was conducted according to the

manufacturer's instructions. TNF-α result was presented in pg/g tissue.

2.7. Histopathological evaluation

Liver tissues samples of all rats in study groups were kept in 10% formaldehyde solution for 24 hours. Then these tissues were embedded in paraffin, 5 μm sections were obtained and stained with haematoxylin and eosin stain to verify histological features using light microscopy.

2.8. Statistical analysis of data

The data were represented as mean ± SEM and analyzed using SPSS software (IBM-SPSS version 22.0). One-way analysis of variance and Tukey's post hoc test were used to analyze data of this study. Differences were considered significant if p < 0.05.

3. Results

3.1. Effects of VER, FAM and their combination on liver function markers

In this study, our results revealed that single administration of MTX induced rise in elevation of liver enzymes (AST and ALT) significantly when compared to normal control group, as well as a significant decrease in serum albumin and total protein levels when compared to normal control group ($p < 0.001$). Treatment with VER and FAM in single and combined administration for 10 days lowered serum levels of AST and ALT and increased serum levels of albumin and total protein significantly ($p < 0.05$ for all) when compared to the MTX group (**Table1**).

The ameliorative effect of VER, FAM and their combination has been compared. VER administration induced significant improvement of serum AST and ALT levels ($p < 0.05$ for all) with insignificant change in serum albumin and total protein level when compared to MTX group.

Combined administration of VER and FAM induced significant improvement of all parameter when compared to separate administration of VER or FAM (**Table 1**).

3.2. Effects of VER, FAM and their combination on hepatic SOD level

When compared to normal control group, the acquired results in **Figure 1** indicate a significant reduction of hepatic level SOD after single intraperotineal injection of MTX (5 mg∕kg) ($p < 0.001$). Treatment with VER (150 mg∕kg) and FAM (40 mg∕kg) in single and combined administration for 10 days increase hepatic SOD level as compared to MTX group ($p < 0.001$). Administration of VER induced significant elevation of hepatic SOD level ($p < 0.001$) when compared to FAM group. Combined administration of VER and FAM induced significant increase in hepatic level of SOD ($p < 0.05$) when compared to FAM group and induced insignificant change when compared to VER group (**Figure 1**).

3.3. Effects of VER, FAM and their combination on hepatic NO level

Single administration of MTX (5 mg∕kg) significantly increases hepatic level of NO when compared to normal control group ($p < 0.001$). Treatment with VER (150 mg∕kg) and FAM (40 mg∕kg) in single and combined administration of VER and FAM for 10 days lowered hepatic level of NO when compared to MTX group ($p < 0.05$).

The protective effect of VER, FAM and their combination has been compared. Treatment with VER induced significant decrease of hepatic level of NO ($p < 0.05$) when compared to FAM group. Combined administration of VER and FAM induced significant reduction of hepatic NO level ($p < 0.05$) when compared to separate administration of VER or FAM (**Figure 2**).

Table 1: Effects of VER, FAM and their combination on liver function markers

Groups	ALT (U/L)	AST (U/L)	Albumin (gm/dL)	Total protein (gm/dL)
I (Normal control)	36.25±1.51	44.25± 0.45	5.22± 0.34	7.25± 0.94
II (MTX)	89.34±2.5[a]	97.5±1.5[b]	3.11±0.76[b]	4.32±0.34[a]
III (VER+ MTX)	45.12 ± 1.3[bd]	50.34±0.54[bd]	4.72±0.54[b]	5.20±0.34[b]
IV (FAM+ MTX)	50.22± 1.6[b]	56.11±0.33[b]	4.52±0.44[b]	5.05±0.11[b]
V (VER + FAM+ MTX)	41.22± 0.33[bcd]	48.15± 1.2[bcd]	5.03± 0.82[bcd]	5.85±0.22[bcd]

Data represent mean ± SEM of 8 observations. MTX =methotrexate, VER=Verapamil, FAM=Famotidine, [a] refers to significant difference from normal control group, [b] from MTX group, [c] from VER group, and [d] from FAM group.
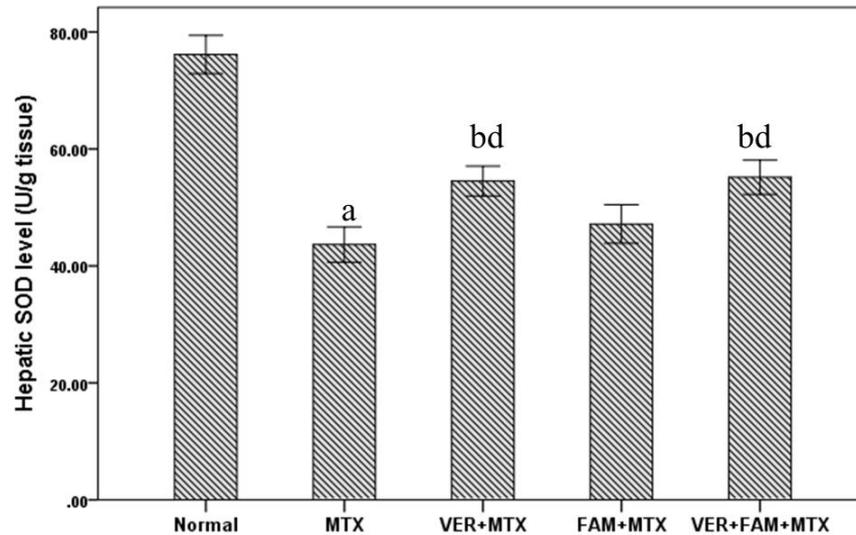
Figure 1: Effect of Effects of VER and FAM on hepatic level of SOD. The data were reported as mean ± SEM (n=8). MTX =methotrexate, VER=Verapamil, FAM=Famotidine, [a] refers to significant difference from normal control group, [b] from MTX group, [c] from VER group, and [d] from FAM group.
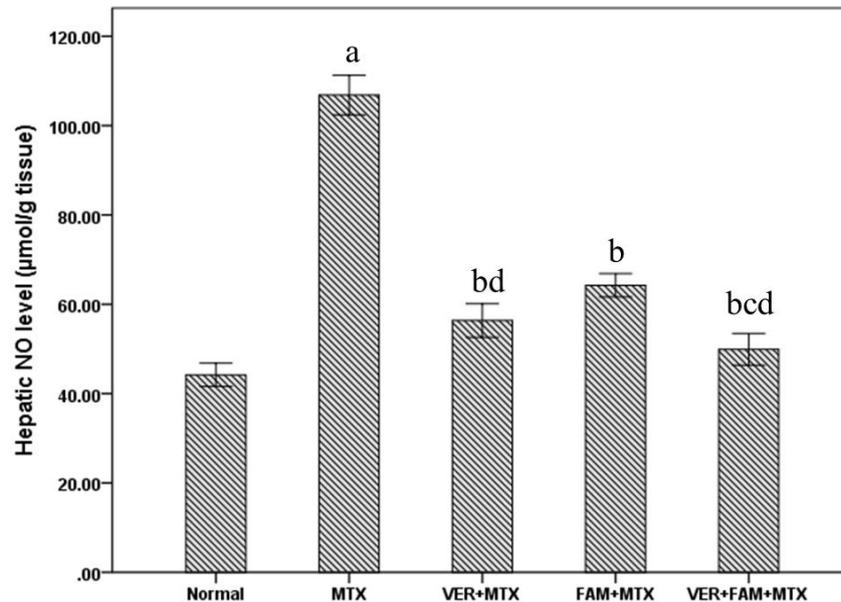

Figure 2: Effects of VER and FAM on hepatic level of NO. The data were reported as mean ± SEM (n=8). MTX =methotrexate, VER=Verapamil, FAM=Famotidine, [a] refers to significant difference from normal control group, [b] from MTX group, [c] from VER group, and [d] from FAM group.

3.4. Effects of VER, FAM and their combination on hepatic lipid peroxidation level

As shown in **Figure 3**, there was a significant increase in hepatic level of MDA after single administration of MTX ($p < 0.001$). Treatment with VER (150 mg⁄kg) and FAM (40 mg⁄kg) in single and combined administration for 10 days lowered hepatic levels of MDA when compared to MTX group ($p < 0.05$). Administration of VER induced significant elevation of hepatic MDA level ($p <$

0.05) when compared to FAM group. Combined treatment with VER and FAM induced significant increase in hepatic level of MDA ($p < 0.05$) as compared to FAM group and induced insignificant change when compared to VER group **(Figure 3)**.

3.5. Effects of VER, FAM and their combination on hepatic TNF-α level

Figure 4 demonstrates that the injection of a single administration of MTX (5 mg⁄kg) significantly rises of hepatic TNF-α level as compared to normal control group ($p < 0.001$). Treatment with VER

(150 mg⁄kg) and FAM (40 mg⁄kg) in single and combined administration for 10 days lowered hepatic level of TNF-α when compared to MTX group ($p < 0.05$). Administration of VER induced no significant difference of hepatic level of TNF-α ($p < 0.05$) when compared to FAM group. Combined administration of VER and FAM induced significant decrease in hepatic TNF-α level ($p < 0.05$) when compared to FAM group and induced insignificant change when compared to VER group (**Figure 4**).

3.6. Histopathological results

In general, liver tissue of all rats had preserved architecture with identified hepatic cording. Administration of MTX induced few microscopic spots of necrosis in addition to numerous scattered apoptotic hepatocytes. In addition; hepatocytes showed cloudy swelling and granular cytoplasm with almost central uniform nuclei. Other changes included congested central venules and congested hepatic sinusoids. There was mild to moderate portal and lobular inflammatory reaction mainly infiltration by lymphocytes and few neutrophils. No encountered fatty changes of hepatocytes (**Figure 5A and 5B**).

Rats treated with FAM showed scattered apoptotic hepatocytes with no identified necrosis. Hepatocytes showed granular cytoplasm with central normal looking nuclei. Additionally; congested central venules and scattered portal and lobular inflammatory reaction were identified (**Figure 5C and 5D**).

Alternatively; rate treated with VER showed relatively few apoptotic bodies and less prominent inflammation (**Figure 5E**). Combined treatment with FAM and VER protected liver tissue from damaging effect of MTX in terms of absent necrosis, rare apoptotic bodies and minimal inflammatory reaction. However, focal cloudy swelling of hepatocytes and few congested venules were identified (**Figure 5F**).

4. Discussion

In the current study, parameters of liver function were evaluated in normal control group and remained within normal levels; normal architecture with no pathological findings. Single administration of MTX induced a significant impairment in liver functions parameters indicated by a major rise in serum live enzymes (AST and ALT) when compared to normal control group, these findings may be attributable to toxic effect of MTX on the liver. In addition to, serum albumin and total proteins levels were significantly reduced as compared to normal rats. Our findings were in harmony with previous researches that reported an increase in serum levels of AST and ALT, as well as a reduction in serum albumin and total protein levels in MTX treated rats (**Salliot and van der Heijde, 2009; Ali et al., 2014; Yucel et al; 2017**).

Antioxidant enzymes play important roles in detoxifying oxygen free radicals and decrease their over synthesis and concentration in the cells (**Aristatile et al., 2009**). The present study revealed
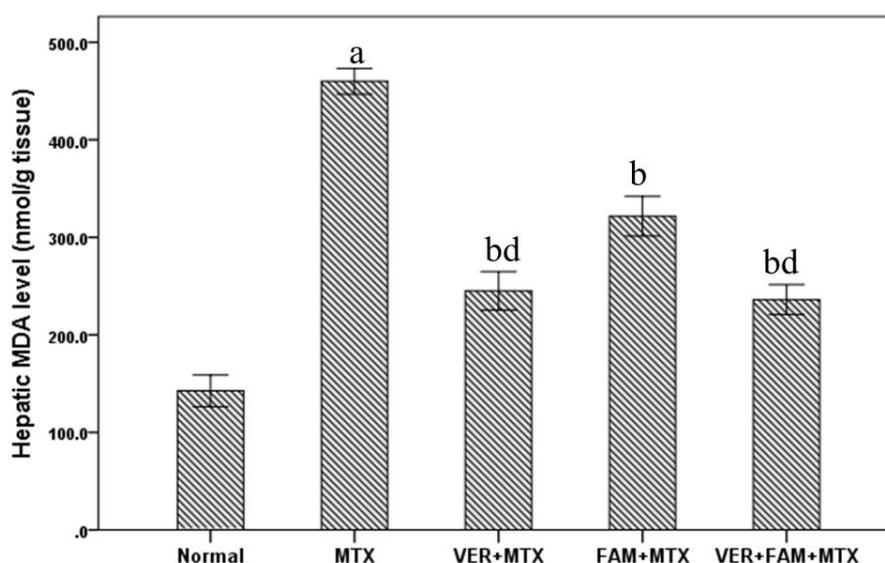

Figure 3: Effects of VER and FAM on hepatic level of MDA. The data were reported as mean ± SEM (n=8). MTX =methotrexate, VER=Verapamil, FAM=Famotidine, [a] refers to significant difference from normal control group, [b] from MTX group, [c] from VER group, and [d] from FAM group.
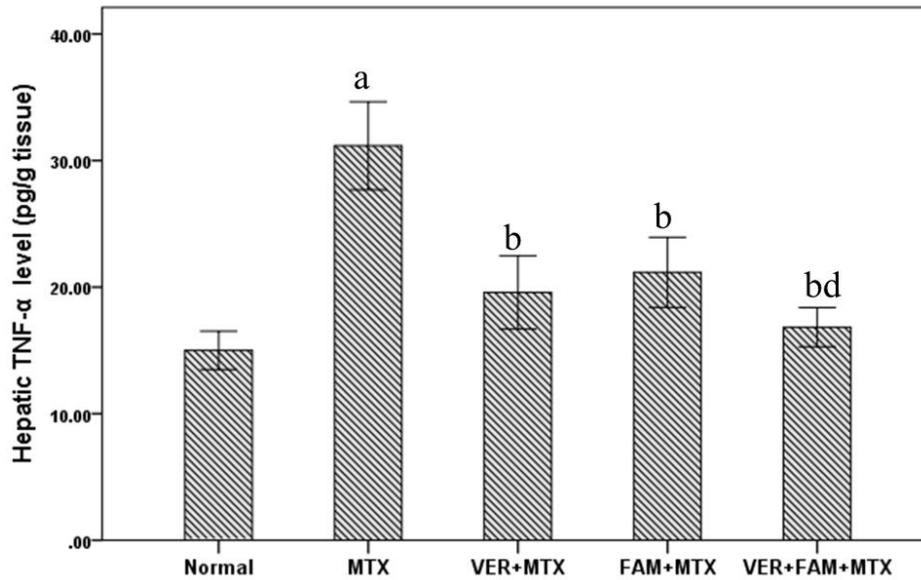
Figure 4: Effects of VER and FAM on hepatic level of TNF-α. The data were reported as mean ± SEM (n=8). MTX =methotrexate, VER=Verapamil, FAM=Famotidine, [a] refers to significant difference from normal control group, [b] from MTX group, [c] from VER group, and [d] from FAM group.
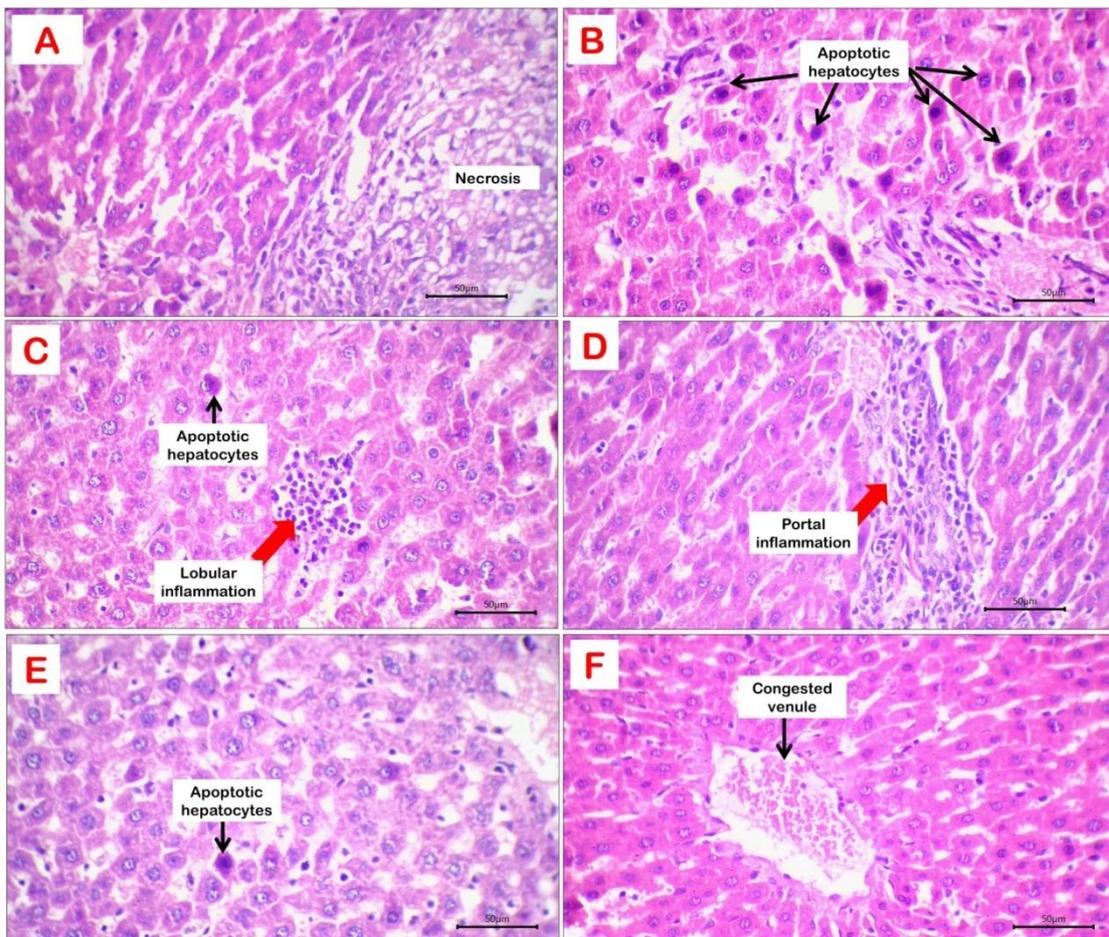

Figure 5: Sections of liver tissue of MTX treated rats showed focal necrosis (**A**) and numerous apoptotic hepatocytes (**B**). FAM treated rats showed cloudy swelling of hepatocytes, scattered apoptotic bodies with lobular and portal inflammation (**C and D**). VER treated rats showed cloudy swelling of hepatocytes with occasional apoptotic bodies (**E**) while rats treated with FAM and VER showed normal liver architecture with congested central venules (**F**). H&E stained sections; magnification is x400 for all.

that administration of MTX induced a highly significant decrease of hepatic SOD level and highly significant increase in hepatic levels of NO and MDA when compared to normal control group. These results were in line with previous studies (**Uraz et al., 2008; Gautam et al., 2016; Abo-Haded et al., 2017; Hassanein et al., 2019**); where MTX administration elevates ROS and MDA production that are considered as oxidative stress markers. Other report recorded that high NO level and lipid peroxidation is an important factor of MTX hepatotoxic effect. Famurewa and colleagues reported that ROS/RNS have a role in pathogenesis of hepatotoxic effect of MTX. When these reactive species interact with biological macromolecules to produce lipid peroxides and cause mutations of DNA (**Famurewa et al., 2018**).

Single administration of MTX induced significant increase in hepatic level of TNF-a in comparison to normal rats. In addition to apoptosis, necrosis of liver cells and inflammatory reaction. These results were in line with Famurewa study that demonstrated hepatotoxic effect of MTX which induce inflammation and oxidative toxicity in rats (**Famurewa et al., 2018**).

Administration of VER significantly decreases serum liver enzymes (AST and ALT) and increases serum levels of total protein and albumin as compared to MTX group. Additionally, VER administration markedly improved oxidative stress biomarkers (MDA, SOD and NO) when compared to MTX group. Hepatic level of TNF-α was significantly increases in VER group when compared to MTX group. VER administration ameliorates biochemical and histopathological deterioration induced by MTX in the current study. These findings were in consistent with **Huber et al. (2009)** and **Sato et al. (2009)** who reported that elevation of intracellular calcium deteriorates mitochondrial function in the cell through oxidative stress, reduces intracellular production of ATP and causes hepatic cell damage. Blocking calcium channel solves these problems and protects the liver cell. Additionally, Ray and colleagues recoded explanation for hepatoprotective effect of VER which may be explained by its calcium channel blocking influx to hepatocytes (**Ray et al., 1993**). **Murata et al. (1994)** showed an association between high intracellular calcium level and injury of oxidative stress.

Calcium has an important role in mitochondrial membrane permeability transition and liver cells damage which can be inhibited with calcium

channel blocker (VER) (**Bruschi and Priestly, 1990**). Elevation of calcium level in the cytoplasm stimulates production of proteases which is calcium dependent. Blocking of calcium channel at this stage will protect liver cell from damage. Nucleus destruction is also calcium dependent endonuclease production. Calcium channel blocker will inhibit fragmentation of nucleus and DNA (**Oliveira et al., 2004**). Administration of VER was associated with moderate improvement of histological liver cell damage caused by MTX by ameliorating inflammation, necrosis and congestion of hepatocytes.

The present study revealed hepatoprotective effect of FAM by significant reduction of serum liver enzymes (AST and ALT) when compared to MTX group. Additionally, serum albumin and total protein levels were increased after treatment with FAM, these results were in agreement with Halliwell research (**Halliwell et al., 1992**). Treatment with FAM induced significant elevation in hepatic SOD and reduction of hepatic NO and MDA levels when compared to MTX group. These findings were proved by improvement of histopathological deterioration which induced by MTX in our results by mild reduction of hepatic necrosis and inflammation. These results were in agreement with **Bindu and Babu (2001)**. Previous study revealed that FAM has many pharmacological actions in vivo and in vitro. Antioxidant effects of FAM have been reported in patients with peptic ulcers and in patients with ischemia perfusion of gastric mucosa (**Ching et al., 1993**). FAM counteracts effect of NO production and, this prevents adverse effects of excessive NO production in human body (**Dehpour et al., 2009**). Furthermore, FAM Scavenges hydrogen peroxide by its phenolic component, which can neutralize to hydrogen peroxide into water by adding electrons to it (**Ahmadi et al., 2011**). Oxygen free radicals mediate lipid peroxidation which is an important cause of deterioration and damage of cell membrane by degradation of its fatty acids. Peroxidations of cell membrane may causes changes in membrane permeability and fluidity and also induce degradation of protein which ended by cell lysis (**Yoshikawa et al., 1993**). These previous facts explain hepatoprotcitive effect, antioxidant activity and inflammatory effects of FAM that occurred in our study. Combined VER and FAM administration showed more protective effects on the liver than single administration of VER or FAM as regard biochemical and histological findings.

5. Conclusion

MTX has a hepatotoxic effect in rats by it oxidative damage ability. VER administration recorded better result than FAM in most parameters and their combination recorded the best results in all parameters.

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Conflicts of Interest

No competing interests were declared by the authors.

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