Effect of methotrexate on the liver of male albino rats and possible protective role of Barley\'s grains

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Abstract: Introduction: The most common side effects associated with the use of methotrexate (MTX) in the commonly used doses is hepatic toxicity. As a herbal alternative *Hordeum vulgare* seeds (Commonly known as Barley) commonly used by traditional medical practitioners in the treatment of many diseases including liver diseases. **Aim of the work:** The aim of this study was to study the potential protective effect of Barley\'s grains on MTX induced liver injury. **Materials and methods:** Adult male rats were used. The animals were divided into three groups each of them consists of 10 rats: Group I: (control group): were injected with IP saline. Group II: were IP injected with MTX in a dose of 6mg/kg /day for 5 days. Group III: Barley grains were given at a dose of 200g/kg/day for 30 days plus intraperitoneal injections of MTX on the day 25, at the dose 6mg/kg /day for 5 days. The rats were sacrificed by decapitation then liver pieces for light microscopic study were obtained. **Results:** MTX markedly affected the liver tissue as appeared by light microscopic, immunohistochemical examinations and morphometric studies. Pretreatment of Barley\'s grains could attenuate some of these changes. **Conclusion:** Pretreatment of Barley\'s grains had a possible protective effect against MTX induced liver injury.

Keywords: liver, MTX, Barley\'s grains.

1. Introduction

Methotrexate (MTX) is an effective treatment modality largely used in rheumatoid arthritis, psoriasis, leukemias and some other autoimmune disorders for more than 40 years (Naldi and Griffiths, 2005). It is an anti-metabolite drug used against a broad range of neoplastic disorders. It interferes with the synthesis of nucleic acids (Ortiz et al., 1998).

MTX is minimally metabolized by the human species. It is converted in the liver and other cells to higher polyglutamate forms (Mueller-Koch et al., 2005). About 20% of the drug is eliminated in the bile; so one of the most serious side effects of MTX therapy is hepatic toxicity (AlMotabagani, 2006).

Barley (*Hordeum vulgare* L.), is widely consumed cereal, Barley meals and fractions renewed interest as ingredients for the production of functional foods, due to its bioactive compounds, such as β-glucans and tocopherols (Dabina Bicka et al., 2011). The antioxidant role of Barley is because of its higher content water-soluble, fat-soluble, and insoluble antioxidants especially for selenium, which is an essential component of several major metabolic pathways antioxidant defense systems. (Badr et al., 2000). As methotrexate is essential drug for treatment of many diseases and we could not dispense it continuous trials were done to attenuate its side effects especially on the vital organs. The aim of this study is to know how far the Therapeutic feeding is essential nowadays to counter the side effects of different drugs.

2. Materials and Methods

**Materials:**

MTX was brought from CIPLA LTD Verna industrial estate pharmaceutical company. Barley was purchased from local market at Sohag, Egypt.

**Animals:**

Thirty adult male rats weighting 150-200 gm/each were used in the present study. All animals were housed under the same conditions and allowed food and water. Rats were randomly divided into three equal groups.-

**Group I (Control group):** was given the usual food and injected intraperitoneally with saline. **Group II (MTX group):** given the usual food with intraperitoneal injections of MTX at the dose 6mg/kg/day for 5 days (Kesik et al., 2009).-

**Group III (Barley + MTX group):**

Barley grains were given at a dose of 200g/kg/day beside the usual food for 30 days (Rebolé et al., 2010) with intraperitoneal injections of MTX on the day 25, at the dose 6mg/kg /day for 5 days (Kesik et al., 2009) and at the end, the animals were sacrificed by cervical dislocation. Specimens from the liver were obtained and subjected to light microscopic studies.

**For light microscopic study:**

Specimens were fixed in 10% formalin, processed and embedded in paraffin. Serial sections (5 microns) were prepared and stained by hematoxylin
and eosin stain to verify histological details, Masson’s trichrome to demonstrate the collagen fibers and Periodic Acid Schiff (PAS) for detection of glycogen (Bancroft and Stevens, 1996).

For Immunohistochemistry:
Expression of CD68 and P53 was detected using avidin–biotin complex (ABC) method (Sternberger, 1979). Dewaxed and rehydrated sections were washed in distilled water for 5 min, rinsed in phosphate buffered saline with Tween-20 (PBST) for 10 min and incubated with 10% normal goat serum for 15 min. Then, the sections were incubated with anti-mouse P53 or CD68 anti-mouse monoclonal antibody (Dako 1:100 and 1:800, respectively) for 1–2 h at room temperature (Holness and Simmons, 1993; Tousson et al., 2011).

Morphometric study:
The mean number of P53+ and CD68+ cells as well as the density of PAS reaction and area% of the collagen fibers were measured in five fields from each animal on high power field at magnification 400 from equally magnified images captured from the stained sections. The digital photos were captured in the Microscopic Photography Unit (Histology Department, Faculty of Medicine, Sohag University) using a Fifty different relevant fields from each animal group were selected randomly. The measurements were performed using Digimizer PC image analysis and image j software (Leica Q 500 MC program, Wetzlar, Germany).

Statistical analysis:
Statistical analysis were done using SPSS software version 16. Variables were represented by mean ± SD (mean ± standard deviation of mean). One way ANOVA was used to compare the means of these variables between different groups. Finally the significance was considered according to the level of significance $p$ value as follows: $p > 0.05$ non significant, $p \leq 0.05$ significant *, $p \leq 0.01$ highly significant** and $p \leq 0.001$ (***) → Very high significant difference.

3. Results
Group I (Control group)
H & E stained sections:
In the control group (Group I), the ordinary architecture of the hepatic tissue was clear. The hepatocytes were arranged in cords radiating from the central vein. They were polygonal cells with acidophilic cytoplasm having granular basophilia, vesicular nuclei and prominent nucleoli (Fig. 1).

PAS stained section showed intense reaction with glycogen content diffusely distributed throughout the hepatic lobules. The glycogen granules appeared as heavy magenta red deposits filling the cytoplasm of the hepatocytes (Fig. 2 & Histogram1).

Masson’s trichrome stained Sections revealed very thin strands of collagen fibers around the central veins and portal tracts (Fig. 3 & Histogram 1).

Immunohistochemical stains:
Expression of cytoplasmic P53 and the incidence of apoptotic cells were very low in the control which showed few p53 positive hepatocytes and vonkupffer cells (Fig. 4 & Histogram 2). Expression of CD68 was high in with normal distribution of a liver macrophages (intense reaction for CD 68 in Vonkupffer cells ) (Fig. 5 & Histogram 3).

Fig (1): A section of control liver showing the central vein (c ), cords of hepatocyte separated from each other by blood sinusoids (S), hepatocytes are polygonal acidophilic with basophilic granules and vesicular nucleus (arrow). (H & E x 400).

Fig (2): A section of control liver showing Intense PAS positive reaction in hepatocytes (arrow) (PAS X400)
Fig (3): A section of control liver showing few strands of collagen fibers around central vein (arrow) (Masson Trichrome X200)

Fig (4): A section of control liver showing minimal reaction for p53 in hepatocytes (irregular arrow) and vonkupffer cells (arrow). (p53 X400)

Fig (5): A section of control liver showing, intense reaction for CD 68 in Vonkupffer cells (arrow). (CD 68X400)

Group II (Methotrxate treated group)

H & E stained sections showed numerous remarkable histological changes in this group compared to the control. There was distortion of the normal hepatic architecture. Most of hepatocytes were degenerated with central faint nuclei and vacuolated cytoplasm. The wall of central vein was destroyed with leakage of blood (Fig.6). PAS stained sections showed depletion of the glycogen stores of the liver. Hepatocytes showed a cytoplasm either partially or completely devoid of glycogen (Fig.7 & Histogram 1). There was increase in the amount of collagen fibers around the central veins, portal area and blood sinusoids. (Fig.8 & Histogram 1)

Immunohistochemical stains: There was significant increase in positive cells of the P53 in both hepatocytes and vonkupffer cells compared with the control group (Fig.9 & Histogram 2). There was significant decrease in the CD68 positive cells compared with the control group (Fig.10 & Histogram 3)

Fig (6): A section of Methotrxate treated liver showing: most of hepatocytes are degenerated (arrow head). The wall of central vein is destroyed with leakage of blood (arrow). (H & E X400)

Fig (7): A section of methotrexate treated liver showing, depletion of the glycogen stores of the liver. The cytoplasm of hepatocytes either partially or completely devoid of glycogen (arrow) (PAS X400)
Fig (8): A section of MTX treated liver showing, increased collagen fibers around central vein (arrow) and around blood sinusoids (arrow head) (Masson Trichrome X 200)

Fig (9): A section of methotrexate treated liver showing, intense reaction of p53 in hepatocytes (arrow) and Vonkupfer cells (arrow head). (P53 X400)

Fig (10): A section of Methotrexate treated liver showing, minimal reaction for cd68 in Vonkupfer cells (arrow) (CD 68 X400)

**Group III (The Barley + MTX group)**

**L.M in H & E stained sections:**

Hepatocytes restored their cytoplasmic appearance as the control group. The endothelium of the central vein were intact (Fig. 11). Mild reaction of PAS for glycogen content was observed (Fig. 12 & Histogram 1).

Masson’s trichrome sections showed decreased amount of collagen fibers around the central veins and the portal areas compared to the previous group (Fig. 13 & Histogram 1).

**Immunohistochemical stains:**

There was decrease of P53 positive hepatocytes and vonkupfer cells compared to the previous group (Fig. 14 & Histogram 2). Increase expression of positive cells of CD68 observed compared to the previous group (Fig. 15 & Histogram 3).

Fig (11): A section of Barley treated liver showing: most of hepatocytes are more or less as the control (arrow head) with prominent intact nucleus (arrow). The wall of central vein (C) and sinusoids (S) are intact. (H & E 400)

Fig (12): A section of Barley treated liver showing intense PAS reaction in hepatocytes (arrow). (PAS X400)
Morphometric Study:
- Density of PAS reaction in liver in Methotrexate treated animals showed Highly significant decrease (< 0.01) in comparison to control and Barley treated group. Table (1), Histogram (1).
- Area % of collagen fibers in liver in Methotrexate treated animals showed significant increase (< 0.01) in comparison to control and Barley treated group. Table (1), Histogram (1).
- Expression of P53 (in hepatocytes and Von Kupffer cells) in Methotrexate treated animals showed Highly significant increase (P ≤ 0.01) in comparison to control and Barley treated group. Table (1), Histogram (2).
- Expression of CD68 (in Von Kupffer cells) in MTX treated animals showed highly significant decrease (P ≤ 0.01) in comparison to control and Barley treated group. Table (1), Histogram (3).

<table>
<thead>
<tr>
<th></th>
<th>P53 Hepatocytes</th>
<th>P53 Von Kupffer</th>
<th>CD68</th>
<th>PAS density</th>
<th>Area % of collagen fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.3±.33</td>
<td>4.6±.32</td>
<td>42.3±.82</td>
<td>187.91±1.5</td>
<td>21.19±0.34</td>
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<tr>
<td>MTX group</td>
<td>26.7±.73**</td>
<td>10.3±.43**</td>
<td>12±.52**</td>
<td>135.29±1.7**</td>
<td>49.05±0.62**</td>
</tr>
<tr>
<td>MTX+ Barley group</td>
<td>11.4±.53</td>
<td>6.32±.42*</td>
<td>23.3±.62*</td>
<td>170.81±1.8</td>
<td>32.41±0.46</td>
</tr>
</tbody>
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Values are represented by means ±SD (standard deviation)**: highly significant (P ≤ 0.01)
Histogram (1): showing expression of PAS density and area percent of collagen fibers in different groups.

Histogram (2): showing expression of P53 (in hepatocytes and Von kuppfer cells) in different groups.
4. Discussion

In the present work we tried to study the protective effect of Barley's grains on the histological changes of the liver caused by MTX in adult male rats. Many histological changes were induced by MTX; Both of degeneration and apoptosis were observed in group treated with methotrexate. Destruction of the central veins with leakage of blood also was detected. These results were in agreement with previous studies Al-Ali et al. (2005) who studied the effects of MTX on liver cells and stated that MTX caused observed vacuolation of the cytoplasm of liver cells which was explained by accumulation of toxic metabolites caused damage of the cell membranes with subsequent hydropic degeneration and vacuolation of the hepatocytes. Cutolo et al., (2001) reported that MTX inhibits lipoxygenase enzyme resulted in accumulation of lipid droplets in the cytoplasm of cells. Davis (1984) and Rubin (2001) reported that accumulation of lipids within the hepatocytes could be related to impaired protein synthesis as a result of rER damage and accordingly inhibition of lipoprotein manufacture results in the accumulation of fats liver cell cytoplasm.

MTX impair the normal structural organization of the hepatic lobules. (Abd El- Azim, 2014). As the metabolism of the drug mainly take place in the liver with conversion of the drug into its major extracellular metabolite, 7-hydroxymethotrexate (Chladek et al., 1997). MTX is stored inside the cells in a polyglutamated form (Galivan et al., 1983). Long term drug administration can cause accumulation of MTX polyglutamates and decreased folate levels (Prey and Paul, 2009) that is considered as a mechanism for MTX hepatotoxicity (Kamen et al., 1981; Kremer et al., 1986). The present work showed increased amount of collagen fibers around the central veins, portal area and blood sinusoids. This observation was consistent with previous study (Sener et al., 2006). In a previous study it was proved that MTX could activate myofibroblast with subsequent pulmonary fibrosis (Ohbayashi et al., 2014). MTX cause vascular endothelial dysfunction by causing hyperhomocysteinemia, direct injury to endothelium or by increasing the oxidative stress. (Sankrityayan and Majumdar, 2016).

The vascular lesions that might affect the blood supply to the liver cells resulting in their degeneration and necrosis (Yamamoto et al., 1995). The present study showed depletion of the glycogen stores of the liver cells which might be occurred as a result of mitochondrial damage. Some authors explained glycogen depletion due to the inhibition of mitochondrial energy metabolism and to inability of the liver to store glycogen and to convert lactate and private to glycogen (Hsu et al., 1997). In the current study, the immunohistochemical observations of the liver tissues showed a significant increase in the apoptotic proteins P53 and decrease in CD68 reaction after Methotrxate treatment compared to the control group. Methotrxateacts as a dihydrofolic acid analogue that binds to the dihydrofolic acid reductase enzyme by inhibiting the synthesis of tetrahydrofolate, which is required for DNA synthesis. Inhibition of purine and pyrimidine synthesis leads to DNA defects, which results in apoptosis (Tsurusawa et al., 1997). It also inhibits RNA and protein synthesis and prevents cells from entering the S phase of the cell cycle. Therefore
Methotrexate affects not only tumor cells (kolli et al., 2007) and (Mueller-Koch et al., 2005).

In the present study, the light microscopic results revealed that rats treated with Methotrexate and Barley showed partial restoration of the normal hepatic architecture. The central vein of Barley treated group regained its intact endothelium, a previous study proved that intake of Barley had antioxidant properties could increase angiogenesis in vitro and in vivo (Agostini et al., 2015).

Hepatocytes showed partial restoration of glycogen content. There was mild increase in the amount of collagen fibers after treatment with Barley compared to the control group. These results are in agreement with previous studies which reported that barely is rich in antioxidants and explained the protective effect of Barley which was noticed in the liver of Barley treated diabetic animals (Khalaf and Mohamed, 2008).

Raw Barley contains catalase, cellobiase, cytaste, diastase, lichenase, mannase, mannobiase, oxidase, peroxidase, and phytsas with active proteolytic enzymes (Kanauchi et al., 2001). This antioxidant effect of Barley because of its higher content of water-soluble, fat-soluble, and insoluble antioxidants. The long list of cereal antioxidants includes vitamin E, selenium, phenolic acids, and phytic acid. These multifunctional antioxidants come in immediate release to slow-release forms and thus are available throughout the gastrointestinal tract over a long period after being consumed (Slavin et al., 1999).

In the current study, the immunohistochemical observations of the liver tissues a significant decrease in the expression of the cytoplasmic P53 and increase in CD68 was observed. The hepatoprotective activity of methanolic extract of Barley was tested against Ethanol-induced liver damage in rats. Pretreatments with it produced significant reversal in the expression of nuclear proto-oncogenes during methotrexate polyglutamate accumulation in-vitro: effects of cellular folate content. Biochemical Pharmacology; 46(5):371-379.

Conclusion:

Pretreatment of Barley's grains had a possible protective effect against MTX induced liver injury. It is recommended to use Barley's grains as a basic constituent of the meals in patients receiving MTX. More researches should be done to study the effect of different constituents of Barley's grains separately.

References