Protective Effect of Vitamin A against Oxidative Stress Caused by Methotrexate

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Abstract
Methotrexate (MTX), a folate antagonist agent, is mainly used in treatment of malignant tumors and autoimmune diseases. The present study was undertaken to determine whether antioxidant vitamin (vitamin A) could ameliorate methotrexate-induced oxidative stress in male rabbits. Twenty male rabbits were randomly assigned into four groups. Group 1: control group, Group 2: MTX-treated group (received 20 mg/kg MTX intraperitoneally), Group 3: Vit.A treated group received 5000 IU Vit.A orally) and Group 4: MTX+Vit.A treated group received MTX 20 mg/kg plus 5000 IU vit.A). After 4 weeks of treatment, blood samples were collected by cardiac puncture to determine the serum malondialdehyde (MDA), as a good indicator for lipid peroxidation and oxidative stress. The results showed that there was a significant increase (p<0.05) in serum MDA level in MTX-treated group compared with control group. Also, the results revealed that there was a significant decrease (p<0.05) in serum MDA level in MTX+Vit.A treated group in comparison with MTX-treated group. The present study suggests that the administration of vit.A with MTX is associated with reduction in oxidative stress, and therapeutic role in reduced toxicity of MTX.

Keywords: methotrexate, vitamin A, malondialdehyde, oxidative stress.

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**Introduction:**

Methotrexate (MTX), a folate antagonist agent, is mainly used in the treatment of malignant tumors; it has also been found to have a major therapeutic role in non-neoplastic diseases as an anti-inflammatory and immune-suppressive agent [1]. Methotrexate acts 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 [2].

Strong evidences support a role for reactive oxygen species (ROS) in the pathogenesis of MTX damages [3]. It is known that cells are protected against oxidative stress by the action of certain enzymes, vitamins, and other substances, collectively known as antioxidants [4].

Antioxidants have been reported to ameliorate MTX injury, Somi *et al.* [1] found the use of lipoic acid as an antioxidant reduced the toxic effect of MTX, also the same effect found by Ramadan *et al.* [5] found use melatonin as antioxidant attenuate toxicity of MTX. Vitamin A is a potent antioxidant and acts as a scavenger of free radicals either independently or as a part of large enzyme system [6]. Ciaccio *et al.* [7] has been reported that Vit.A may act as a physiological antioxidant in cell membranes where it is localized.

Lipid peroxidation in biological membranes causes impairment of membrane functioning, decreased fluidity, inactivation of membrane-bound receptors and enzymes, and increased non-specific permeability to ions [8].

Malondialdehyde (MDA), it is the end product of lipid peroxidation by free radicals. As it is generally accepted that lipid peroxides (ROO•) may play an important role in the pathogenesis of free radicals induced cellular injury [9 and 10]. So it consider a good marker of free radical mediated damage and oxidative stress [11]. This compound is reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress in cells and for advanced glycation end products [12].

**Aim of study**

The present study aims to investigate the effects of Vit.A on lipid peroxidation product malondialdehyde, in MTX-treated rabbits.

**Materials And Methods**

**Animals and Experimental Design**

Twenty local male rabbits weighing 1250-1480 gm were obtained from Biotechnology researches center of Al-Nahrain University. All the rabbits were housed in metal cages in a room under standard environmental conditions, with a range temperature 21-23 °C and 12/12 hours light/dark cycle. Pellet and tap water were provided *ad libitum*. The animals were allowed to acclimatize for two weeks before beginning the experiment. The rabbits were divided into 4 groups, each group including 5 animals, as following:-

**Group 1:**

Rabbits had been given 1ml of Normal saline intraperitoneally.

**Group 2:**

Rabbits were given methotrexate at a dose level of 20 mg/kg intraperitoneally on alternative days for 4 weeks (1).

**Group 3:**

Rabbits were given Vitamin A 5000 IU orally on alternative days for 4 weeks [13].

**Group 4:**

Rabbits were given methotrexate 20 mg/kg intraperitoneally and Vitamin A (5000 IU) orally on alternative days for 4 weeks.

**Blood Collection**

At the end of the experiment and after overnight food deprivation, blood samples (10 ml) were collected by cardiac puncture. The serum was prepared by centrifuging the blood samples at 3000 rpm for 15 minutes and collected by pipetting. The serum was kept in -20 °C until used for biochemical analysis.

**Determination of Malondialdehyde (MDA):**

Determination of the level of MDA, which is an index of lipid peroxidation, is based on the reaction with thiobarbituric acid (TBA) forming TBA-MDA product, which is a pink coloured product, according to the standard method of Guidet and Shah [14].

- **Procedure:**
  1. TBA solution (0.6) % was prepared by adding (0.6) gm of TBA powder to cylinder tube and add distilled water (D.W) gradually until reach final volume (100) ml, and put in water bath at (37)°C for (24) hour to facilitate dissolve of the powder.
  2. TCA solution (70) % was prepared by adding (70) gm of TCA crystal and put in cylinder tube and add D.W gradually until reach final volume (100) ml.
  3. TCA solution (17.5) % was prepared by adding (17.5) gm of TCA crystal and put in cylinder tube and add D.W gradually until reach final volume (100) ml.
4. Test tubes were prepared according to the number of serum samples and one test tube for blank.
5. (1) ml from each sample was added to espicilazed test tubes, (1) ml of D.W was added to blank.
6. (1) ml from TCA (17.5) % was added to all tubes.
7. (1) ml from TBA (0.6) % was added to all tubes.
8. The tubes were mixed by vortex, incubated in water bath at (100) °C for (15) minute.
9. After that tubes lefted for (10) minute in order to cooling.
10. (1) ml from TCA (70) % was added to tubes and shaker well and lefted to (20) minute at room temperature, in order to reaction occure.
11. Test tubes were put in centrifuge for (15) minute, at 2000 rpm.
12. Supernatant was separate from participitate, and supernatant colour was read it absorbance by spectrophotometer at wave length (534) nm.
13. Calculation of MDA concentration:

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\text{MDA concentration} = \frac{0.156 \times 10^5 \times 100}{\text{OD sample} - \text{OD blank}}
\]

Extension coefficient= 1.56*10^5 ml^-1 cm^-1

Statistical Analysis
The results were analyzed statistically by using the Statistical Analysis System- SAS (2004), completely randomized design-CRD (ANOVA table) and Duncan multiple range were used to comparative between means.

Results:
Serum malondialdhyde (MDA)
The statistical analysis of the results (Table 1 and Figure 1) showed that there were a significant (p<0.05) increase in serum MDA level in MTX treated group (6.42±1.21 nmol/ml when compared with the control group (1.24 ± 0.11) nmol/ml.

The result also revealed that there was a non-significant (P>0.05) increase in Vit.A treated group when compared with control, while there was a significant (P< 0.05) increase in MTX+Vit.A treated group (4.75±0.41) nmol/ml compared with control group.

The results showed that there was a significant (P< 0.05) decrease in serum MDA level in MTX+Vit.A treated group in comparison with MTX treated group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Malondialdhyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.24 ± 0.11</td>
</tr>
<tr>
<td>MTX</td>
<td>6.42 ± 1.21</td>
</tr>
<tr>
<td>Vit.A</td>
<td>1.98 ± 0.72</td>
</tr>
<tr>
<td>MTX+Vit.A</td>
<td>4.75 ± 0.41</td>
</tr>
</tbody>
</table>

Values are means± SE, Different letters refer to significant differences (P<0.05)

Figure 1- Effect of methotrexate (MTX) and Vit.A on serum malondialdehyde (MDA) concentration in male rabbits.

Discussion:
The results of this study showed a significant increase in serum MDA level in the MTX-treated group when compared with control, this result is in agreement with several reports demonstrated that MTX could MTX cause oxidative damage and increase in serum MDA level [15,16 and 17].
This effect of MTX could be due to the fact that MTX-treatment cause oxidative stress or oxidative cellular damage with it dual of free radical generation and profound lipid peroxidation [3].
Asci et al. [18] mentioned that oxidative stress due to abnormal production of ROS has been accused in the etiology of MTX- hepatotoxicity. Somi et al. [1] mentioned that lipid peroxidation mediated by free radical is believe to be an important cause of destruction and damage to cell membrane caused by MTX-treatment.
Malondialdehyde is a natural product of peroxidation of unsaturated fatty acids with three or more double bond [19]. Peroxidation of lipids is a binding process connected with the formation of aldehydes. One of them is MDA [20].
As MDA is one of the final products of polyunsaturated fatty acids peroxidation in the cells, increase in free radicals causes overproduction of MDA. Malondialdehyde level is commonly known as a marker of oxidative stress and the antioxidant status [21]. So an increase in lipid peroxidation and free radicals generation caused by MTX-treatment lead to increase MDA level. The results showed a significant decrease in MDA level in MTX+Vit.A treated group when compared with MTX treated group, demonstrating the protective effect of Vitamin A to reduced lipid peroxidation product. Al-Zeiny [22] mentioned the supplementation with vitamin A significantly reduced Glutathione (an important marker of lipid peroxidation) caused by treatment with methotrexate. Study by Fakher et.al. [23] showed that supplementation with Vit.A caused a decrease of MDA level of plasma in diabetic patients. Because vit. A is a potent Vitamin and act as scavenger of free radicals either independently or as a part of large enzyme system [24]. Also antioxidant Vitamin such Vitamin A act as the first line of defense against free radicals attack and lipid peroxidation, by it's ability to stabilize highly reactive free radicals [25]. Gey et al. [26] showed that Vit.A interact with singlet oxygen and can thus prevent the oxidation of polyunsaturated fatty acid, by scavenges O₂⁻ and reacts directly with peroxy radical [27]. Vitamins A provide antioxidant defenses by their ability to exist in reversible oxidized and reduced forms. Antioxidants, which are working against the oxidative damage within the cell, consist of preventive and chain breaking mechanisms [28]. By these activities of Vit.A as an antioxidant lead to decrease lipid peroxidation and MDA level which caused by MTX treatment.

So Vit.A play important role in protecting cell membrane against oxidative damage caused by MTX because of it is ability for scavenging free radicals and reduced lipid peroxidation. The results also showed there were a non significant increase in Vit.A treated group compared with control, while a significant increase in MTX-Vit.A treated group compared with control may be due to that duration of treatment with Vit.A did not sufficient to reduce the cytotoxic effect of MTX.

References


